BACTERIAL DEGRADATION OF ETHYL TERT-BUTYL ETHER AND STUDY OF THE MOLECULAR MECHANISMS UNDERLYING ITS BIODEGRADATION

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DOCTORAL THESIS

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CERTIFY

That the present study, entitled “Bacterial degradation of ethyl *tert*-butyl ether and study of the molecular mechanisms underlying its biodegradation” presented by Vijayalakshmi Gunasekaran for the award of the degree of Doctor, has been carried out under my supervision at the Chemical Engineering Department of the University Rovira i Virgili, and that it fulfils the requirements to obtain the Doctor International Mention.

Tarragona, 23rd July 2013

Dr. Magdalena Constanti Garriga

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Summary

Ethyl tert-butyl ether (ETBE) is used as a fuel additive mostly in Europe and Japan to achieve complete combustion of automobile fuels and to control air pollution. It can be potentially toxic on continued usage and can be a possible threat to the human system as it can contaminate water bodies.

This Ph.D. work has been divided into two parts, the first part includes the aim to enrich and identify bacterial consortia in order to potentially degrade ETBE, to study the degradative abilities of the isolated bacterial consortium to degrade other compounds, and also to study the molecular mechanisms underlying ETBE degradation. The second part focuses on studying the role of cytochrome P450, a key enzyme that can participate in ETBE or MTBE degrading pathway in *Achromobacter xylosoxidans* MCM2/2/1 which has been an proven ETBE and MTBE degrading single strain.

The foremost objective of the study was to enrich and isolate bacterial consortia capable of degrading ETBE. With this aim in hand, the enrichment was done by seeding two different water samples collected at two different sites in a gasoline contaminated water body located in the Western part of Spain. ETBE was given as source of carbon and energy (in case of consortium B), and as co-metabolic substrate along with methanol (in case of consortium A) which resulted in the identification of two different bacterial consortia namely consortium A and consortium B that can degrade ETBE. The degradation abilities of the two enriched bacterial consortia were studied. Chapter 2 describes the ETBE degradation capacity of the enriched bacterial consortium A. This consortium degraded about 51% of 50 mg/L of ETBE in 9 days. *Xanthomonas* sp., *Methylibium* sp., *Methylobacillus* sp., and *Methylovorus* sp were identified as the participating bacteria during ETBE degradation by DGGE - 16S rDNA analysis. In addition to ETBE, this consortium degraded benzene, toluene and xylene isomers (BTX) when they were present as the sole carbon source. But, the degradation efficiency increased predominantly when ETBE was included as an additional carbon source. Interestingly, the presence of BTX had a negative effect on the degradation of ETBE. This is a crucial observation for ETBE degradation in the natural environment.

The other bacterial consortium B was able to degrade and grow on ETBE as sole carbon source. This consortium was able to degrade approximately 95% of the added ETBE in 16 days. The degradation product of ETBE, *tert*-butyl alcohol (TBA) was identified and characterized. Chapter 3 demonstrates the potential of the isolated bacterial consortium B to degrade higher concentrations of the recalcitrant pollutant ETBE. Finally in the last subculture, only two colonies corresponding to *Xanthomonas sp*. and *Pseudomonas sp.* were identified as the participating bacteria by 16S rDNA identification. This chapter demonstrates the potential of the bacterial consortium B that can be useful for bioremediation of sites heavily contaminated with ETBE.

Bacterial consortium B with the proved potential to degrade higher concentrations of ETBE was selected for further study at the molecular level to understand better, the effects and the mode of ETBE degradation and for the same a gel based proteomic analysis was chosen. The proteomic analysis revealed difference at the expression level in about 241 proteins which respond differently with respect to the bacterial consortium propagated in glucose and ETBE. Interestingly we were able to identify aldehyde dehydrogenase which plays an important role in the degradation pathway of ETBE and found to be upregulated in presence of ETBE indicating its participation in ETBE degradation pathway. Proteins related to amino acid and energy metabolism were found to be up-regulated. We also observed induction of a number of transport proteins, metabolism-related proteins and chaperons. The use of two different higher concentrations of ETBE reflected in the protein profiles of the bacterium grown in 500 mg/L and 1000 mg/L of ETBE. The expression profiles of five of the identified protein spots differed significantly with an increasing pattern in higher concentration of ETBE used, of which the aldehyde dehydrogenase was one. This chapter (Chapter 4) demonstrates that the presence of ETBE significantly altered the metabolic profile of the bacterial consortium B and also confirms that increase in ETBE concentration increases the expression profiles of certain proteins. This study gains more significance as it is the first proteomic analysis conducted on a bacterial consortium that can degrade ETBE.

Chapter 5 includes the study about the ETBE and MTBE degrading *Achromobacter xylosoxidans* MCM2/2/1 which describes the other objective of the thesis. Cytochrome P450 (CYP) is an enzyme that has been reported to initiate the ETBE/MTBE degradation pathway. The presence of CYP in *A. xylosoxidans* MCM2/2/1 was confirmed by PCR and was suspected to participate in degrading ETBE and MTBE degradation pathway. The

identification and sequencing of full length cytochrome P450 gene in this organism was accomplished. The identified CYP was cloned and expressed in *E. coli*. The expressed CYP was functionally active which was tested with Co-difference spectra. Further the expressed CYP will be studied for its ability to use in *in-vitro* bio-transformation of ETBE and other xenobiotic compounds in order to study its role in ETBE and/or MTBE degrading pathways.

Resumen

El etil *tert*-butil éter (ETBE) se utiliza como aditivo de combustibles preferentemente en Europa y Japón para obtener una combustión completa de los combustibles de automóviles y para controlar la contaminación del aire. Puede ser potencialmente tóxico cuando se usa continuamente y convertirse en una amenaza humana como consecuencia de la contaminación del agua.

El presente estudio se ha dividido en dos partes: la primera parte incluye el enriquecimiento y la identificación de un consorcio bacteriano para potencialmente degradar el ETBE, para estudiar la capacidad degradadora de otros compuestos mediante el consorcio bacteriano aislado, así como estudiar los mecanismos moleculares subyacentes de la biodegradación del ETBE. La segunda parte se centra en el estudio de la enzima citocromo P450, una enzima clave que puede participar en la vía de degradación del ETBE o MTBE en Achromobacter xylosoxidans MCM2/2/1, cepa que ha sido previamente demostrada como degradadora de estos compuestos.

El objetivo principal de este estudio ha sido el enriquecimiento y aislamiento de un consorcio bacteriano capaz de degradar el ETBE. Para esta tarea, el enriquecimiento se ha realizado mediante la siembra de dos muestras de agua distintas recogidas en dos emplazamientos distintos de agua contaminada en Cataluña. El ETBE se subministró como fuente de carbono y energía, en el caso del consorcio B; y como cometabolito con metanol, en el consorcio A, dando lugar a la identificación de dos consorcios bacterianos distintos (consorcio A y consorcio B) capaces de degradar el ETBE. Se ha estudiado la capacidad de degradación de ambos consorcios. El capítulo 2 describe la biodegradación del ETBE mediante el consorcio A. Este consorcio degrada un 51% de 50 mg/L de ETBE en 9 días. Las bacterias participantes han sido identificadas como *Xanthomonas* sp., *Methylibium* sp., *Methylobacillus* sp., y *Methyloborus* sp. durante la biodegradación del ETBE mediante el análisis DGGE – 16S rDNA. Además del ETBE, este consorcio degrada benceno, tolueno y xileno (BTX) cuando están presentes como única fuente de carbono. La eficiencia de degradación incrementó significativamente cuando se añadió ETBE como fuente de carbono adicional. Resulta interesante que la presencia de BTX afecta negativamente a la

biodegradación del ETBE. Esta observación es crucial para la biodegradación del ETBE en ambientes naturales.

El consorcio bacteriano B es capaz de degradar y crecer en presencia de ETBE como única fuente de carbono. Este consorcio degrada aproximadamente un 95% del ETBE añadido en 16 días. Se ha identificado y caracterizado el producto de degradación del ETBE, ter-butil alcohol (TBA). El capítulo 3 demuestra la potencialidad del consorcio bacteriano B aislado para degradar elevadas concentraciones del contaminante recalcitrante ETBE. Finalmente, en el último subcultivo, sólo se han identificado dos cepas, *Xanthomonas* sp. y *Pseudomonas* sp., como participantes del consorcio mediante la identificación del fragmento 16S rDNA. Este capítulo demuestra el potencial del consorcio bacteriano B para la bioremediación de emplazamientos altamente contaminados con ETBE.

El consorcio bacteriano B, degradador de elevadas concentraciones de ETBE, ha sido seleccionado para posteriores estudios moleculares para comprender mejor los efectos y el modo de degradación de ETBE mediante un análisis proteómico. El análisis proteómico ha revelado diferencias a nivel de expresión en 241 proteínas, las cuales responden diferencialmente al cultivo del consorcio en presencia de glucosa o ETBE. Entre las diferentes proteínas se ha identificado la aldehído deshidrogenasa, la cual tiene un papel importante en la vía de degradación del ETBE y su expresión está aumentada en presencia de ETBE indicando su participación en esta vía. Otras proteínas relacionadas con el metabolismo de amino ácido y energético también están incrementadas. Del mismo modo hemos observado la inducción de proteínas transportadoras, proteínas relacionadas con el metabolismo y chaperonas. La aplicación de dos concentraciones elevadas distintas de ETBE se refleja en los perfiles proteicos de la bacteria crecida en presencia de 50 mg/l y 100 mg/L de ETBE. Los perfiles de expresión de cinco de los spots proteicos identificados difieren significativamente mostrando un patrón incrementado a las elevadas concentraciones de ETBE utilizadas, como por ejemplo la aldehído deshidrogenasa. Este capítulo (capítulo 4) demuestra que la presencia de ETBE altera significativamente el perfil metabólico del consorcio bacteriano B y también confirma que un aumento en la concentración de ETBE incrementa los perfiles de expresión de ciertas proteínas. Este estudio adquiere más importancia teniendo en cuenta que es el primer análisis proteómico realizado con un consorcio capaz de degradar el ETBE.

El capítulo 5 incluye el estudio sobre la bacteria degradadora de MTBE y ETBE *Achromobacter xylosoxidans* MCM2/2/1, el cual describe el otro objetivo de la tesis. La citocromo P450 (CYP) es una enzima que participa en las reacciones iniciales de la vía de degradación de ETBE/MTBE. La presencia de CYP en *A. xylosoxidans* MCM2/2/1 estaba previamente confirmada mediante PCR y se sospechaba su participación en la vía de biodegradación de ETBE y MTBE. Se ha identificado y secuenciado la total longitud del gen citocromo P450 en este microorganismo. Una vez identificada CYP, se ha clonado y expresado en *E. coli*. La expresión de CYP ha sido funcionalmente activa, lo que se ha comprobado con el espectro de CO-CYP. En un futuro, se estudiará la capacidad de esta CYP para la biotransformación *in vitro* de ETBE y otros compuestos xenobióticos para así elucidar su función en las vías de biodegradación de ETBE y/o MTBE.

List of publications

1. Vijayalakshmi Gunasekaran, Luke Stam, and Magda Constanti. The effect of BTX compounds on the biodegradation of ETBE by an ETBE degrading bacterial consortium. *Accepted in Biotechnology and Bioprocess Engineering.*
2. Vijayalakshmi Gunasekaran, Emre Donmez, Marco Girhard, Vlada B. Urlacher, Magda Constantí. Biodegradation of fuel oxygenates and their effect on the expression of a newly identified cytochrome P450 gene in *Achromobacter xylosoxidans* MCM2/2/1. *Accepted in Process Biochemistry.*
3. Vijayalakshmi Gunasekaran, Magda Constanti. Biodegradation of ethyl *tert*-butyl ether by the bacterial consortium B in a minimal medium. *Manuscript in preparation*.
4. Vijayalakshmi Gunasekaran, Magda Constanti. Proteomic analysis of the Ethyl *tert*-butyl ether degrading bacterial consortium. *Manuscript in preparation.*

Abbreviations

ALKB	Alkane Monoxygenase B
BTX	Benzene, Toluene, and Xylene
CAA	Clean Air Act
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CYP	Cytochrome P450
DGGE	Denaturing gradient gel electrophoresis
DIGE	Difference gel electrophoresis
DIPE	Di-isopropyl ether
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EPA	Environmental Protection Agency
ETBE	Ethyl *tert*-butyl ether
GC-MS	Gas chromatography – Mass Spectrometry
HIBA	hydroxyisobutyric acid
ICM	Isobutyryl-CoA mutase
IEF	Isoelectric focussing
IPG	Immobilized pH gradient
IRAC	International Agency on Research on Cancer
MALDI-TOF	Matrix-assisted laser desorption/ionization- time-of-flight
MEGA	MOLECULAR EVOLUTIONARY GENETICS ANALYSIS
MM	Minimal medium
MTBE	Methyl *tert*-butyl ether
NCBI	National Center for Biotechnology Information
OD	Optical density

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDMS/DVB	Polydimethyl siloxane/divinylbenzene fibre
PI	Isoelectric point
PMF	Peptide mass fingerprinting
PTM	Post-translational modifications
RFG	Reformulated gasoline
RH	Rehydration buffer
RNA	ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis
SPME	Solid phase micro extraction
TBA	*Tert*-butyl alcohol
USEPA	United States Environmental Protection Agency
16S rRNA	16S ribosomal ribonucleic acid
2D-PAGE	Two-dimensional polyacrylarnide gel electrophoresis
2-M1,2-PD	2-methyl 1, 2-propanediol

Contents

Index of figures and tables

List of figures

List of Tables

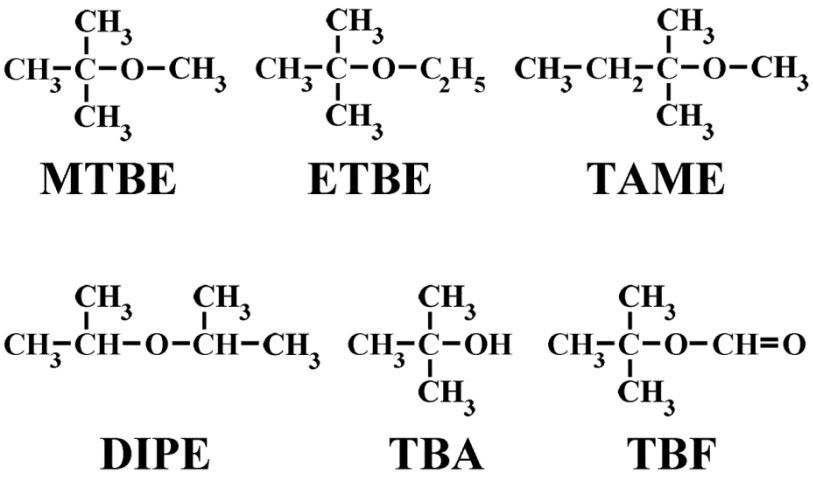
Chapter 1

Introduction

1.1. Introduction

Fuel oxygenates are oxygen-rich compounds that are added to reformulated gasoline (RFG) to enhance the octane number, and also to increase the combustibility of the gasoline so as to reduce toxic emissions. The octane rating of a gasoline is a measurement of the gasoline resistance to the auto ignition in internal combustion engine. Previously, during 1920 almost all the gasoline used worldwide contained lead, specifically, lead tetra methyl. Until the 70s, use of lead had been the cheapest way for increasing the octane rating. But later the emission of lead particles to the air caused serious problems of public health. The introduction of oxygenates additives in gasoline, started in some states of USA at the end of the 70s in order to improve the octane number in the reduction phase of leaded additives. It was in the 90s when their use was expanded spectacularly around of the country with the Clean Air Act (CAA) in 1990 (USEPA, 1990; USEPA, 2006; Barcelo et al., 2006). The objective of this act was to reduce the emissions of carbon monoxide (CO) in the urban zones. In order to achieve the objective two different programs were elaborated. The winter oxygenated fuel (Oxyfuel) Program, started on November of 1992, where the gasoline must have more than 2.7 % of oxygen during winter. The other, Reformulated Gasoline Program (RFG), aimed to solve the problem related with ozone was started in 1995 to be enforced all year round where the reformulated gasoline should have more than 2% of oxygen in weight and with inferior levels of benzene and other aromatic compounds dangerous for the humans (USEPA, 1998).

Figure 1.1. Chemical structures of fuel oxygenates



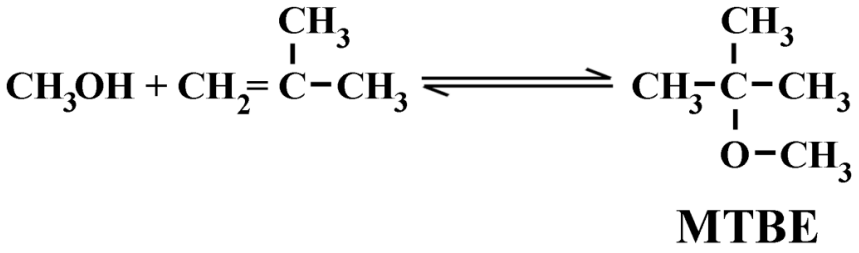
Introduction of fuel oxygenates such as methoxy-2-methylpropane, or methyl *tert*-butyl ether (MTBE) and 2-ethoxy-2-methylpropane, or ethyl *tert*-butyl ether (ETBE), *tert*-amyl ether (TAME) and diisopropyl ether (DIPE), *tert*-butyl alcohol (TBA), ethanol and methanol (Figure 1.1) in the gasoline, replaced the toxic lead thereby reducing the emission of carbon monoxide, unburned hydrocarbons, polycyclic aromatics, oxides of nitrates and particulate carbon (Barceló, 2007).

1.2. Fuel oxygenates: production, usage and advantages

Fuel oxygenate is defined as "An oxygen containing ash less organic compound such as an alcohol or ether, which can be used as fuel or fuel supplement" according to Standard Specification for Automotive Spark-Ignition Engine Fuel (ASTM D4814). Although the CAA amendments did not specify which oxygenate must be added to gasoline to meet the oxygen, MTBE was preferred and widely used. MTBE was added in 15% by volume in OXY gasoline whereas RFG containing 11% by volume was added to the gasoline to achieve the recommendations. In 1999, over 200,000 barrels per day of MTBE were being produced in the United States (USEPA, 2006). MTBE market showed that the demand for MTBE increased gradually from 20.6 million tons in 1994 (Nihlén et al., 1999) to 21.0 million tons and 22.0 million tons in 1999 and 2002, respectively (Donahue et al., 2002). MTBE was produced by a reaction between isobutene (contained in C4-fractions) and methanol (Figure 1.2a). MTBE production peaked in US (61% of total use) and became the most commonly used octane enhancer (Rosell et al., 2003). MTBE addition, in the gasoline averaged around 2% in Europe. Particularly in Spain, MTBE was used in different types of gasoline at the range of 2.8 – 4.3% in 95% unleaded gasoline and up to 6.9 – 10% in 98% unleaded gasoline (Pérez Pascual, 2001). Whilst, other fuel oxygenates such as TAME, ETBE and ethanol (except in Europe) were used in substantial amounts (Schmidt et al., 2002). ETBE was produced from ethanol (47% v/v) and isobutene 12 (53% v/v) with heat over a catalyst (Figure 1.2ba) (Alcantara et al., 2000; Bisoworno and Tade, 2000). ETBE can also be produced directly from TBA and ethanol in liquid phase (Ozbay and Oktar 2009). Addition of fuel oxygenates to the gasoline benefit by reducing the toxic emission such as carbon monoxide.

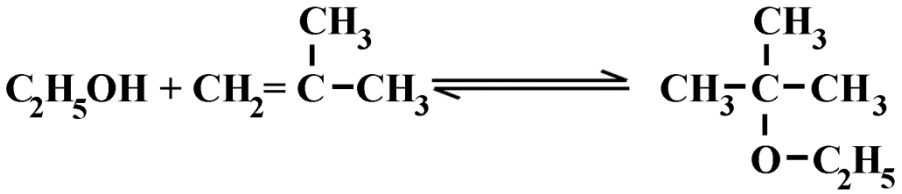
Figure 1.2. Chemical synthesis of MTBE and ETBE

a. Chemical reaction of methanol and isobutene to produce MTBE



MTBE

b. Chemical reaction of ethanol and isobutene to produce ETBE



ETBE

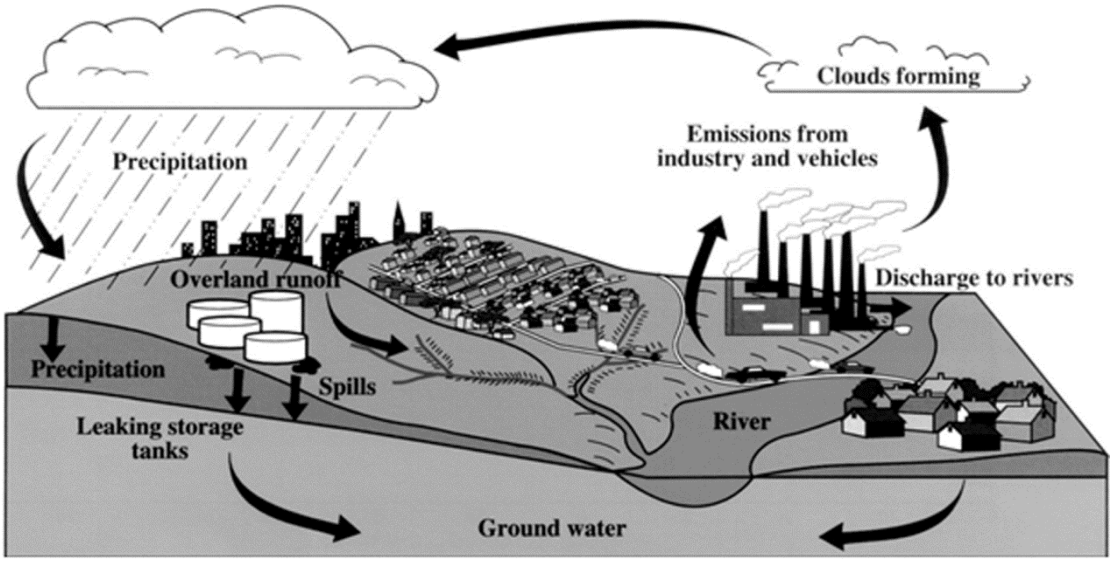
Source: Ancillotti and Fattore (1998)

1.3. MTBE distribution and phase out

Although MTBE has the benefits of increasing combustion efficiency and improving air quality, its wide usage poses an indirect risks to the environment. MTBE enters the environment in different ways such as auto emissions, evaporative losses from gasoline stations and vehicles, storage tank releases, pipeline leaks, and accidental spills, and refinery stock releases. When gasoline is released into air, a significant portion exists in air and a small portion enters into soil and water (Ahmed et al., 2001). When it is released into water a significant portion remains dissolved in surface water, with some partitioning into air and a much smaller amount into soil (WHO, 1998). Because of its high solubility, it moves through the soil and into the groundwater more rapidly than other chemicals (Ahmed et al., 2001). These unique properties allow it to move quickly and easily through the water column with minimal retardation and also make it difficult to be removed. Once these contaminants move below the surface (Figure 1.3), they can have even longer life spans due to anaerobic conditions (Bradley et al., 1999). Widespread gasoline spills and storage tank leaks have contaminated the groundwater systems, making it the second most common water pollutant (USEPA, 2008). As a result of all these drawbacks, elimination of the use of MTBE as a

gasoline additive was initiated by California Air Resources Board (CARB) and banned completely in USA in 2004 (Lidderdale, 2003).

Figure 1.3. Movement of fuel oxygenates in the environment



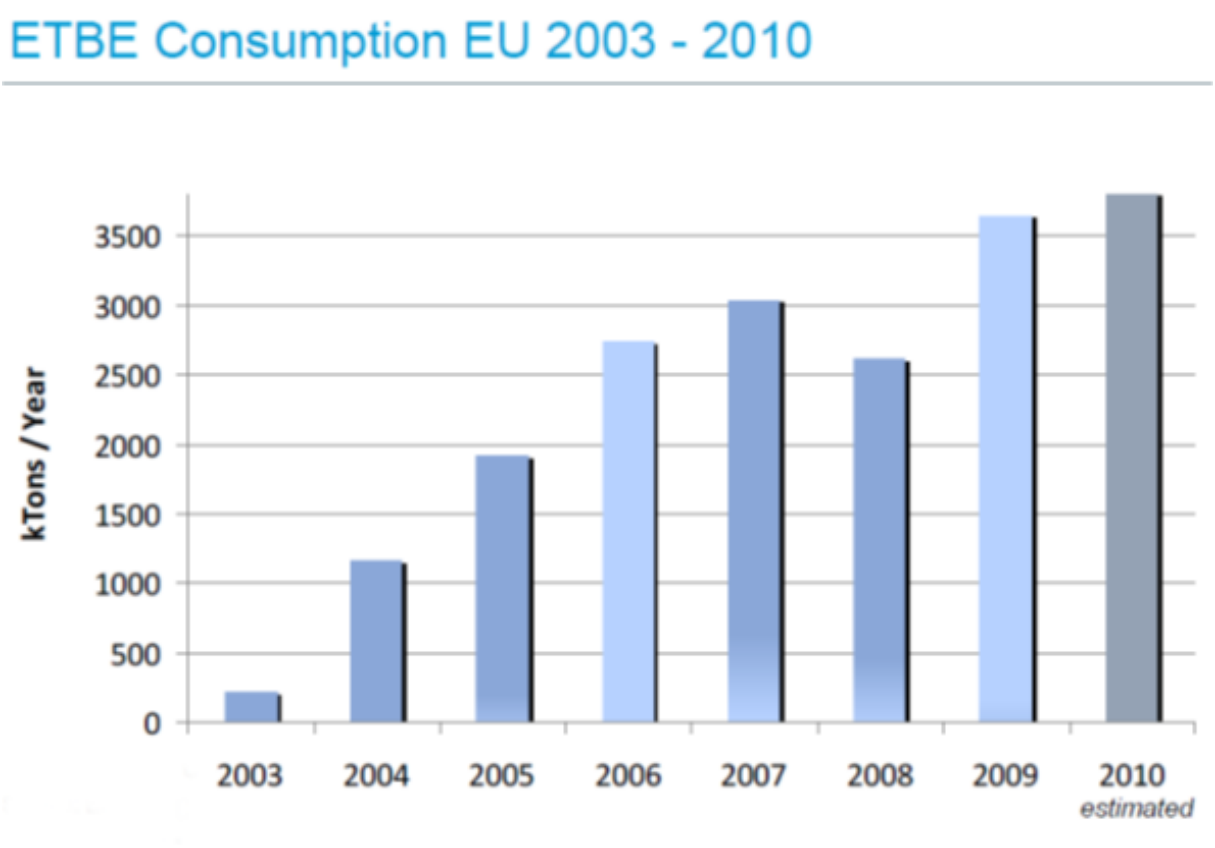
Source: http://epw.senate.gov/105th/zog_12-9.htm, 1997

1.4. ETBE as fuel oxygenate

European countries such as France, Spain, Netherlands, Germany and Belgium (Zereshki et al., 2011; Maladain et al., 2010; Auffret et al., 2009) switched to ETBE after the phase out of MTBE and USA adopted ethanol over MTBE which was once the second mostly used compound in USA. ETBE was first used in France in 1992. In 2002, France and Spain contributed a total of 568,000 t to the ETBE production capacity in the European Union (Demirbas et al., 2006). The ETBE production capacity increased from 2 million tons to 4 million tons from 2005 to 2007 (Vlasenko et al., 2009; Zereshki et al., 2011). Figure 1.4 illustrates the consumption of ETBE from 2003 to 2010. According to Digabel et al., 2013, MTBE has been replaced by ETBE mainly because it can be produced from bio-based ethanol and it has been reported in 2011 that, the total global consumption of ETBE was about three million metric tons, with about two million metric tons consumed in Western Europe and most of the rest in Japan and Eastern Europe (Digabel et al., 2013). The use of

ETBE also increased in Japan (Matsumoto et al., 2009) to meet the mandates set by the Kyoto protocol to reduce the greenhouse gases (GHG) emission which agreed to use 840,000 kL/year of ETBE. In Japan, ETBE-blended (7%) gasoline is being sold as "bio-gasoline" (Fujii et al., 2010). It is utilized in Europe where no modification is required for vehicles and distribution equipment in comparison to direct blend of ethanol.

Figure 1.4. ETBE consumption in Europe



Source: European fuel oxygenate association (2009)

1.4.1. Physical and chemical properties

Comparing ETBE with MTBE, there are various related aspects needed to be considered, including octane contribution, technical convenience, available supply, economic consideration, as well as their environmental impact. Since the fate and transport of organic compounds in the environment are primarily determined by their chemical and physical

properties, a fundamental comparison of these properties is of great significance. Table 1.1 summarizes the key properties of ETBE against other oxygenates.

After the disadvantages of MTBE were revealed, ETBE was found to be an alternative and environmentally friendly octane oxygenate. ETBE has higher boiling point and lower vapour pressure than MTBE, which ensure that ETBE is compatible to be blended into gasoline, allowing a more efficient blending and the mixed gasoline can be transported via pipeline without any problem. Despite of all these technical convenience and supply available, ETBE also presents some advantages and disadvantages concerning environmental protection and sustainable development.

With high octane number, either MTBE or ETBE has significant function of combustion enhancement. Besides, their high oxygen content leads to more complete gasoline combustion, resulting in lower emissions of many air pollutants such as carbon monoxide, VOCs, nitrogen dioxide and particulates thereby improving air quality. Although both of them provide the above mentioned benefits for air quality, there are slight differences between them. ETBE shows advantage over MTBE in terms of formaldehyde, sulphur oxides, hydrocarbons and methane emissions. However, ETBE appears to be not as advantageous as MTBE in terms of particulates, nitrogen dioxide, acetaldehydes and ammonia emissions.

Bio-ETBE can be synthesized by reacting bio-ethanol produced from feed stocks (Thiel et al., 1997, Yang et al., 2000; Ozbay and Oktar 2009;), and isobutene and it can be considered a “bio-fuel” which makes it attractive as part of the raw material in gasoline that can be replaced with a renewable material. As a result, it provides potential economic and environmental benefits in the sense that it opens the door for an acceptable fuel component to absorb the growing surplus of grain and other biomass, ensuring a sustainable development. However, the higher cost of grain-derived ETBE makes it uneconomic compared with the use of MTBE as a gasoline additive.

1.4.2. ETBE distribution

There are comparatively very few reports on distribution of ETBE as ETBE has not been studied extensively like MTBE. MTBE in groundwater due to contamination by gasoline was estimated in both the USA and in Europe (Achten et al. 2000, 2002a, b; Johnson et al. 2000; Rosell Linares 2003); however, the level of ETBE contamination is not generally

known. ETBE and TAME have been found mainly in groundwater and surface waters in the USA, UK, Denmark, Belgium, Netherlands and Germany, and others (Bartling et al 2011). Previously, Rosell et al., 2006 have done extensive study on MTBE distribution in the regions around Catalunya, Spain. Extensive study of ETBE distribution in Europe and other parts of the world should be done where ETBE use is reaching higher levels with its recent addition in the fuels (Rosell et al., 2006). Only one study has been documented due to a leaking storage tank below a gas station in France in which the underground water was contaminated with a higher concentration of about 300 mg/L of ETBE (Fayolle-Guichard et al., 2012).

Table 1.1. Physico-chemical properties of ETBE in comparison with other fuel oxygenates

Compound	Abbreviation	CAS number	MW (g/mol)	Boiling point (°C)	Water solubility (mg/L)
ETHER OXYGENATES					
Methyl tert-butyl ether	MTBE	1634-04-4	88	55	51000
Ethyl tert-butyl ether	ETBE	637-92-3	102	73	12000
tert-Amyl methyl ether	TAME	994-05-8	102	86	2640
Diisopropyl ether	DIPE	108-20-3	102	69	8800
ALCOHOL OXYGENATES					
Methanol	MeOH	67-56-1	32	65	complete
Ethanol	EtOH	64-17-5	46	78	complete
DEGRADATION PRODUCTS					
tert-Butyl alcohol	TBA	75-65-0	74	82	complete
tert-Butyl formate	TBF	762-75-4	102	83	11200

Source: http://epw.senate.gov/105th/zog_12-9.htm, 1997

1.5. ETBE and other fuel oxygenates toxicity

1.5.1. Odour and Taste threshold levels

Ethers oxygenate give contaminated water an unpleasant odour and taste, making it undrinkable (Inal et al., 209). MTBE is described as having a turpentine-like taste, bitter and nauseating (Fayolle et al., 2001). In 1997 in the USA, EPA issued an advisory taste threshold of 40 ppb and an odour threshold of 20 ppb for MTBE (USEPA, 1997). In a Dutch study (van Wezel et al., 2009), the odour and taste threshold for MTBE was found to be 7 µg/L and 15 µg/L respectively. The researchers commented that this is comparable to literature values found previously. The study also found the thresholds for ETBE to be 1 µg/L for odour, and 2 µg/L for taste. TAME was found to be 8 µg/L for odour, and 16 µg/L for taste (Table 1.2).

Table 1.2. Taste and odor threshold levels of ETBE and other fuel oxygenates

	ETBE	MTBE	TBA	TAME	Reference
Taste (µg/L)	47	20 – 40 25 – 60	nd	128	Johnson et al., 2000 Schmidt et al., 2004
	2	14	nd	16	Van Wezel et al., 2009
Odour (µg/L)	13	53 40 – 70 14 – 46	21000	27	Johnson et al., 2000 Schmidt et al., 2004 Fayolle et al., 2001
	1	7	nd	8	Van Wezel et al., 2009

1.5.2. Animal toxicity studies

Studies have been conducted on the potential toxicity of MTBE and ETBE over the last 20 years. Some bodies such as the EPA warn that MTBE is a potential carcinogen based on animal testing. The majority of studies over the years were done with MTBE. Although there has been limited studies undertaken on the toxicology of ETBE, the structures of MTBE and ETBE are very similar, so it is generally accepted in the literature that they would also have similar toxicological properties (McGregor, 2007). MTBE contamination of freshwater poses a toxic risk to humans and marine animals in very high concentrations which are not

found in nature. But, the presence of MTBE can potentially increase the toxicity of pesticides and other pollutants present in the same environment (Rosell et al., 2007). Single dose toxicology tests show that ETBE has low toxicity and is essentially a non-irritant to eyes and skin. Exposure to 50ppm of ETBE was observed to cause some irritation on subjects who recorded responses to discomfort on a questionnaire. Only very high concentrations have shown neurological effects. ETBE has no effect on reproduction, development, or genetic material (McGregor, 2007). ETBE was found to have low acute toxicity in rats and rabbits. ETBE exposed rats were shown to have an oral LD50 value greater than 5000 mg/kg (McGregor, 2007). The EPA has classified MTBE as a potential carcinogen, based on animal studies and MTBE inhalation. There were uncertainties and limitations in the data, so there was no conclusive estimate at what level of exposure could be a risk in carcinogenicity. Nevertheless this provided evidence that MTBE is a potential animal carcinogen (USEPA, 1997) (Ahmed et al., 2001). The UN International Agency on Research on Cancer (IARC) however, concluded that there was inadequate evidence that MTBE is a carcinogen (IARC, 1999). Although its risk to human health has not been proved (Dekant et al., 2001), yet, the undesirable taste and odour of ETBE can be an issue.

1.6. Biodegradation of ETBE

Bioremediation is considered to be potentially the best method to remove oxygenates from water because conventional techniques are neither efficient nor cost-effective (Lopez-Ferreira et al., 2006). Bioremediation is the exploitation of bacterial, fungal or plant metabolic pathways to degrade harmful contaminants. Bioremediation's major advantage is that it can completely mineralise MTBE to harmless products such as carbon dioxide, biomass, and water in a cost-effective, non-invasive manner (Eixarch and Constanti, 2010).

Bacterial degradation of ETBE and MTBE have been observed under aerobic (Squillace et al., 1996) and anaerobic (McGregor et la., 2010) conditions since the last decade. Although degradation of MTBE and other compounds have been reported either under aerobic condition or anaerobic condition, the degradation rate has showed to be comparatively very slow in anaerobic degradation (Schmidt et al., 2004).

Only few reports have described ETBE degradation by pure bacterial cultures that can utilize ETBE as sole carbon and energy source. They were *Rhodococcus ruber* IFP2001 (Hernandez-Perez et al. 2001), *Rhodococcus wratislaviensis* IFP2016 (Auffret et al., 2009),

Variovorax paradoxus CL-8 (Zaitsev et al., 2007), and *Aquincola tertiaricarbonis* L108 (Lechner et al. 2007).

1.7. Degradation pathway of ETBE

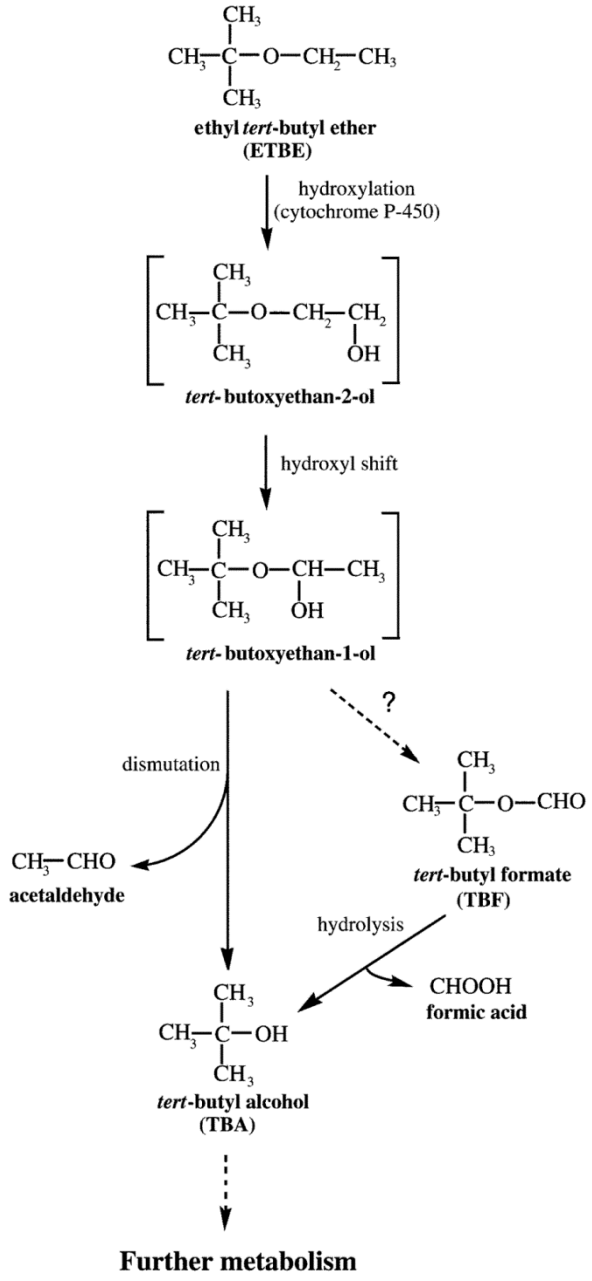
ETBE and it close relative MTBE are relatively stable compounds because the ether bond is difficult to cleave, resisting the microbial attack of the tertiary carbon structure (Barbera et al., 2011). A hypothetical metabolic pathway for ETBE degradation has been proposed by Kharoune et al. (2001) after studies conducted on bacterial strains E1 and E2 (Figure 1.5). The first step in ETBE degradation is the cleavage of the ether bond which involves the participation of cytochrome P450 (CYP). This is similar to MTBE degradation pathway. ETBE is catabolised to produce an intermediate called tert-butoxyethan-2-ol. This is transformed into a hemiacetal called tert-butoxyethan-1-ol. It rapidly dismutates into the alcohol, TBA, and the aldehyde, acetaldehyde.

TBA could also be formed via the hydrolysis of TBF. It is unknown how ETBE is degraded to TBF. Two hypotheses were formulated from the study conducted by Kharoune et al., 2001, each proposing different routes of metabolism for ETBE: the degradation of ETBE after dismutation of *tert*-butoxyethanyl-ol to form TBA and acetaldehyde, and/or the formation of TBF, which rapidly hydrolysed to TBA and formic acid. The authors suggest that a major limiting step to the oxygenate degradation was the accessibility and cleavage of the ether bond. The same strain used was able to degrade ETBE, MTBE, and TAME which also suggests that the same or very similar metabolic pathway is used by the bacteria to degrade the fuel oxygenates.

1.8. Role of genes and enzymes involved in ETBE and MTBE degradation pathway

Exploring the molecular mechanisms by identifying the genes and enzymes responsible for degradation by microorganisms could be one of the best ways to understand the mechanism of degradation.

Figure 1.5. Metabolic pathway of ethyl *tert*-butyl ether biodegradation



Source: Hypothetical metabolic pathway of ETBE (Kharoune et al., 2001)

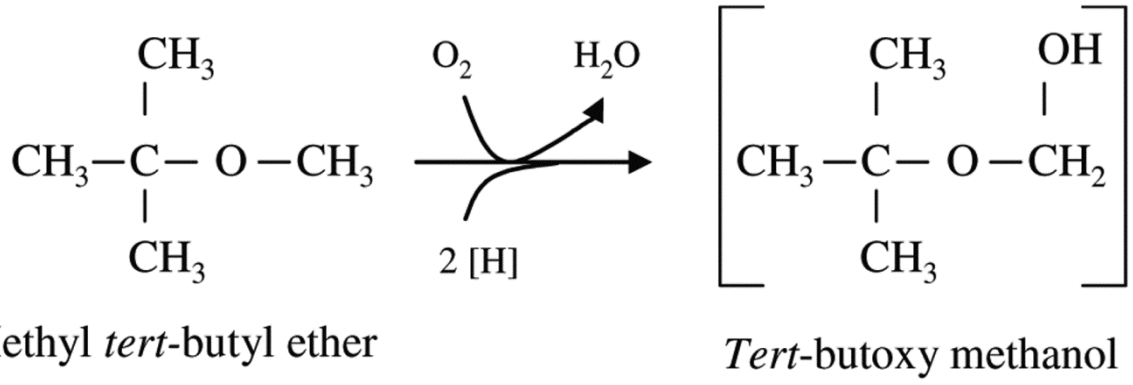
1.8.1. CYP Monoxygenases

Cytochrome P450s (CYPs) are one of the most probable candidate enzymes for carrying out these characteristic reactions. CYPs are heme proteins, that constitute a large family of monooxygenases, which are commonly found to perform a number of difficult oxidative reactions, such as C-H bond hydroxylation, *N*-dealkylation, *N*-hydroxylation, *O*-dealkylation, *S*-oxidation, epoxidation of numerous endogenous and exogenous compounds

(Urlacher et al., 2012). Biodegradation of either MTBE or ETBE has been reported to start with the cleavage of the ether bond (o-demethylation) (Youngster et. al, 2008; Malandain et al., 2010) mediated by a monooxygenase, (Figure 1.6) which produces respective tertiary alcohols. CYPs are apparently involved in the detoxification of a broad spectrum of environmental chemical pollutants (Karlson et al., 1993; Chauvaux et al., 2001).

TBA, the primary measurable compound in MTBE detoxification, may also undergo oxidation by CYP as increased concentrations of acetone were observed in rats exposed to *tert*-butanol (McGregor, 2007). Hatzinger et al. (2001) showed that monooxygenases were involved in TBA oxygenation in *Hydrogenophaga flava* ENV735 which could lead to 2-methyl 1, 2-propanediol (2-M1, 2-PD) (Lopez-Ferreira et al., 2006). And later, a cluster of *mpd* genes was found to be involved in the conversion of 2-M1, 2-PD to hydroxyisobutyric acid (HIBA) which was isolated from *Mycobacterium austroafricanum* IFP 2012 (Hatzinger et al., 2001).

Figure 1.6. Oxidation of MTBE by cytochrome P450 to produce t*ert*-butoxy methanol



Source: Lopez-Ferreira et al., 2006

Cytochrome P450 is encoded by the *eth* genes (Figure 1.7) in the following strains: *Rhodococcus rubus* IFP2001, *Rhodococcus zopfii* IFP2005, and *Mycobacterium sp.* IFP 2009, which were induced and were able to grow on MTBE and ETBE. These P450 are involved in the initial step to degrade MTBE to *tert*-butoxy methanol (Lopez-Ferreira et al., 2006).

Figure 1.7. Participation of *Eth* genes in ETBE degradation.

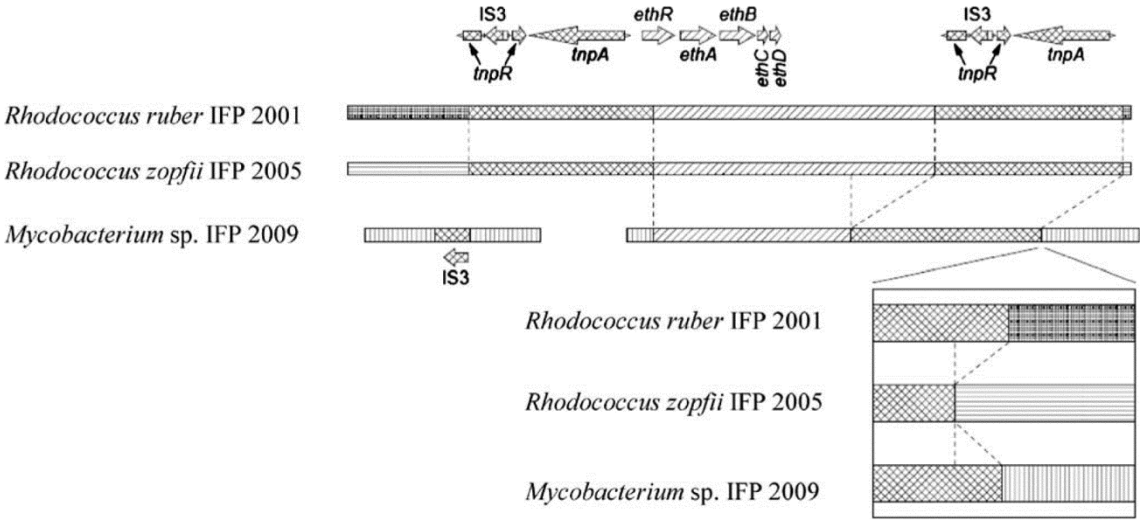


Figure illustrates Eth gene coding for cytochrome P450 present in the three bacterial strains: Rhodococcus rubus IFP 2001, Rhodococcus zopfii IFP2005, and Mycobacterium sp. IFP 2009 (Lopez-Ferreira et al., 2006).

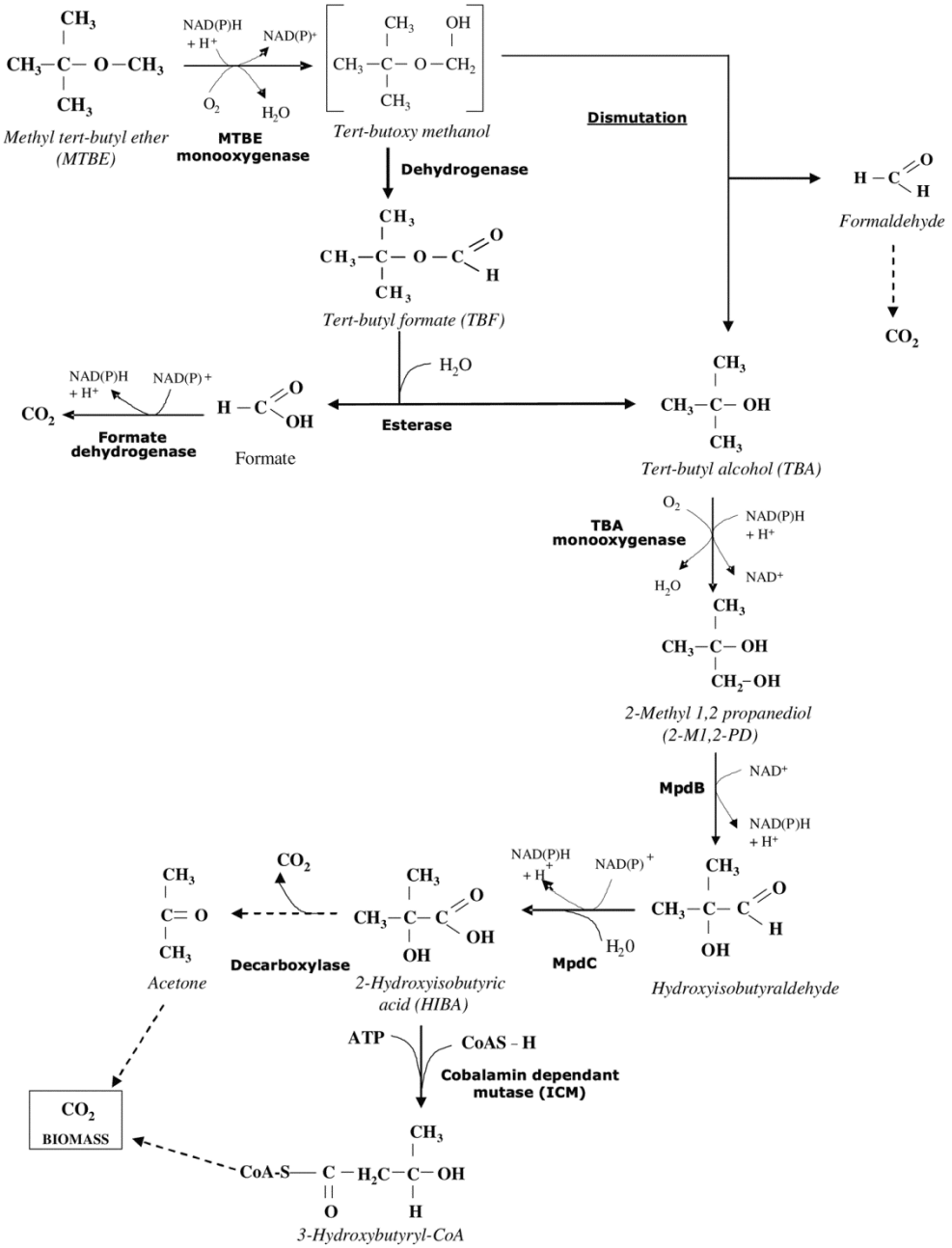
1.8.2. Other monoxygenases in ether degradation

Alkane monoxygenases identified in *Pseudomonas putida* GPo1 in which with two operons, *alkBFGHJKL* operon (Eggink et al., 1987) and the *alkST* region (Eggink et al., 1998) are found to participate in MTBE degradation. The non-heme monooxygenase (AlkB), located in the membrane, is involved in an electron transfer chain, including also a rubredoxin with a non-heme iron atom and a FAD-dependent rubredoxin reductase which transfer electron from the dioxygen to the substrate. This enzyme system has been reported to oxidize a wide range of substrates (van Beilen et al., 1994).

1.8.3. Esterases

An esterase is known to hydrolyse TBF to TBA (Figure 1.8) and is important in the early steps of MTBE degradation (Lopez-Ferreira et al., 2006). TBA is a key intermediate of both the MTBE and ETBE catabolic pathway, and is often found in MTBE/ETBE contaminated aquifers as a result of partial degradation of the ethers (Babe et al., 2007).

Figure 1.8. Enzymes and genes involved in MTBE degradation pathway



(Source: Lopez-Ferreira et al., 2006)

1.8.4. Dehydrogenases and other enzymes

Two alcohol dehydrogenases, MpdB and MpdC were shown to be involved in the conversion of 2-M1, 2-PD to HIBA (Figure 1.8 & 1.9). These were isolated from the bacteria *M. austoafricanum* IFP 2012, cloned and expressed in *M. smegmatis* mc2 155 (Lopez-Ferreira et al., 2006). MpdB and MpdC are part of a cluster of mpd genes which induce and

regulate the conversion of 2-M1, 2-PD to HIBA in the metabolic pathway. This gene cluster includes a regulator that controls the activity of the two dehydrogenases. They were shown to be strongly induced during the growth of IFP2012 on MTBE (Lopez-Ferreira et al., 2006). Rohwerder et al., (2006) identified an isobutyryl-CoA mutase subunit in strain L108 (wild type) and in its variant L10, which was unable to grow on MTBE but still able to grow on TBA. The authors state that HIBA is converted into 3-hydroxybutyrate in the presence of ATP and acetyl CoA in the strain L108 via cobalamin dependent mutase encoded

Figure 1.9. Participation of mpd gene clusters in the MTBE degradation pathway

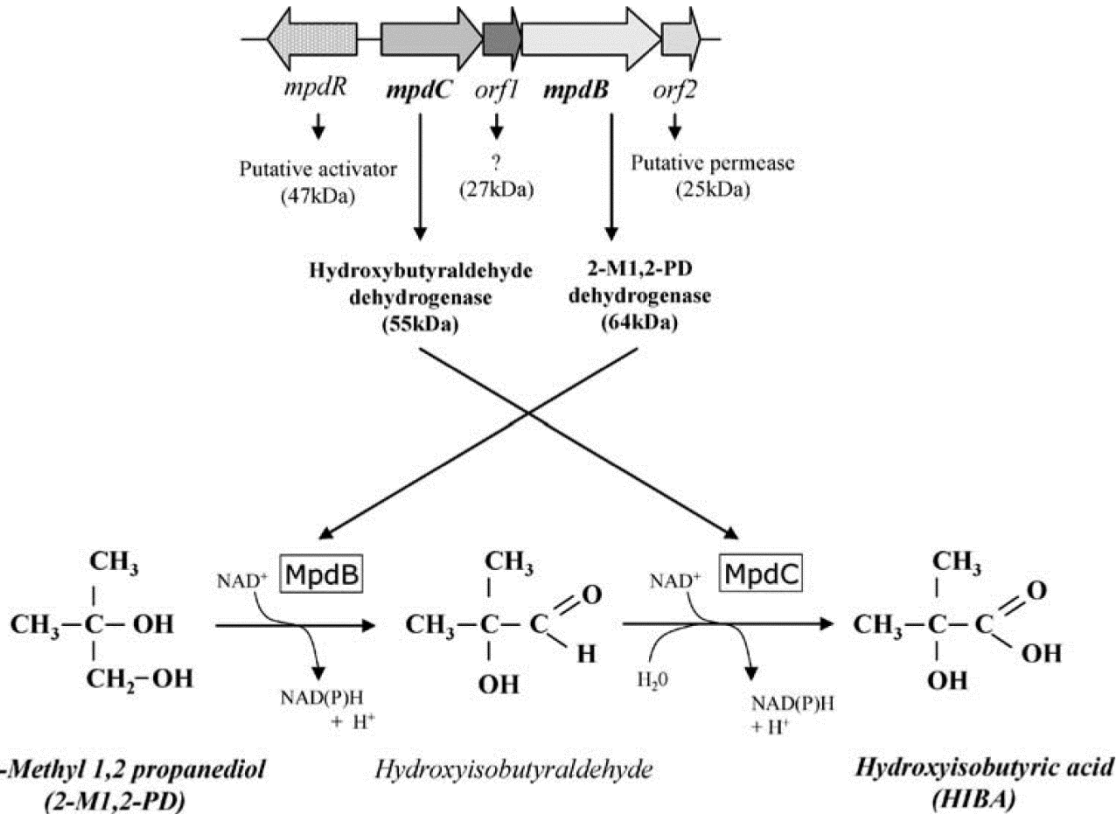


Figure illustrates the role mpd genes from M. austroafricanum IFP2012 in metabolising MTBE (Lopez-Ferreira et al., 2006).

by isobutyl cobalamin dependent mutase (icm) genes. Similar icm genes were found to be highly conserved in another bacterial strain called *M. petroleiphilum* PM1.

1.9. Genomic and proteomic approaches

Some of the important methods that were used for this study are described below

1.9.1. 16S rDNA analysis

16S rDNA identification is a standardised and invaluable tool in microbiology for the identification of isolated prokaryotes. Ribosomal RNAs are ancient molecules that are ubiquitously expressed and conserved between organisms that are phylogenetically distant. Bacterial RNA has three subunits namely 5S, 16S, and 23S rRNAs and these subunits of ribosomes are necessary for the translation of messenger RNA. There is higher genetic variance in the non-conserved regions of 16S rRNA subunit than in 5S and 23S rRNA which makes it an ideal choice for making a phylogenetic classification system for prokaryotic species (Pontes et al., 2007). 16S rRNA is highly conserved between different species of bacteria and archea but contain hypervariable regions that are species specific and can be exploited to identify bacteria under study. In addition, rRNA genes being less likely than other genes to be deleted or acquired through horizontal transfer, the method provides important information about the taxonomic status of the microorganisms detected (Beguin et al., 2003). rRNA's are organised in operons with conserved coding regions interspersed with semi conserved and non-conserved regions. The conserved regions are useful as primer targets for PCR amplification of the entire gene (Edwards et al., 1989). Synthetic oligonucleotide primers are designed to amplify 16S rRNA gene via PCR. The amplified DNA is sequenced and the nucleotide sequence is then compared to a 16S rRNA database of known bacteria. If an identical match cannot be found, and the bacterium under study has less than 97% 16S rRNA gene sequence homology with known species, it is regarded as a criterion for the identification of a new species (Pontes et al., 2007).

1.9.2. DGGE-16S rDNA

Denaturing gradient gel electrophoresis (DGGE) is a commonly used molecular technique for rapid fingerprint analysis of microbial community composition, diversity, and dynamics. Analysis of 16S rRNA genes using DGGE represents a powerful tool to study bacterial community structure in complex environments as well as in enrichment cultures (Brito et al., 2006; Luo et al., 2009). DGGE of PCR-amplified 16S ribosomal DNA (rDNA)

fragments has been frequently applied to the fingerprinting of natural bacterial populations. Species identification and population enumeration are critical in the study of microbial communities.

Traditionally, microbial species are cultured and then characterized by their physiological and biochemical properties. This method, however, has a serious drawback because most of the bacteria cannot be readily isolated and cultured. It was estimated that only less than 1% of bacteria in the natural environment can be cultured (Amann et. al., 1995).

In the last few years, the development of molecular typing methods offered the possibility to advance more rapidly and efficiently on bacterial identification; moreover, direct sampling in complex matrices may avoid biases related to traditional methods. 16S rDNA fragments from different microbial species have the same length but different DNA sequences therefore the species can be identified by the band positions on the DGGE gel. DGGE allows the simultaneous analysis of multiple samples and the comparison of microbial communities based on temporal and geographical differences (Muyzer et al., 1998). Phylogenetic identification of individual members of the bacterial communities can be obtained either by excising DGGE bands from gel and their subsequent sequencing, or by the construction of the 16S rDNA clone libraries which are screened by DGGE (Muyzer et al., 1993).

1.9.3. Genome walking: Inverse PCR

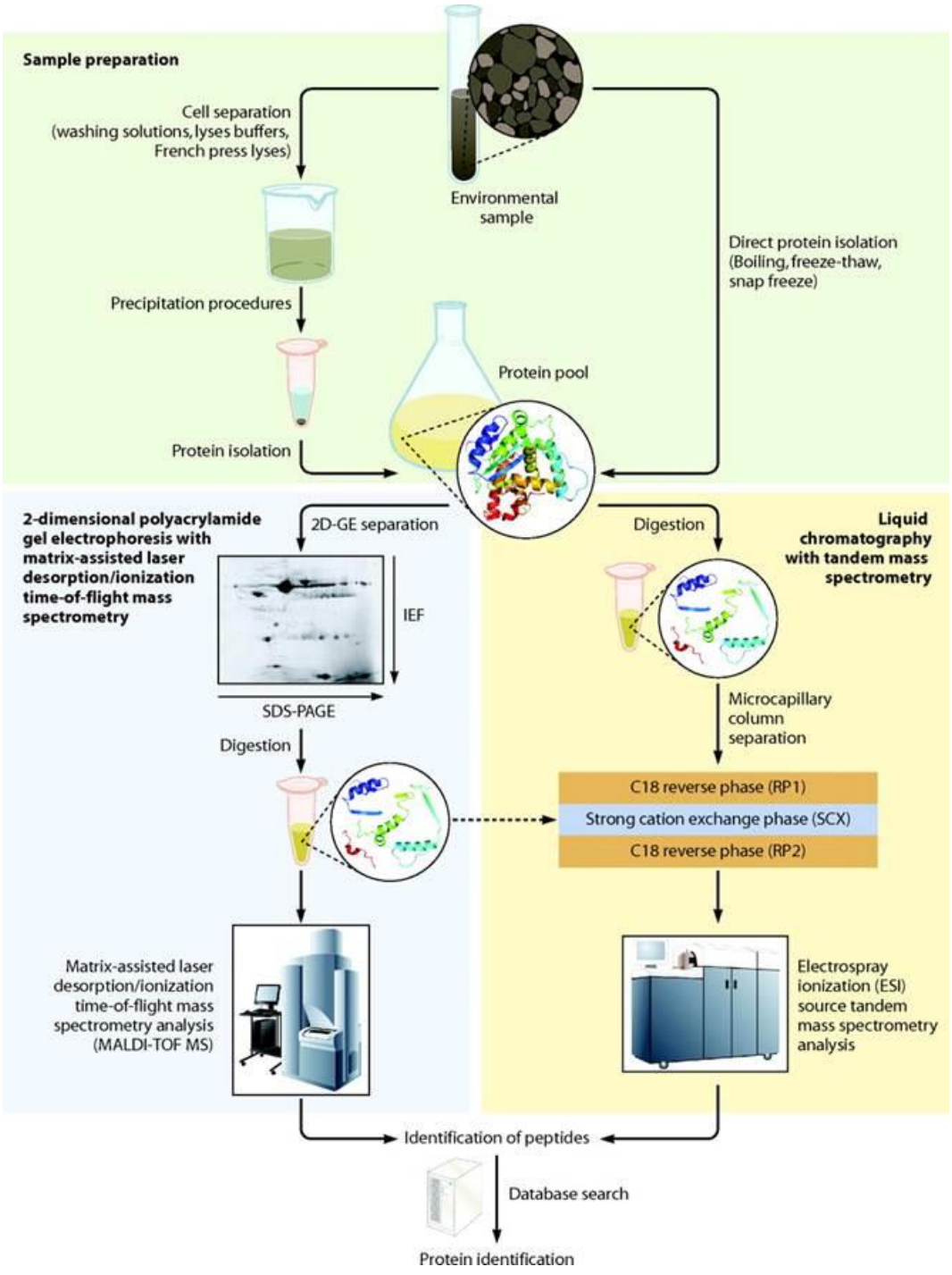
The traditional approach for “walking” from regions of known sequence into flanking DNA sequences involved the successive probing of libraries with clones obtained from prior screenings. This method, besides being laborious, was also time consuming. Though PCR is an effective method for selectively amplifying specific DNA segments, conventional PCR only allows the amplification of sequences within known boundaries. Therefore, sequence information at the extremities of the DNA fragment to be amplified is a prerequisite for selective amplification of specific DNA, thus posing a major limitation on the use of the PCR in the amplification and isolation of these unknown regions. Numerous modifications have been made to existing protocols for the amplification of an unknown DNA sequence that flank regions of known sequences, by PCR.

Inverse PCR (Ochan et al., 1998) was done in our study to walk and sequence the unknown sequence targets of CYP gene. PCR was usually carried out using restriction enzyme-digested-genomic DNA fragments as template, which was circularized by self-ligation with locus-specific primer(s) from known sequences to amplify a unknown fragment contiguous to the known sequence. The other genome walking methods include, panhandle PCR (Shymala et al., 1989), vectorette PCR (Arnold et al., 1991), anchored PCR (Roux et al., 1990) AP-PCR (Trueba and Johnson, 1996), capture PCR (Lagerstrom etal., 1991), and adapter-ligated PCR (Williems, 1998).

1.9.4. Proteomics

Microbes such as bacteria, fungi, and viruses are omnipresent and play an essential role in biogeochemical cycles and can decompose virtually all natural compounds and xenobiotics, thereby exerting a lasting effect on biosphere and climate. Microbial activity and physiology in a certain environment is strongly dependent on the composition of the communities present and the interactions of the community members during nutrient competition, predation, and cellular signalling (Brock, 1987). However, the fact that more than 90% of the microorganisms in a given environment are not readily cultured using standard methods (Amann et al., 1995) hampered investigations aimed towards a deeper insight into the structure and function of biological systems for a long time and individual contributions of different species to a certain environment remained largely unknown. Proteomics is one of the newest emerging technologies in functional genomics and arguably the most daunting omics approach which can fill the gap and help in understanding the role of microbial proteins in a given environment. Proteomics approach is used not only to identify and quantify the differentially expressed of the proteins, but also in determining their localization, modifications, interactions, activities and importantly, their functions. Proteomics methods allow qualitative and quantitative assessment of the protein complement in a given environment (López-Barea et al., 2006; Keller and Hettich, 2009; Wilms and Bonds, 2009; VerBerkmoes et al., 2009; Lacerda et al., 2009). Proteomic analysis is more challenging than genomic analysis, but is also more rewarding, because it captures regulatory effects at all levels of gene expression (i.e., transcriptional, translational, and post-translational).

Figure 1.10. Diagrammatic representation of proteomics work flow

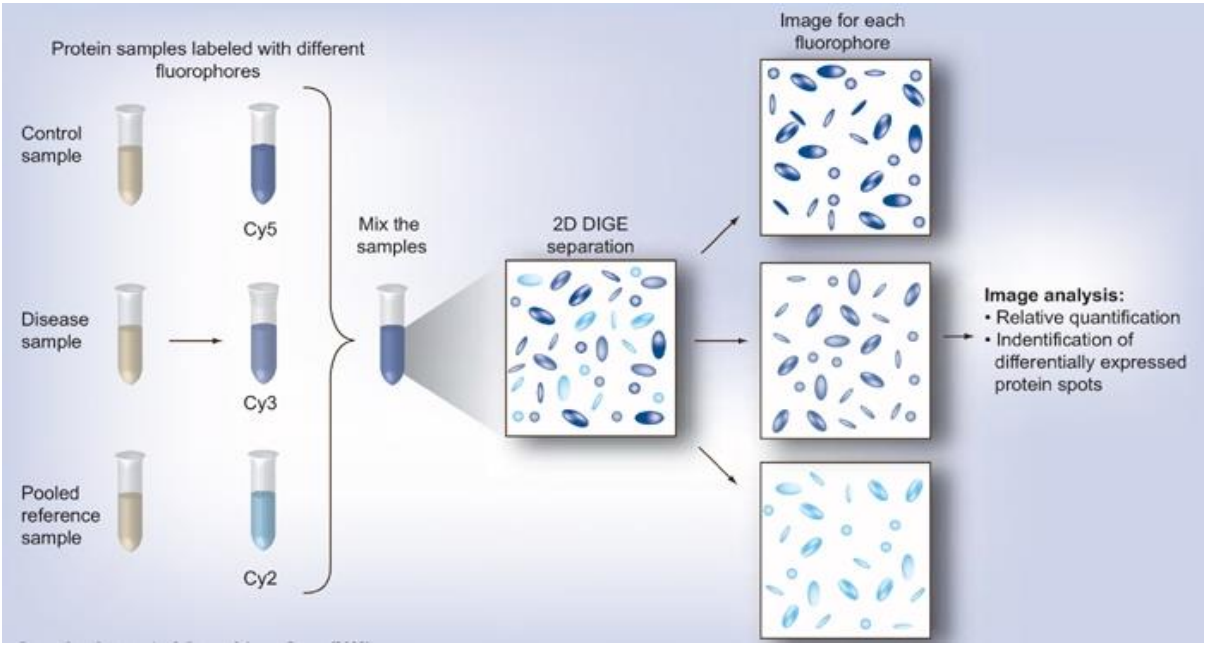


Overview of two major environental proteomic strategies. In one strategy (top left), proteins are separated by 2D-GE followed by MS analysis with either peptide mass mapping or LC-MS approaches. An alternate strategy (top right) involves protein digestion followed by online liquid LC separation and MS characterization. There are dramatic differences in measurement throughput, automation, and depth of protein identification for these two related but distinct approaches.

1.9.4.1. Gel-based proteomics

Depending on the gel size and pH gradient used, 2-DE has been demonstrated to have a resolving capacity of more than 5000 proteins simultaneously in a single gel at less than 1 ng of protein per gel spot (Görg and Dunn, 2004). Immobilized pH gradient techniques for IEF improve gel reproducibility, rendering quantitative comparison between gels feasible (Harrison et al., 2002). Figure 1.10 illustrates a standard 2D-DIGE work flow. Proteins were extracted sequentially with Tris-base, urea/CHAPS/DTT and then a combination n of urea, thiourea and zwitterionic surfactants, where even membrane proteins have been identified. Coomassie Brilliant Blue (CBB) staining and silver staining are the predominant protein detection methods in 2-DE. However, their low sensitivity or limited linear dynamic range may impede accurate quantitative analysis.

Figure 1.11. Diagrammatic representation of Difference gel electrophoresis (DIGE) labelling



Procedures for performing a 2D DIGE experiment. CY: Cyanine.

Source: Expert Rev Proteomics© 2009 Expert Reviews Ltd.

Multiplexing technology using two-dimensional difference gel electrophoresis (2D-DIGE) where proteins are covalently labelled by reaction of cyanine dyes with cysteine or lysine residues (Figure 1.11) is another approach to overcome the limitations in quantitative

analysis (Shaw et al., 2004). In 2D-DIGE, the simultaneous separation, detection and quantification of multiple samples tagged with fluorescent dyes could be performed in a single gel surpassing the gel to gel variability (Schneider Riedel, 2010). Compared with conventional 2-DE method, with fewer gel replicates, quantitative data with high-confidence levels for expression profiling could be obtained statistically with 2D-DIGE.

In addition, proteins with post-translational modifications (PTMs) can easily be detected as separate spots in 2D gels. The proteins isolated from the gels can be further analyzed using MS, where Mr and pI estimated from the 2-DE map are helpful for positive identification in many cases in contrast to non-gel-based methods in which Mr and pI are lost information (Schneider and Riedel, 2010). Proteome coverage and ‘‘resolving power’’ of environmental proteomics analyses strongly depend on size and quality of reference protein databases against which MS and/or MS/MS data have to be searched.

Nevertheless, as a widely used technique in proteome analysis, 2-DE has its advantages. The extremely high-resolving power for complex protein mixtures and the straightforward visualization of separation and quantification results are its major strengths.

1.10. Objectives

The main objective of the thesis is to focus on bacterial degradation of ETBE and to understand and identifiy the moleclar basis of ETBE degradation. To accomplish this, the following work has been carried out.

I. Enrichment and isolation of bacterial cultures to degrade ETBE.
II. Identification of the key bacterial isolates participating in ETBE degradation.
III. Examination of the potential of the isolated ETBE degrading bacterial consortium A and B to degrade other fuel oxygenates such as MTBE, TBA and other compounds such as benzene, toluene and xylene (BTX).
IV. Proteomic anaysis of the bacterial consortium B that can degrade higher concentrations of ETBE.
V. Identification of cytochrome 450 gene (the probable candidate enzymes to initiate ETBE degradation) in ETBE and MTBE degrading *Achromobacter xylosoxidans* MCM2/2/1. Cloning and expression and characterization of the newly identified cytochrome P450 gene in *E.coli*.

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Chapter 2

The effect of BTX compounds on the biodegradation of ETBE by an ETBE degrading bacterial consortium A

2.1. Abstract

Ethyl *tert*-butyl ether (ETBE) is a fuel oxygenate that is commonly used in Europe to achieve complete combustion of automobile fuels and to control air pollution. It is potentially toxic and can enter the human system via contaminated water bodies. In the present study, we have identified and enriched bacterial consortium from a gasoline-contaminated site that can degrade ETBE. Bacterial consortium A was able to degrade 47% of the added ETBE in 4 days and it continued to degrade up to 51% in 9 days. Consortium A consisted of *Xanthomonas* sp., *Methylibium* sp., *Methylobacillus* sp., and *Methylovorus* sp. which were identified as the participating bacteria during ETBE degradation by DGGE-16S rDNA analysis. In addition to ETBE, this consortium degraded benzene, toluene and xylene isomers (BTX) when they were present as the sole carbon source. The degradation efficiency increased predominantly when ETBE was included as an additional carbon source. Interestingly, the degradation of ETBE decreased to 14% in 9 days when present with BTX compounds. We report that ETBE degradation is slowed down or inhibited when BTX compounds are present. This is a crucial observation for ETBE degradation in the natural environment.

2.2. Introduction

Fuel oxygenates are compounds that were introduced into automobile fuels to increase the octane number and to achieve complete combustion. Methyl *tert*-butyl ether (MTBE) was one of the most extensively used fuel oxygenates which was later found to be the most common pollutant in many water bodies in the United States mainly because of its high solubility in water. In addition to MTBE, ethyl *tert*-butyl ether (ETBE), an ethyl derivative, was used in fuels to reduce carbon monoxide and ozone emissions (Inal et al., 2009; Streger et al., 2002). Later, in the 1990s, ETBE was used as an alternative fuel additive by some European countries in reformulated gasoline. Because its chemical properties are similar to those of MTBE, it may also pose a threat as a ground water pollutant if used continuously or if there are accidental spillages (Rosell et al., 2007). According to Rosell et al. (Rosell et al., 2007) ETBE will be the next emerging fuel-derived contaminant in Europe in the near future. Since ETBE affects the taste and odour of water even at comparatively very low concentrations of about 1-2 μg/L (Van-Wezel et al., 2009) and can pollute water bodies, it should be extensively studied to determine under which conditions it can be successfully biodegraded. The risk of ETBE or MTBE being a human carcinogen is still under debate. Although the potential risks of ETBE have not been extensively studied, MTBE, a similar compound, has been studied and labeled as a potential human carcinogen by USEPA 1997 (Ahmed, 2001). Therefore, it can be expected that ETBE is as toxic as MTBE. Consequently, there is an urgent need to develop remediation methods to eliminate ETBE from the environment.

Bioremediation is the best option since it can completely mineralize compounds to harmless products such as carbon dioxide, and water in a cost-effective, non-invasive manner (Eixarch and Constanti, 2010). Although several bacterial strains have been reported to degrade ETBE (Hernandez-Perez et al., 2001; Pannier et al., 2010; Purswani et al., 2008), few reports have described ETBE degradation by a consortium of bacteria. Another important problem to degrade ETBE in the natural environment is the possibility that ETBE co-exists with other compounds. Little is known about the coexistence of ETBE but previous studies done on MTBE could give us some idea. Such studies have shown that MTBE often co-exists in the environment with benzene, toluene, ethyl benzene, and xylene (BTEX) compounds (Al Kuisi et al., 2012; Schmidt et al., 2004).

In this study, we aim to select and identify a bacterial consortium from a gasoline-contaminated water body that can degrade ETBE, as well as to study the population dynamics during ETBE degradation. As the gasoline-polluted water sample used for enriching the consortium consisted of significant amounts of BTEX together with ETBE, we examined the ability of consortium A to degrade BTX compounds individually and also in the presence of ETBE.

2.3. Materials and Methods

2.3.1. Materials

Microbiological reagents were purchased from Scharlab, Barcelona, Spain. ETBE, benzene, toluene, ethyl benzene and xylene (*meta* and *para* isomers) (purity 99%) were purchased from Sigma-Aldrich, Madrid, Spain. Oligonucleotides, polydimethyl siloxane/divinylbenzene fibre (PDMS/DVB), solvents (HPLC grade), and all other chemicals, were also purchased from Sigma-Aldrich and were used as received.

2.3.2. Growth medium

All the cultures were grown in minimal medium (MM) containing KH_2PO_4 - 0.225 g/L, K_2HPO_4 - 0.225 g/L, $(NH_4)_2SO_4$ - 0.225 g/L, $MgSO_4 \cdot 7H_2O$ - 0.050 g/L, $CaCl_2 \cdot 2H_2O$ - 0.005 g/L, $FeSO_4 \cdot 7H_2O$ - 0.005 g/L along with trace elements ($ZnCl_2$ - 0.1 g/L, $MnCl_2 \cdot 4H_2O$ - 0.03 g/L, H_3BO_3 - 0.3 g/L, $CoCl_2 \cdot 6H_20$ - 0.2 g/L, $CuCl_2 \cdot 2H_2O$ - 0.01 g/L, $NiCl_2 \cdot 6H_2O$ - 0.02 g/L, $NaMoO_4 \cdot 2H_2O$ - 0.03 g/L (9). The trace elements were prepared in 1 L and 1 mL was added to every 1000 mL of MM prepared. The medium was adjusted to pH 7 with 1 N NaOH. ETBE and BTEX were sterilized using 0.2 µm sterile filter before they were added to the medium.

2.3.3. Enrichment of bacterial consortium

Ground water sample contaminated with gasoline was collected from the northeastern part of Spain. This was inoculated in 20% in MM along with 50 mg/L of ETBE (from a stock solution prepared in 0.25% of methanol) as carbon source and incubated at 30°C at 180 rpm for 9 days (this was known as consortium A enrichment culture 1 (ECA1)). The bacterial

growth was monitored by continuously measuring absorbance (λ=600 nm) using a Varian Cary 100 spectrophotometer. After 9 days of incubation when the optical density (O.D.) was significant (O.D. = 0.53), subcultures were made by inoculating 20% of the ECA1 into fresh minimal medium along with 50 mg/L of ETBE. All these subcultures were made and incubated in the same conditions and referred to with successive numbers such as ECA2, ECA3, etc.

2.3.4. Isolation and identification of cultivable bacterial strains

The initial subculture ECA1 was serially diluted onto washed agar plates amended with 50 mg/L of ETBE. The populations of bacteria were isolated and identified using 16S rDNA identification. Each colony were picked from the plate and transferred to another agar plate and used for further steps. Washed agar was prepared by washing about 20 g of agar with 1000 mL MM and allowed to stand for 20 min. The clarified supernatant was discarded followed by addition of new MM. The wash was repeated for three times and finally resuspended in 800 mL of MM. The final yield of agar would be approximately 1.5% (Okeke et al., 2003). The agar solution was autoclaved and cooled to 50°C after which 50 mg/L of ETBE was added. The plates were allowed to incubate at 30°C until visible colonies appeared on the plates.

2.3.5. 16S rDNA identification

The colonies were inoculated in LB broth and grown for overnight which were then used for isolation of genomic DNA using GenElute^{TM} bacterial genomic DNA kit (Sigma, Madrid, Spain. Partial 16S rRNA gene sequences of bacterial strains were amplified by PCR using the universal primers 616 V (5'-AGA GTT TGA TYM TGG CTC AG-3') and 699R (5'-RGG GTT GCG CTC GTT-3') (Barbera et al., 2011). A 1,000 bp region of the gene was amplified. PCR reaction mixtures contained 1X GoTaq Flexibuffer (Promega), 3 mM $MgCl_2$, 200 µM (each) dNTP, 0.4 µM of each primer, 100 ng of genomic DNA, and 1 unit of GoTaq (Promega) in a total volume of 50 µl. The optimized PCR conditions were initial denaturation at 95°C for 2 min; 25 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 25 s; extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were

sequenced and the 16S rRNA gene sequence results were analyzed using BLAST and ribosomal database to identify the microorganisms.

2.3.6. Biodegradation experiments

2.3.6.1.ETBE

About 30 mL of ECA6 (20%) was centrifuged and the pellet was washed twice with 0.9% NaCl and resuspended in 1 mL of MM. This was inoculated in a 250 mL screw-cap bottle with 50 mg/L of ETBE along with 150 mL of MM and incubated in a rotary shaker at 30°C at 180 rpm for 9 days. The growth of the bacterial culture was monitored by measuring the O.D. at λ=600 nm and 10 mL samples were collected from each flask at different time points in headspace vials sealed with crimp caps which were used to estimate the ETBE. Similarly, control samples were prepared in the same conditions but in the absence of bacteria.

2.3.6.2. BTX

About 150 mL of MM was inoculated with 20% of ECA6 (centrifuged and pellet resuspended in 1 mL) in three different 250 mL screw-cap flasks each of which contained 50 mg/L of benzene, toluene, and xylene respectively. Ethyl benzene could not be quantified with the method used in this study so it was not included in all the degradation experiments. Controls were maintained for each compound without the bacterial consortium being added. Growth was measured by taking O.D. readings at regular intervals. Samples were taken from each flask at regular intervals to monitor the concentration of each compound.

2.3.6.3.ETBE and BTX

The consortium was also examined for the degradation of ETBE in the presence of BTX compounds. Flasks containing 50 mg/L of each compound - benzene, toluene, xylene, together with ETBE were inoculated with 20% of ECA6 (centrifuged and pellet resuspended in 1 mL) in 150 mL of MM. Controls were maintained for each compound without the bacterial consortium being added. Growth was measured by taking O.D. readings at regular intervals. Samples were taken from each flask at regular intervals to monitor the

concentration of each compound. A number of three replicates were used in all degradation assays. The data were statistically analyzed using student's T test.

2.3.7. Analytical methods

The amount of ETBE was quantified by preparing the samples using solid phase micro extraction (SPME). The headspace vials were heated in a water bath for 30 min at 75°C for ETBE and then the gaseous phase was absorbed on an SPME fiber by incubating the fiber into the headspace vials for 30 min at the same temperature. The fiber was then manually injected into the sample inlet of a Hewlett Packard HP6890 capillary gas chromatograph with a DB-624 column (J&W Scientific, 30 m × 3320 mm and a film 1.8 mm thick). The FID detector and injector were maintained at 250°C. The column was held at 55°C initially for 3 min and increased to a final temperature of 85°C at a rate of 7°C/ min with helium as the carrier gas. ETBE and TBA as low as 1 mg/L was found to be the minimum concentration that can be detected using this method.

To quantify the BTX compounds, the samples were prepared by liquid-liquid extraction using dichloromethane as a solvent. About 2 µL of the extracted sample was then injected into the inlet of a Hewlett Packard HP6890 capillary gas chromatograph with FID detector at 250°C with a pressure of 16 psi. Compounds were separated on an Agilent 190915-433 HP5 5% phenyl methyl siloxane capillary column (30.0 m x 250 µm x 0.25 µm) with helium as a carrier gas. The injector was held at 250˚C with a split ratio of 100:1 and a split flow of 14.9 mL/min. The oven temperature was initially held at 45˚C for 1 min and then increased to a final temperature of 65°C at a rate of 5°C/ min for 7 min.

2.3.8. Denaturing gradient gel electrophoresis identification

About 20 mL of bacterial culture was collected during degradation at the same time points at which ETBE was quantified. The samples were centrifuged at 4075 g for 15 min and washed three times with 0.9% NaCl. The pellet was used to extract DNA. The total genomic DNA was isolated using the Ultraclean Microbial DNA kit (MO BIO laboratories Inc., Carlsbad, CA) as per the manufacturer's instructions. The 16S rDNA of about 588 bp in length in the V3-V5 region was amplified using the primer pairs: namely, 16F341-GC (5'-CGCCCGCCGCGCCCCGCGCCCGGCCGCCGCCCCCGCCCGCCTACGGGAGGCAGC

AG-3') and 16R907 (5'-CCGTCAATTCCTTTRAGTTT-3') (Yu and Morrison, 2004). The PCR reaction was carried out with GoTaq Flexi PCR reagents (Promega Biosciences, Madrid, Spain) in a total volume of 50 µL containing 50 ng of isolated genomic DNA, 1 µM of primers, 1.5 mM of $MgCl_2$, 200 µM of dNTP, 1X GoTaq buffer and 2.5 units of Taq polymerase. The PCR conditions were: initial denaturation at 95°C – 5 min followed by 20 cycles of denaturing at 95°C - 1 min, annealing at 55°C - 1 min, extension at 72°C - 1.5 min and a final extension step at 72°C - 10 min. Approximately 100 ng of amplified PCR products was analyzed by running the samples in a 6% polyacrylamide gel with denaturing gradients ranging from 30%-70%. The denaturing gradient gel was prepared by mixing two solutions: solution A containing 100% denaturant (7 M urea and 40% formamide) and solution B without the denaturant. The gel was run in a denaturing gradient gel electrophoresis (DGGE) system (DGGE-2001, CBS Scientific Company, Inc.) with a constant voltage of 100 V for 18 h. Gels were stained with GelRed nucleic acid stain (Biotium, Hayward, USA) according to the manufacturer's instructions and visualized in Bio-rad gel imaging system.

Each DGGE band was excised with a sterile razor blade and resuspended in 20 µL of nuclease free Millipore water. The DNA was extracted from the polyacrylamide gel using the QIAEX II Gel Extraction kit (Qiagen Iberia, Madrid, Spain), according to the manufacturer's protocol. About 5 µl of extracted DNA was used to re-amplify the fragments using the same primer pairs with the forward primer 16F341 and without the GC clamp (5'-CCTACGGGAGGCAGCAG - 3'). The amplified products were purified and sequenced. The phylogenetic analysis was done by generating a phylogenetic tree using the MEGA 4.0 software using the Neighbor Joining method with bootstrap replicates of 1000.

2.4. Results and Discussion

2.4.1. Growth and Identification of ETBE utilizing colonies from the bacterial consortium enrichment culture A

The growth of enrichment culture is slow but reached to 0.5 OD in 7 days which then dropped in later days.

The growth of initial inoculated subculture (ECA1) was higher with an O.D. of 0.5 in 6 days. However, the subculture ECA6, which was used for all degradation experiments,

showed a maximum growth of only 0.3 O.D. in 6 days. This may be due to the presence or absence of other compounds in the initial gasoline-contaminated water sample which was used for inoculation. However, the subsequent cultures contained only ETBE as a sole carbon source.

Table 2.1. Identification of bacterial strains from enrichment culture 1 (EAC1) by 16S rDNA identification

Colony Name	Strain (Genbank)[a]	Accession number[b]	Strain (RDP)[c]	Accession number[b]	Class	Characteristics
a2 II	*Microbacterium.* sp	JQ897420.1	*Microbacterium* sp	S000009268	Actinobacteria	aerobic, gram-positive, rod-shaped
a5	*Microbacterium* sp	JQ897420.1	*Microbacterium* sp	S000108546	Actinobacteria	aerobic, gram-positive, rod-shaped
a7	*Microbacterium* sp	JQ897420.1	*Microbacterium* sp	S000009268	Actinobacteria	aerobic, gram-positive, rod-shaped
a9	*Xanthomonas* sp	GQ250436.1	*Stenotrophomonas*	S000357128	Gamma proteobacteria	aerobic, gram-negative, rod-shaped
a10	*Microbacterium* sp	JQ897420.1	*Microbacterium* sp	S000009268	Actinobacteria	aerobic, gram-positive, rod-shaped
a12	*Microbacterium* sp	JF262932.1	*Microbacterium* sp	-	Actinobacteria	aerobic, gram-positive, rod-shaped

[a]*As obtained from the sequence match from Genbank Blast*

[b]*Nearest hit found in the respective databases*

[c]*As obtained from the sequence match from the RDP 10 database*

Thirteen colonies appeared in washed agar plates amended with ETBE as sole carbon source in the ECA1 subculture.

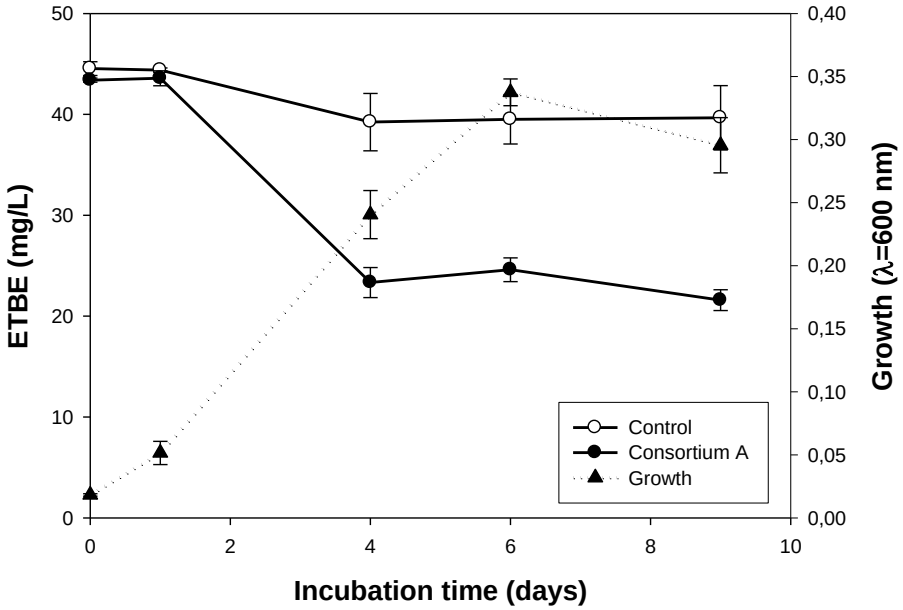
The colonies showed three distinctly different morphologies. Colonies appeared on washed agar plates within 2–3 days with white to cream-coloured, flat colonies of 1–2 mm approximately in diameter. Among them five morphologically different colonies namely a2II, a5, a7, a9, and a 12 were identified through 16SrDNA identification (Table 2.1). Four of the identified colonies are found to belong to *Microbacterium* sp. through Genbank RDP databse

analysis. All the four identified *Microbacterium* 16S rDNA sequences share more than 94% identity when analyzed by ClustalW2. One colony was identified as *Xanthomonas* sp. as analyzed by BLAST genbank and as *Stenotrophomonas* sp. through Ribosomal Database Project (RDP). This colony appeared as yellow colonies in the washed agar plates.

2.4.2. Degradation of ETBE

A significant degradation of about 47% occurred on the fourth day with consortium A (ECA6) and the control experiment showed relatively few losses of ETBE compared to the samples with bacteria in the biodegradation assay over the course of 9 days (Figure 2.1).

Figure 2.1. ETBE degradation by bacterial consortium A



ETBE was used at an initial concentration of 50 mg/L. Error bars indicate standard deviation from independent experiments (n=3).

The degradation of ETBE was about 51% with a measured ETBE concentration of 21.5 mg/L on day 9. However, the degradation of ETBE was monitored beyond 9 days and it was found to be constant. Hence, the degradation experiments were observed only for 9 days. Furthermore, the degradation was highest at the end of the exponential growth phase as observed from the increase in optical density of the culture medium (Figure 2.1).

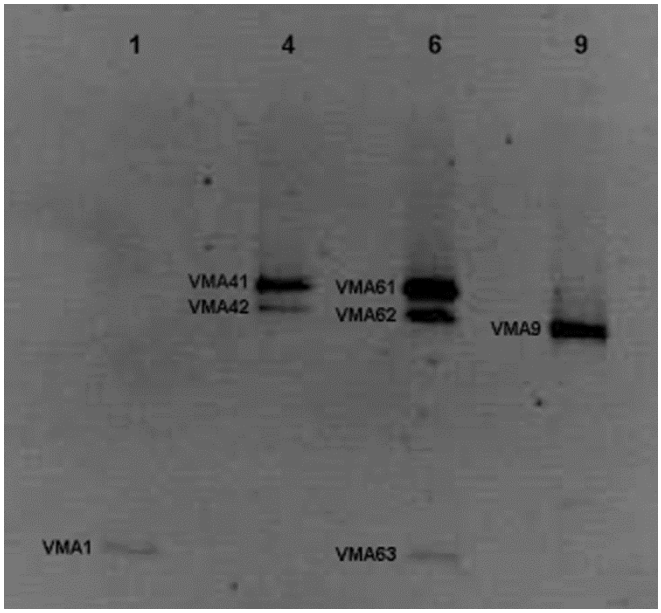
ETBE was degraded by consortium A during growth and the O.D. increased by up to 0.3, which indicated that consortium A utilizes ETBE as a carbon source for its survival.

About fifty percent of ETBE was removed in about 9 days. However, *tert*-butyl alcohol (TBA), the primary metabolite of ETBE biodegradation could not be identified in consortium A. There may be two reasons for this: either TBA was present in trace amounts, which could not be identified by our methods, or TBA was also metabolized by the consortium leading to complete mineralization of the compound.

2.4.3. Population dynamics during ETBE degradation

About seven bands appeared in the DGGE gel during the nine day degradation study (Figure 2.2). The bands were named A1-1, A4-1, A4-2, A6-1, A6-2, A6-3, and A9-1. On the first day of inoculation only one band (A1-1) appeared, and it was identified as *Xanthomonas* sp. by 16S rDNA identification. On the fourth day, two bands A4-1, A4-2 showed different migration patterns in DGGE gel. A4-1 was identified as *Methylovorous menthalis* by Genbank and as *Methylobacillus* sp. by RDP (Table 2.2).

Figure 2.2. DGGE identification of ETBE degrading strains.



Lanes marked 1, 4, 6 and 9 indicate the day on which the degradation was monitored. Each DGGE band was given a specific name. For example VMA41, where A indicates the consortium used, followed by a number indicating the day on which degradation was observed, and then by another number indicating the number of bands that appeared in the same lane in the DGGE gel.

The band A4-2 was confirmed to be *Methylophilus* sp. by both databases. The bands A4-1 and A4-2 migrated at the same position in the gel as A6-1 and A6-2, respectively, confirming that the corresponding bacteria were maintained from day 4 to day 6. The band A6-3 was also identified as *Xanthomonas* sp. but interestingly, only the band A9-1 was found on the 9th day when half of the ETBE concentration had been removed. The corresponding organism was identified as *Methylophilus* sp. The 16S rDNA nucleotide sequences were submitted to Genbank and their accession numbers are indicated in Figure 2.3. The sequences of V3 region of 16S rRNA gene for VMA42 and VMA62 are 100% similar from blast results and the similarity is 99% for the pairs of VMA42, VMA9 and VMA62, VMA9.

Table 2.2. Identification of ETBE degrading strains by DGGE

DGGE DNA Band	NCBI			RDP		
	Organism Identified[a]	**Accession[b] Number**	**Percent of Similarity**	**Organism identified[c]**	**Accession[b] Number**	**Percent of Similarity**
A1-1	Uncultured *Xanthomonas* sp.	JF736649.1	99	*Xanthomonas oryzae*	S000497875	99
A4-1	*Methylovorus menthalis* strain MM	HQ380796.1	99	*Methylobacillus* sp.	S001046723	99
A4-2	*Methylophilus* sp. u33	EU375653.1	99	*Methylophilus* sp.	S000358576	99
A6-1	*Methylovorus menthalis* strain MM	HQ380796.1	99	*Methylobacillus* sp.	S001046723	99
A6-2	*Methylophilus* sp. u33	EU375653.1	99	*Methylophilus* sp.	S000358576	99
A6-3	*Xanthomonas* sp. clone CFC54	JF736649.1	99	*Xanthomonas oryzae*	S000497875	99
A9-1	*Methylophilus* sp. u33	EU375653.1	99	*Methylophilus*	S000358576	99

[a]*As obtained from the sequence match from Genbank Blast*

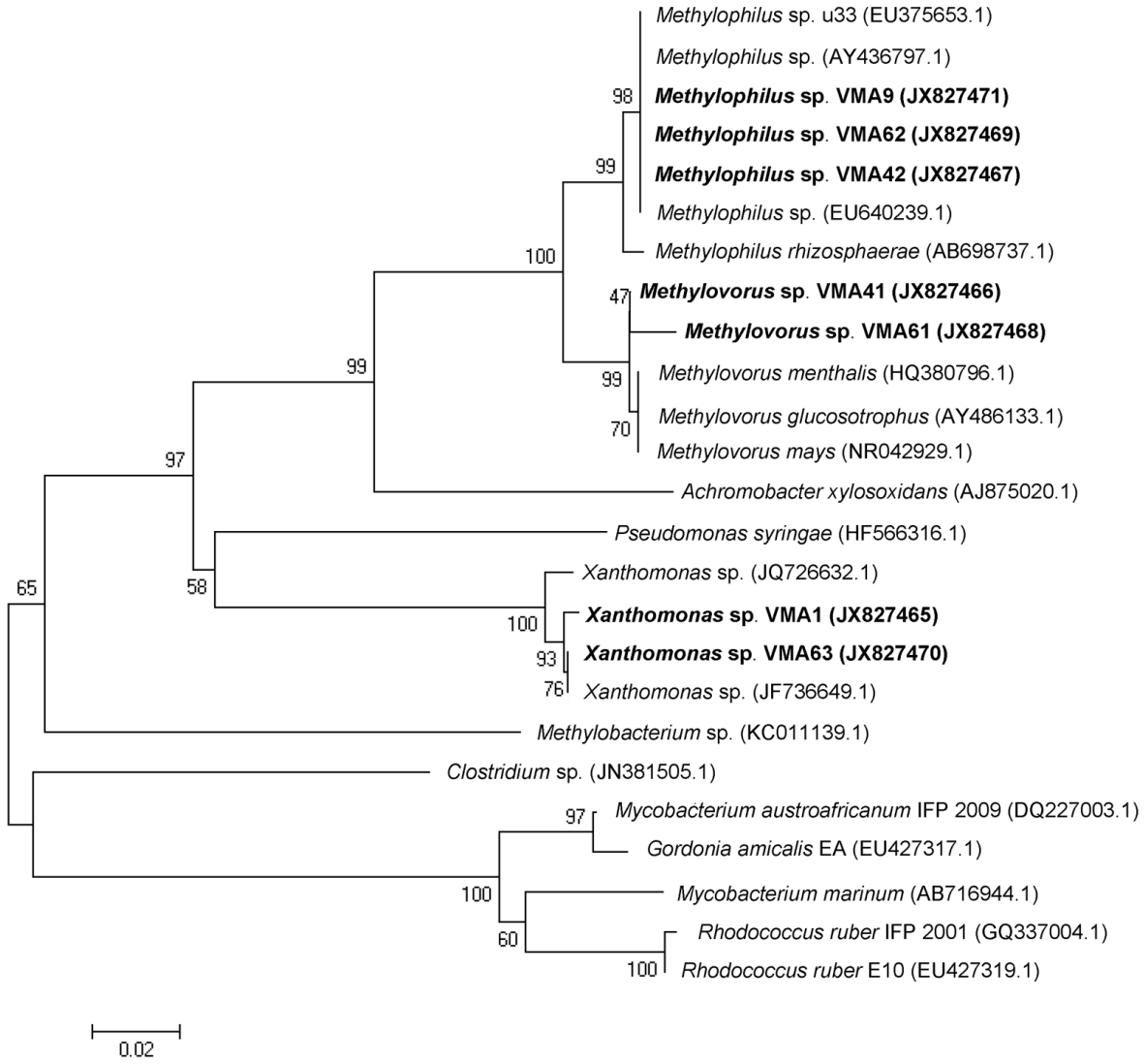
[b]*Nearest hit found in the respective databases*

[c]*As obtained from the sequence match from the RDP 10 database*

The phylogenetic tree constructed with reference strains and with ETBE/MTBE degrading strains derived from Genbank (Figure 2.3) indicated that the strains VMA42,

VMA62, VMA9 could be similar strains appearing in the same node. Similarly the strains VMA41, VMA61 (95% similarity) and VMA1, VMA63 (99% similarity) aggregated in the same clade.

Figure 2.3. Phylogenetic tree of strains identified by DGGE.

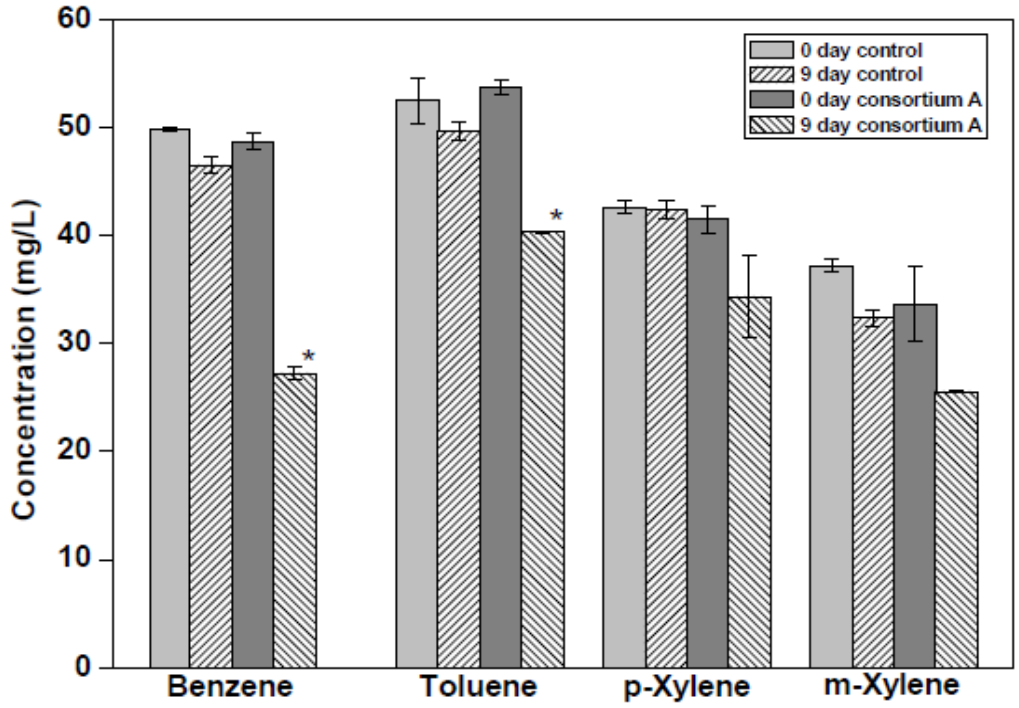


Tree was constructed by maximum likelihood with MEGA 4.0 with bootstrap replicates of 1000. Taxon samplings include representative sequence of strains from Eubacteria and some ETBE/MTBE degrading strains. The accession number for each strain is given in brackets after the strain name. Branch lengths are shown to scale, indicating relatedness.

2.4.4. Degradation of BTX

Each of the compounds benzene, toluene, and xylene was found to be individually degraded by ETBE utilizing bacterial consortium A (Figure 2.4). Benzene was degraded the most with 37.7% removal (statistically significant; P value of 0.026) followed by toluene with 18.5% significant removal (P value of 0.028) which measured 27 mg/L and 40.3 mg/L respectively on day 9. The xylene isomers: *p*-xylene was degraded by 17.6% (P value of 0.158) and *m*-xylene by 16.9% (P value of 0.192) with corresponding measured concentrations of about 34.3 mg/L and 25.5 mg/L remaining on day 9.

Figure 2.4. Degradation of benzene, toluene and xylene by consortium A.

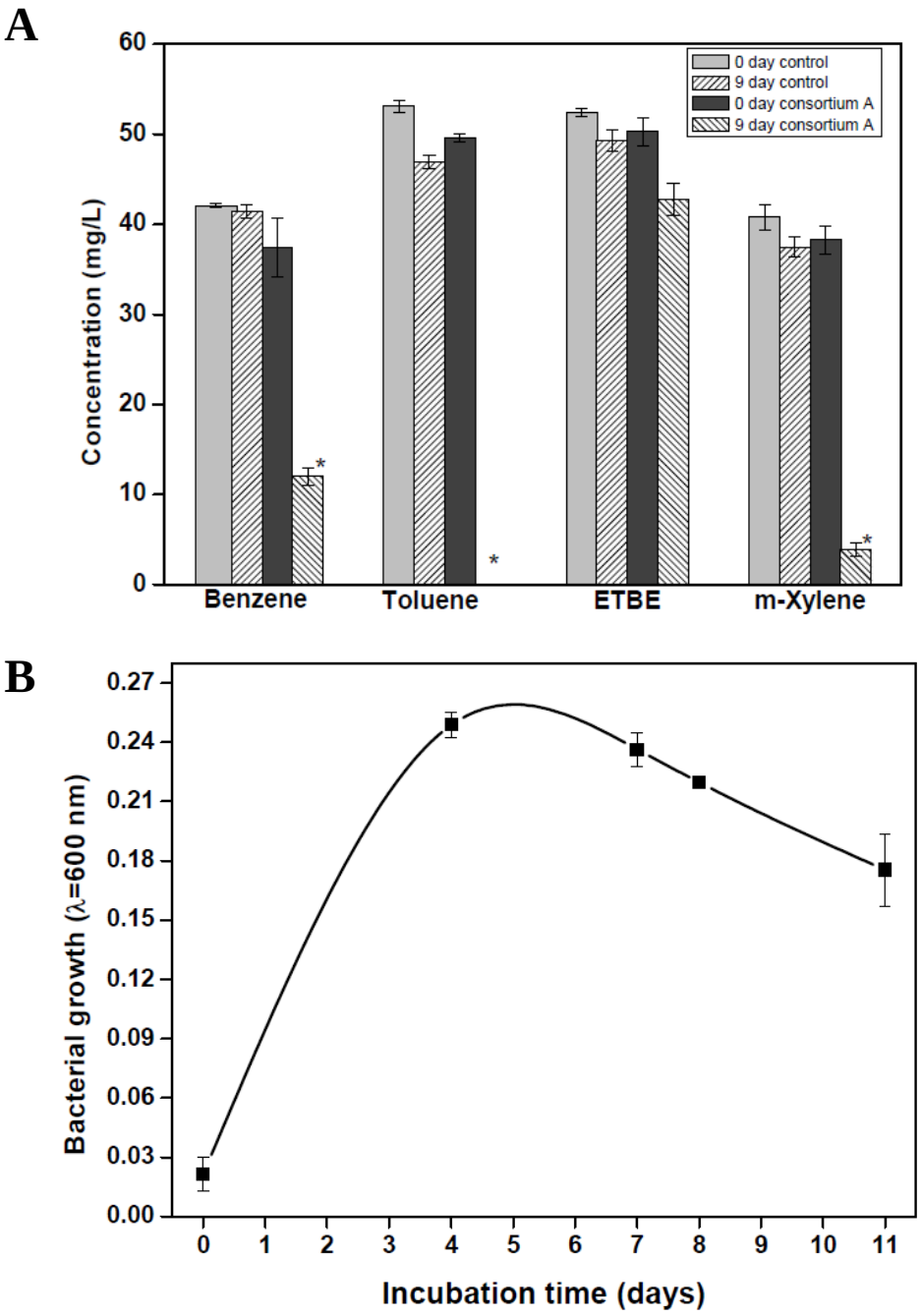


Benzene, toluene, p- xylene, and m-xylene were used at an initial concentration of 50 mg/L. Error bars indicate standard deviation from independent experiments (n=3). The asterisks indicate that the decrease is statistically significant with a significance level of 0.05.

2.4.5. Degradation of ETBE in the presence of BTX

Consortium A was tested for its ability to utilize ETBE in the presence of BTX (Figure 2.5A).

Figure 2.5. Degradation of ETBE in the presence of BTX



(A) Co-metabolic degradation of benzene, toluene, xylene and ETBE by consortium A. Each compound was used at an initial concentration of 50 mg/L.

(B) Growth of consortium A in the presence of benzene, toluene, and xylene with ETBE. Error bars indicate standard deviation from independent experiments (n=3). The asterisks indicate that the decrease is statistically significant at a significant level of 0.05.

The degradation of toluene was higher with 100% significant removal on day 9 with no detectable toluene measured (P value of 0.004). Xylene and benzene were recorded for 82% and 71% significant removal respectively with measured concentrations of about 3.8 mg/L and 12 mg/L (P values are 0.010 for xylene and 0.041 for benzene). On the other hand, consortium A degraded ETBE by only 14% in 9 days with remaining 42 mg/L of ETBE (P value of 0.190). The growth of consortium A in presence of BTX and ETBE was higher than when BTX compounds were present alone (Figure 2.5B). An important observation of this study is that when, ETBE was the sole carbon source, it was degraded by about 51% in 9 days but in combination with BTX compounds, it was degraded by only 14% in 9 days even when inoculated with equal amounts of initial population. On the other hand, toluene degraded by only 18.5% in 9 days when present alone but when ETBE was present as an additional substrate, it degraded completely. A similar increase in the degradation was found with m-xylene (from 16.9% to 82%) and benzene (from 37% to 71%). Degradation patterns show that consortium A follows an order of preference with BTX and ETBE together. The order of preference is as follows: toluene > xylene > benzene > ETBE.

There are not many reports on the role of BTX compounds in ETBE biodegradation. However studies with MTBE could be useful to understand and compare the role of BTX compounds in ETBE degradation. MTBE has been reported to have similar inhibitory behavior with different microbial populations during the co-metabolic degradation of MTBE and BTEX compounds (Deeb et al., 2001; Pruden and Suiden, 2004; Chi-Wen et al., 2007). In the first study, (Deeb et al., 2001) in which *M. petrophilum* PM1 strain was used, MTBE degradation was inhibited due to the preferential utilization of BTEX compounds, which is similar to the findings of our study. This study found that ethyl benzene and xylene isomers, in particular, largely inhibited MTBE degradation by PM1. The authors hypothesized that this was due to the allosteric or non-competitive inhibition of MTBE degrading enzymes, or the fact that the BTEX degradation intermediates were toxic to MTBE metabolizing cells. However, in the case of *Methylibium* strain UC1, MTBE was not inhibited when BTEX was added to the medium (Pruden and Suiden, 2004). These results conflict with the results found in the other study (Deeb et al., 2001) which suggests that there may be other factors inhibiting the degradation of MTBE. Pruden and Suidan (Pruden and Suiden, 2004) suggest that the concentration of BTEX in the medium cannot inhibit MTBE biodegradation and also concluded that BTEX and MTBE can be biodegraded simultaneously. Recently, MTBE biodegradation by *Pseudomonas aeruginosa* was shown to be inhibited with the co-presence

of BTEX (Chi-Wen et al., 2007). The results of the study showed that only after benzene and toluene had been almost entirely degraded, did the level of MTBE degradation increase to the same as when it was the sole carbon and energy source. It should also be noted that in our study, consortium A did not grow when each of the BTX compounds were present alone, except in the case of benzene. But the combined presence of benzene, toluene, xylene, and ETBE led to better growth and degradation. Considering the decrease in ETBE degradation in the presence of BTX compounds, it can be hypothesized that the metabolites from BTX degradation would be toxic for the ETBE degrading population. But it is evident that ETBE is necessary for the consortium to grow. It also suggests that consortium A prefers compounds with aromatic rings with alkyl groups rather than an ether bonded compound. It may also be assumed that enzymes that mediate the degradation pathway of aromatic ring structure are easily inducible or are more available than an ether degrading group of enzymes. In the end, it seems that the biodegradation of chemical compounds by the bacterial consortium is largely dependent upon their chemical structures and the availability of enzymes that can convert them.

Our results propose that the presence of BTX inhibits the degradation of ETBE and this would be the first time that the role of BTX compounds in the biodegradation of ETBE is reported. Further insights are needed to analyse the enzymes that mediate the degradation pathway.

2.5. Conclusion

In this study, the ETBE degrading consortium was enriched using water sample collected from gasoline-contaminated sites. Consortium A degraded about 51% of 50 mg/L of ETBE in 9 days. Information obtained from DGGE analysis during ETBE degradation helped to determine which individual bacterial population was the most important in the ETBE biodegradation process. It can also be concluded that BTX degradation can be positively supported by the presence of ETBE. In other words, the presence of BTX compounds slowed down the degradation of ETBE. To our knowledge, this is the first time that ETBE biodegradation was studied in presence of BTX compounds. This finding is important since these contaminants are found together in contaminated environments which will affect the degradation of individual compounds.

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Chapter 3

Biodegradation of ethyl *tert*-butyl ether by the bacterial consortium B in a minimal medium

3.1. Abstract

A microbial consortium that can degrade ethyl *tert*-butyl ether (ETBE) was isolated from gasoline contaminated water. This consortium named as consortium B (EB) was able to grow using ETBE as sole carbon and energy source. The consortium initially consisted of *Arthrobacter, Herbaspirillum, Pseudoacidovorax, Pseudomonas, and Xanthomonas* and was able to degrade 50% of the added 50 mg/L of ETBE in 9 days. But, *Methylophilus* sp. was identified during the course of degradation with a subculture EB3 by denaturing gradient gel electrophoresis (DGGE) analysis where methanol was used together with ETBE. After prolonged subcultures, the consortium's ETBE degrading efficiency was increased to 95 - 98 % with higher concentrations of ETBE (without methanol) of up to 1000 mg/L without any inhibition. The bacterial consortium B degraded ETBE with the measurable accumulation of tert-butyl alcohol (TBA), the prominent intermediate of ETBE degradation. Further, the consortium neither grows on TBA, methyl *tert*-butyl ether (MTBE) and benzene, toluene, xylene comunds (BTX) as sole carbon source nor degrades them at least at the concentration tested here. Finally, *Xanthomonas* and *Pseudomonas* were identified in the washed agar plates by 16S rDNA identification in the last subculture used for degradation. Further, studies on ETBE degradation should be done with the identified individual strains either as pure strains or in syntrophic associations to be able to use in bioremediation. This study presents a bacterial consortium that can be useful for bioremediation of sites heavily contaminated with ETBE.

3.2. Introduction

Fuel oxygenates are oxygen-rich compounds that are added to reformulated gasoline (RFG) to enhance the octane number, and also to increase the combustibility of the gasoline so as to reduce toxic emissions. Introduction of ether fuel oxygenates such as methoxy-2-methylpropane, or methyl *tert*-butyl ether (MTBE) and 2-ethoxy-2-methylpropane, or ethyl *tert*-butyl ether (ETBE) in the gasoline, replaced the toxic lead thereby reducing the emissions of carbon monoxide, unburned hydrocarbons, polycyclic aromatics, oxides of nitrates and particulate carbon (Barcelo, 2007). These ethers are also a better alternative as octane boosters than other aromatics such as benzene which helps in reducing the vapour pressure of the fuel while refuelling. Addition of fuel oxygenates was reinforced after 1990 Clean Air Act (CAA) which accounted for the addition of MTBE to the gasoline. Following that, MTBE was found to be used in more than 80% of the RFG during 1998 making it the second most produced chemical in US (USEPA, 1998). MTBE is more soluble and has less partitioning coefficient than other hydrocarbons which makes it to move with the same velocity as the ground water in the subsurface thereby contaminating the groundwater (Michael, 1999). The use of MTBE was also phased out in US following the increased contamination of the same in drinking water which led to switch to ethanol in US. In Europe and Japan, ETBE is preferred in RFG and is used about 13-17% in gasoline as it was produced from bio-ethanol. The potential for groundwater contamination by MTBE and ETBE with increased usage and at higher concentrations is more when compared to other components of gasoline such as benzene, toluene, ethyl benzene, and total xylenes. This is because MTBE and ETBE have greater solubility in water than other compounds and are less likely to adhere to soil particles (Deeb, 2001; Auffret, 2009). ETBE is comparatively less water soluble than MTBE but has less oxygenate capacity which require more addition of about 13-17% than MTBE (11-15%) (Barcelo et al., 2007). However, with ETBE the risk for ground water contamination from leaking underground storage tanks could not be expected to be substantially smaller. The data on ETBE distribution and leaking of underground fuel storage tanks are not available whereas, in the case of MTBE have been well documented and there have been numerous reports on the issue of water contamination (USEPA, 1998). Only one study on ETBE distribution has been documented due to a leaking storage tank at a gas station in France in which the underground water was contaminated with 300 mg/L of ETBE (Fayolle-Guichard et al., 2012). MTBE exposure is associated with the induction of rare and

uncommon tumours (Kathleen et al., 2012). Since these two compounds are similar in structure, it is expected that there could be similarity between toxicity effects caused by both of them. Therefore, it is necessary to establish effective methods for removal of ETBE, before it can cause serious contamination problem in the environment.

Microbial bioremediation is considered as the most significant and influential for degradation or detoxification of xenobiotic compounds. Only few reports have described ETBE degradation by pure bacterial cultures such as *Rhodococcus ruber* IFP2001 (Hernandez-Perez et al. 2001), *Rhodococcus wratislaviensis* IFP2016 (Auffret et al., 2009), *Variovorax paradoxus* CL-8 (Zaitsev et al., 2007), and *Aquincola tertiaricarbonis* L108 (Lechner et al. 2007).

This study aims to enrich and select a bacterial consortium that can degrade ETBE during its growth. The community of the selected ETBE degrading consortium was identified and analyzed through 16S rDNA identification and denaturing gradient gel electrophoresis (DGGE). Finally the ETBE degrading consortium was analyzed for its capacity to degrade other compounds such as *tert*-butyl alcohol (TBA), MTBE and benzene, toluene, xylene compounds (BTX).

3.3. Materials and Methods

3.3.1. Growth medium and enrichment of bacterial consortium

Minimal medium (MM) containing KH_2PO_4 - 0.225 g/L, K_2HPO_4 - 0.225 g/L, $(NH_4)_2SO_4$ – 0.225 g/L, $MgSO_4{\cdot}7H_2O$ – 0.050 g/L, $CaCl_2{\cdot}2H_2O$ - 0.005 g/L, $FeSO_4{\cdot}7H_2O$ - 0.005 g/L along with trace elements was used for culturing bacteria. The trace elements containing ($ZnCl_2$ - 0.1 g/L, $MnCl_2{\cdot}4H_2O$ - 0.03 g/L, H_3BO_3 - 0.3 g/L, $CoCl_2{\cdot}6H_20$ - 0.2 g/L, $CuCl_2{\cdot}2H_2O$ - 0.01 g/L, $NiCl_2{\cdot}6H_2O$ - 0.02 g/L, $NaMoO_4{\cdot}2H_2O$ - 0.03 g/L [9]) were prepared in 1 L and 1 mL was added to every 1000 mL MM. The medium was adjusted to pH 7 with 1N NaOH. The chemical compounds that were used for this study were sterilized using 0.2µm sterile filter before they were added to the medium.

Ground water sample was collected from a gasoline-contaminated water body located in the northeastern part of Spain. The sample was inoculated to a ratio of 1:5 in 130 ml of MM along with 50 mg/L of ETBE (as a stock solution prepared in methanol) as the sole carbon

source in a 250 ml conical flask and incubated at 30°C at 180 rpm (termed as consortium B Enrichment culture 1 [EB1]). The increase in biomass was monitored by measuring the absorbance at λ=600 nm using a Varian Cary 100 spectrophotometer. After 7 days of incubation (when the OD was 0.59) about 30ml was transferred to 130 ml of fresh MM along with 50 mg/L of ETBE for the second enrichment culture (EB2). Further subcultures were made and incubated in the same conditions and named with successive numbers such as EB2, EB3, etc. About eleven subcultures were successively made to obtain a stable enrichment culture. After three subcultures ETBE was given as sole carbon source without the dilution in methanol which is illustrated in Figure 3.1.

3.3.2. Isolation and identification of cultivable bacterial strains

The initial subculture EB2 was serially diluted and plated onto washed agar plates amended with 50 mg/L of ETBE. The populations of bacteria were isolated and identified using 16S rDNA identification. Each colony picked from the plate was transferred to another agar plate and used for further steps. Washed agar was prepared by washing about 20 g of agar with 1000 mL MM and allowed to stand for 20 min. The clarified supernatant was discarded followed by addition of new MM. The wash step was repeated for three times and finally resuspended in 800 mL of MM. The final yield of agar would be approximately 1.5% (Okeke, 2003). The agar solution was autoclaved and cooled to 50°C after which 50 mg/L of ETBE was added. The plates were incubated at 30°C until visible colonies appeared on the plates. Similarly, the enriched subculture EB11 which was used for biodegradation assay was also plated on washed agar plates. The bacterial colonies were isolated and identified as before.

3.3.3. 16S rDNA identification

The colonies were inoculated in LB broth and grown overnight. Genomic DNA was isolated using GenElute^{TM} bacterial genomic DNA kit (Sigma, Madrid, Spain). Partial 16S rRNA gene sequences about 1000 bp of bacterial strains were amplified by PCR using the universal primers 616V (5'-AGAGTTTGATYMTGG CTCAG-3') and 699R (5'-RGGGTTGCGCTCGTT-3') (Barbera et al., 2011). The PCR conditions were as follows: initial denaturation at 95°C for 2 min; 25 cycles of denaturing at 95°C for 30 s, annealing at

55°C for 25 s; extension at 72°C for 1 min; and a final extension at 72°C for 5 min with a PCR mixture consisting of 1X GoTaq Flexibuffer (Promega), 3 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each primer, 100 ng of genomic DNA, and 1 unit of GoTaq (Promega) in a total volume of 50 µL. The PCR products were sequenced and the 16S rRNA gene sequences were analyzed using BLAST and ribosomal database to identify the microorganisms.

3.3.4. Denaturing gradient gel electrophoresis identification

About 20 mL of bacterial culture was collected during the degradation studies with consortium subcultures EB3 at the time points in which ETBE was quantified. The samples were centrifuged at 4075 g for 15 min and washed three times with MM. The pellet was used to extract DNA. The total genomic DNA was isolated using the Ultraclean Microbial DNA kit (MO BIO laboratories Inc., Carlsbad, CA) as per the manufacturer's instructions. The 16S rDNA of about 588 bp in length in the V3-V5 region was amplified using the primer pairs: namely, 16F341-GC (5'-CGCCCGCCGCGCCCCGCGCCCGGCCGCCGCCCCCGCC CGCCTACGGGAGGCAGCAG-3') and 16R907 (5'-CCGTCAATTCC TTTRAGTTT-3') [Yu and Morrison, 2004]. The PCR reaction was carried out with GoTaq Flexi PCR reagents (Promega Biosciences, Madrid, Spain) in a total volume of 50 µL containing 50 ng of isolated genomic DNA, 1 µM of primers, 1.5 mM of $MgCl_2$, 200 µM of dNTP, 1X GoTaq buffer and 2.5 units of Taq polymerase. The PCR conditions were: initial denaturation at 95°C – 5 min followed by 20 cycles of denaturing at 95°C - 1 min, annealing at 55°C - 1 min, extension at 72°C - 1.5 min and a final extension step at 72°C - 10 min. Approximately 100 ng of amplified PCR product was analyzed by running the samples in a 6% polyacrylamide gel with denaturing gradients ranging from 30% - 70%. The denaturing gradient gel was prepared by mixing two solutions: solution A containing 100 % denaturant (7 M urea and 40% formamide) and solution B without the denaturant. The gel was run in a DGGE system (DGGE-2001, CBS Scientific Company, Inc.) with a constant voltage of 100 V for 18 h. Gels were stained with GelRed nucleic acid stain (Biotium, Hayward, USA) according to the manufacturer's instructions and visualized in Bio-rad gel imaging system. Each DGGE band was excised with a sterile razor blade and resuspended in 20 µL of nuclease free Millipore water. The DNA was extracted from the polyacrylamide gel using the QIAEX II Gel Extraction kit (Qiagen Iberia, Madrid, Spain), according to the manufacturer's protocol. About 5 µL of extracted DNA was used to re-amplify the fragments using the same primer

pairs with the forward primer 16F341 but without the GC clamp (5'-CCTACGGGAGGCAGCAG - 3'). The amplified products were purified and sequenced in a ABI PRISM® 3100-*Avant*™ Genetic Analyzer (Life technologies, Madrid, Spain).

3.3.5. Biodegradation experiments

3.3.5.1.Degradation of ETBE

Consortium B (EB3) was used to study the degradation of ETBE. About 30 mL of EB3 (in a ratio 1:5) was centrifuged and the resulting pellet was washed twice with MM and resuspended in 1 mL of the same. This was inoculated in 150 mL of MM in a 250 mL screw-cap bottle with 50 mg/L of ETBE and incubated in a rotary shaker at 30°C at 180 rpm. The increase in biomass was monitored by measuring the optical density (O.D.) at λ=600 nm. About 10 mL of sample was collected from each flask at different time points in headspace vials sealed with crimp caps which were used to estimate the amount of ETBE. All experiments were set up in duplicate. Abiotic controls were also included that consisted of bacteria-free media, amended with ETBE and maintained under the same conditions as live bacterial cultures.

The bacterial consortium B was tested for its ability to degrade different and higher concentrations of ETBE. ETBE at different concentrations of 50, 100, 500, 1000, 10000 mg/L was inoculated with the consortium that has undergone eleven transfers (EB11) inoculated in a ratio 1:5. The flasks were inoculated in duplicates including the abiotic controls, for each ETBE concentration and incubated in the same conditions as described above. The ETBE concentration was monitored on the day where, significant increase in growth was observed (i.e. only on 6 and 16 days).

3.3.5.2.MTBE and TBA

Consortium B (EB11) was used to study the degradation of MTBE. About 30 mL of EB3 (in a ratio of 1:5) was centrifuged and the resulting pellet was washed twice with MM and resuspended in 1 mL of the same. This was inoculated in 150 mL of MM in a 250 mL screw-cap bottle with 50 mg/L of MTBE and incubated in a rotary shaker at 30°C at 180 rpm. The increase in biomass was monitored by measuring the optical density (O.D.) at λ=600 nm.

Similarly, control abiotic samples were prepared in the same conditions without the inoculation of the bacteria. The biodegradation assay was done using the same procedure as described above with the inoculation of either TBA or BTEX. For the quantification of MTBE about 10 mL of sample was collected from each flask at different time points in headspace vials sealed with crimp caps. Similarly, about 1 mL of sample from TBA and BTX amended flasks was taken to quantify the concentration.

3.3.6. Analytical methods

The amount of ETBE and MTBE was quantified by preparing the samples using solid phase micro extraction (SPME). The headspace vials were heated in a water bath for 30 min at 75°C for ETBE and then the gaseous phase was absorbed on an SPME fiber by incubating the fiber into the headspace vials for 30 min at the same temperature. The fiber was then manually injected into the sample inlet of a Hewlett Packard HP6890 capillary gas chromatograph with a DB-624 column (J&W Scientific, 30 m × 3320 mm and a 1.8 mm thick film). The FID detector and injector were maintained at 250°C. The column was held at 55°C initially for 3 min and increased to a final temperature of 85°C at a rate of 7°C/ min with helium as the carrier gas.

The accumulation of metabolites (TBA, and HIBA) was identified and confirmed by GC-MS by direct aqueous injection (DAI) with the following conditions. Once confirmed, they were quantified by the GC-FID. To quantify the TBA and BTX compounds, the samples were prepared by liquid-liquid extraction using dichloromethane as solvent. About 1 µL of the extracted sample was then injected into the inlet of a Hewlett Packard HP6890 capillary gas chromatograph with FID detector at 250°C with a pressure of 16 psi. BTX compounds were separated on an Agilent 190915-433 HP5 5% phenyl methyl siloxane capillary column (30.0 m x 250 µm x 0.25 µm) and TBA on a DB-624 column (J&W Scientific, 30 m × 3320 mm and a film 1.8 mm thick) with helium as carrier gas. The injector was held at 250˚C with a split ratio of 30:1 and a split flow of 14.9 mL/min. The oven temperature was initially held at 45˚C for 1 min and then increased to a final temperature of 65°C at a rate of 5°C/ min for 7 min for BTX. For TBA, the oven temperature was initially held at 45˚C for 1 min and then increased to a final temperature of 65°C at a rate of 5°C/ min for 7 min. The retention time of the compounds used for this study was shown in the Table 3.1.

Table 3.1. Methods used and retention time of different compounds used for degradation in this study

Compounds	Retention time (minutes)	Method used	Column used
ETBE	3.9	GC-FID with SPME	DB 624 (J&W Scientific)
MTBE	4.5	GC-FID with SPME	
TBA	2.9	GC-FID with SPME	
Benzene	2.4	GC-FID (liquid extraction)	HP5 (Agilent)
Toluene	3.6	GC-FID (liquid extraction)	
m-xylene	5.6	GC-FID (liquid extraction)	
p-xylene	6.2		

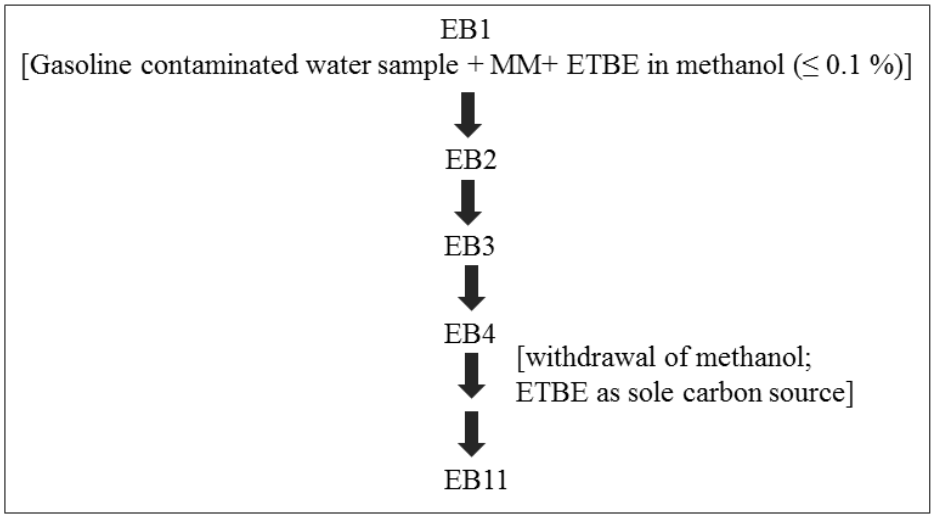
3.4. Results and Discussion

Enrichment cultures were obtained by seeding the samples collected from the groundwater contaminated with ETBE at an initial concentration of 720 µg/L. The other contaminants found together with ETBE in the contaminated samples were MTBE, benzene, toluene, xylene and other hydrocarbons etc. The initial enrichment culture EB1 obtained by inoculating the initial gasoline contaminated water grew readily with ETBE (along with methanol) as sole carbon sources without any additives and reached maximum growth on day 7 (OD_{600} = 0.58). However, methanol was withdrawn during EB4 and the bacterial consortium was forced to use ETBE as sole carbon source in the subsequent subcultures. The bacterial growth decreased to about 0.3 OD on day 7. This may be due to absence of other chemicals (found in the initial gasoline contaminated water sample) which may stimulate the growth of bacteria or due to the absence of methanol. However, the bacterial consortium continued to grow indicating the use of ETBE as sole carbon and energy source.

Many strains were reported to degrade ETBE and MTBE aerobically and anaerobically. But only few bacterial strains have been reported to utilize ETBE as sole carbon source and degrade it completely. The difficulty in enriching an axenic culture to degrade MTBE or TBA may be due to longer incubation time leading to low biomass yield (Barcelo et al., 2007). *Rhodococcus ruber* IFP 2001 (Hernandez-Perez et al. 2001) was studied extensively for its biodegradation capacity and at the molecular level. Another strain *Aquincola*

tertiaricarbonis L108 (Lechner et al. 2007) degraded ETBE completely. More recently a bacterial consortium enriched and isolated with ETBE consisting of *Rhodococcus sp.* IFP2042 and *Bradyrhizobium sp.* IFP 2049 was able to degrade ETBE through a syntrophic association (Le Digabel et al., 2013).

Figure 3.1. Schematic representation of enrichment of bacterial consortium B



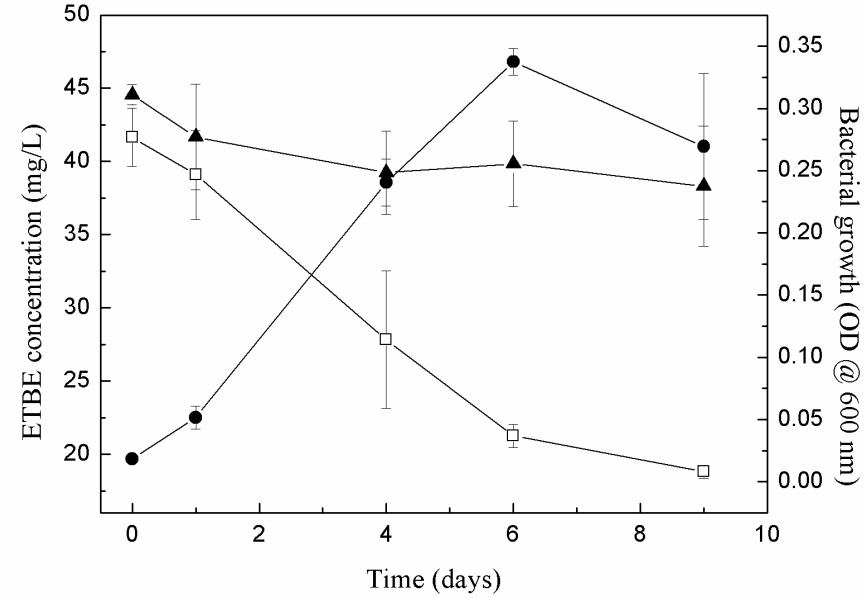
3.4.1. Biodegradation

ETBE degrading capacity of the enriched bacterial consortium was tested at two different stages of enrichment phase (EB3 and EB11) to monitor for any changes in the degradation of ETBE over the subcultures.

The bacterial consortium was later tested for its ability to degrade different and higher concentrations of ETBE. For this, the subculture EB11 was used, as it was assumed to contain the best selected bacterial population to degrade ETBE. The degradation assay was monitored on day 6 where 50% of ETBE was achieved from previous experiment and on 16 day where maximum growth was found. The bacterial consortium EB11 was able to degrade 500 mg/L and 1000 mg/L of ETBE by about 98% in 16 days. The ETBE concentration of 10000 mg/L was found to be inhibitory which impeded the growth of the consortium as measured on 16^{th} day. But, a minimal biodegradation in the ETBE concentration was observed on day 6 and day 16 which was difficult to be calculated by our methods as the fiber got saturated at this concentration. The ETBE degradation efficiency of consortium B is higher than the reported consortium Pz1-ETBE and MC-IFP (composed of *R. wratislaviensis*

IFP 2016, *R. aetherivorans* IFP 2017 and *A. tertiaricarbonis* IFP 2003) whose degradation rates were 0.91 mg/L/h and 0.83 mg/L/h (Fayolle-Guichard et. al., 2012).

Figure 3.2.Degradation of ETBE by bacterial consortium B

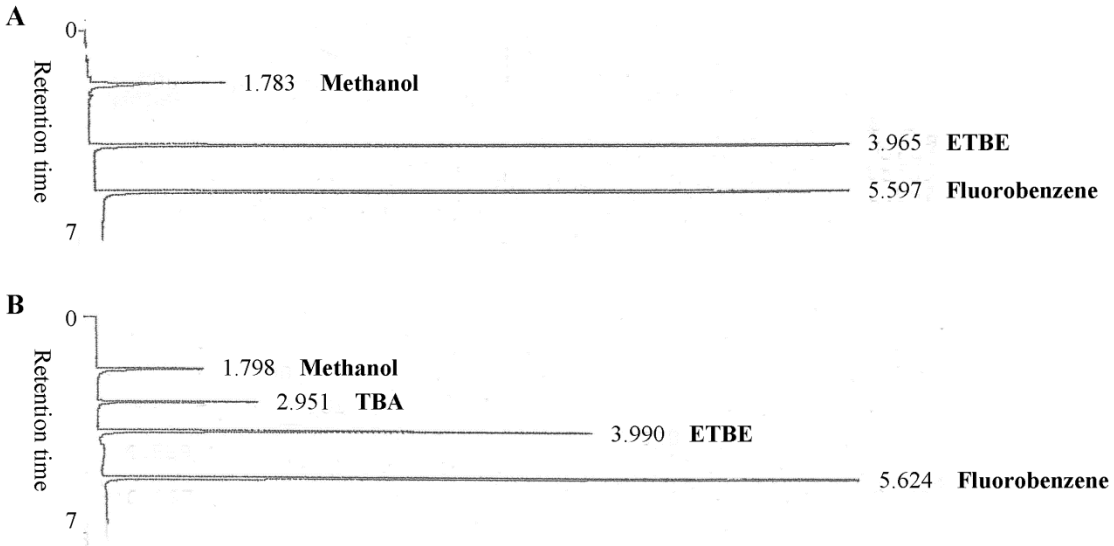


▲ *Control;* □ *B consortium;* ● *Bacterial growth (OD @ 600 nm). The error bars represent the standard deviation between the replicates*

The consortium showed significant growth in samples inoculated with 1000 mg/L with the maximum compared to other concentration tested (OD_{600} of 0.35) on day 16. It should be noted that EB11 was able to grow with ETBE as sole carbon and energy source with no additional substrates added. But in EB3 the same level of growth was reached within 6 days. So it is clear that with subsequent subcultures and with increasing concentration, the lag phase was longer despite using the same concentration of cells for inoculation. The bacterial consortium can also degrade only 36% on day 6 with the 50 mg/L of ETBE which was reduced compared to the previous experiment where 50% removal is recorded. The reason could be the long lag phase observed as previously. This also implies that substances or chemicals that support the bacterial growth which were present more in the beginning were lost in the subsequent subcultures to aid in better growth and degradation. The presence of methanol co-metabolically helped in the growth of the bacterial consortium. On the other hand, the experiments with higher concentrations led to better growth indicating that the bacteria prefers ETBE in higher concentration for its growth. In all the above experiments, the initial bacterial load was kept minimal and constant in order to monitor the ETBE

utilization of the bacterial consortium. Nevertheless, a bacterial consortium capable of degrading higher concentrations of ETBE was enriched which can be of industrially importance in bioremediation of the polluted sites.

Figure 3.3. GC-FID chromatogram of the compounds



Panel A shows the chromatogram obtained in GC-FID using SPME in the control sample. Panel B shows the chromatogram obtained in GC-FID using SPME in the samples with consortium B. Both the figures show definite peaks of retention time of respective compounds. It should be noted that TBA was not detected with the control samples where the bacterial cells were not present. Methanol is a solvent and flurobenzene is an internal standard used for each run.

Bacterial identification

Simultaneously, the bacterial populations (subculture EB1) were also able to grow on washed agar plates amended with ETBE. Fourteen colonies appeared on the plate after 4 days of incubation. Out of fourteen, seven morphologically distinct bacterial colonies (b1, b2, b4, b7, b10, b11, b14) were isolated and identified through 16S rDNA identification (Table 3.2a). All the bacteria identified were aerobic, rod shaped, belonging to β-proteobacteria, γ-proteobacteria and actinobacteria class of bacteria. The identified strains were *Xanthomonas* sp., *Herbaspirillum* sp., *Pseudoacidovorax* sp. *Arthrobacter* sp., and *Pseudomonas* sp. Among them, the genus, *Pseudomonas*, is very well known in degrading many and versatile xenobiotic compounds and also in harbouring the genes involved in the degrading pathways of the same. Several members of this genus have been reportedly involved in the degradation

Table 2a Identification of bacterial strains from initial enriched bacterial consortium B by 16S rDNA

Colony name	Strain (Genbank)[a]	Accession number[b]	Strain (RDP)[c]	Accession number[b]	Class	Characteristics
b1	*Uncultured Xanthomonas* sp	HF678374.1	*Xanthomonas* sp	S000821757	γ-proteo bacteria	aerobic, gram-negative, rod-shaped
b2	*Herbaspirillum* sp. MMD15	JN546222.1	*Herbaspirillum rubrisubalbicans*	S000012852	β-proteo bacteria	aerobic, gram-positive, curved rod-shaped
b4	*Pseudoacidovorax* sp. ptl-2	FJ581042.3	*Pseudacidovorax intermedius*	S000843442	β-proteo bacteria	aerobic, gram-negative, rod-shaped
b7	*Arthrobacter* sp. 2073	KC236846.1	*Arthrobacter nicotinovorans*	S000021578	Actino bacteria	aerobic, gram-positive, rod/cocci shaped
b10	*Pseudoacidovorax intermedius*	NR_044241.1	*Pseudacidovorax intermedius*	S000843442	β-proteo bacteria	aerobic, gram-negative, rod-shaped
b11	*Pseudoacidovorax intermedius*	NR_044241.1	*Pseudacidovorax intermedius*	S000843442	β-proteo bacteria	aerobic, gram-negative, rod-shaped
b14	*Pseudomonas* sp	HE603491.1	*Pseudomonas veronii*	S000004663	γ-proteo bacteria	aerobic, gram negative, rod-shaped

[a]As obtained from the sequence match from Genbank Blast

[b]Nearest hit found in the respective databases

[c]As obtained from the sequence match from the RDP 10 database

of hydrocarbons (Beal et al., 2000; Pornsunthorntawee et al., 2008), aromatic compounds (You et al., 2013), pyrenes (Ma et al. 2013), petroleum tar (Tanti et al. 2013). *Xanthomonas*, a genus from the γ-proteobacteria is an aerobic heterotrophic species, known previously to be

Table 2a Identification of bacterial strains during ETBE degradation by DGGE

Band name	Strain (Genbank)[a]	Accession number[b]	Strain (RDP)[c]	Accession number[b]	Class	Characteristics
VMB3	*Methylophilus* sp. u33	EU375653.1	*Methylophilus methylotrophus*	S000439756	β-proteo bacteria	aerobic, gram negative, rod/curved-shaped
VMB6	*Methylophilus* sp. u33	EU375653.1	*Methylophilus methylotrophus*	S000439756	β-proteo bacteria	aerobic, gram negative, rod/curved-shaped
VMB9	*Methylophilus* sp. u33	EU375653.1	*Methylophilus methylotrophus*	S000439756	β-proteo bacteria	aerobic, gram negative, rod/curved-shaped

[a]As obtained from the sequence match from Genbank Blast

[b]Nearest hit found in the respective databases

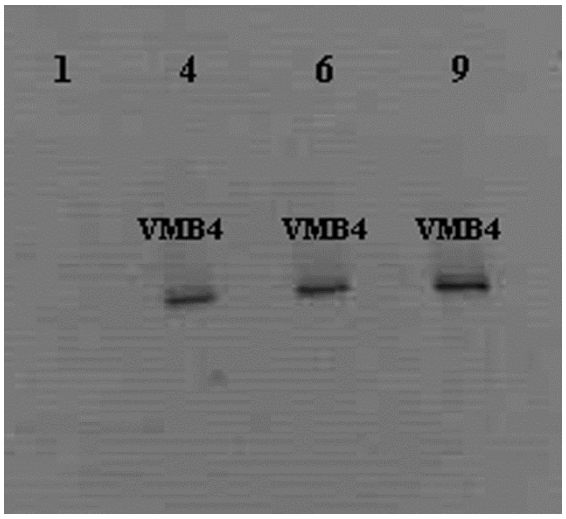
[c]As obtained from the sequence match from the RDP 10 database

able to degrade petroleum hydrocarbons (Li et al., 2000), toluene and xylene isomers (Su and Kafkewitz, 1996), hexachlorocyclohexane (Manickam et al., 2007), and herbicides (Herrera-González et al., 2013). *Arthrobacter* had known before to degrade MTBE (Liu et al., 2001) and other xenobiotics. Previously, *Herbaspirillum* sp. was reported in plants to fix the nitrogen in the soil. But recently, this strain has been identified for its capacity in biodegradation. A new strain of *Herbaspirillum* known as H. *chlorophenolicum* has been reported in chlorophenol degradation (Im et al., 2004). Lately, another species of *Herbaspirillum* similar to *H. chlorophenolicum* was isolated from activated sludge that could utilize fluoranthene as its sole carbon and energy source (Xu et al., 2011). Another recent study reported that a new strain of *Herbaspirillum* which was involved in anthracene degradation (Wang et al., 2012). The other bacterium which was identified is *Pseudoacidovorax* sp. A close relative of this genus, *Acidovorax* has been discussed for its capacity in biodegradation of xenobiotic compounds such as phenantherene, MTBE and BTEX (Singleton et al., 2005). All the bacteria isolated and identified in our study were

previously reported to degrade many xenobiotic compounds which show that the initial population was highly competitive to be able to grow and degrade the xenobiotic compounds.

DGGE was done during degradation so as to get an insight into the strains which participate in the process of biodegradation (with EB3). The results of DGGE (Figure 3.4, Table 3.1b) showed that only one band (VMB3, VMB6, VMB9) appeared in all the degradation time points which upon sequencing had 99% similarity to *Methylophilus* sp. u33 in BLAST analysis. Hence, it can be concluded that *Methylophilus* sp. identified during ETBE degradation in EBE3 participates in ETBE biodegradation. Apart from this, the results with DGGE during the subculture EB3 did not reveal the populations which were identified initially through plating and 16S rDNA identification. This implies that the presence of methanol prior to EB3 cultures stimulated the population that can grow with methanol and also degrade ETBE. *Methylophilus* strain in the initial population could not be identified. This may due to bias in plating and 16S rDNA identification. The subculture EB11 which was used for biodegradation assay was also plated on washed agar plates and bacterial colonies were isolated. Only two, single, pure, morphologically distinct colonies were found on the washed gar plate amended with ETBE in EB11. The colonies were identified using 16S rDNA identification. The 16S rRNA gene sequence was analyzed using BLAST (NCBI) and the sequences matched to *Xanthomanas* and *Pseudomonas* sp.

Figure 3.4. Denaturing gradient gel electrophoresis of ETBE degrading consortium B (EB3).



Lanes marked 1, 4, 6 and 9 indicate the day on which the degradation was monitored. Each DGGE band was given a specific name. For example, in VMB3, B indicates the consortium used, followed by a number indicating the day on which degradation was observed.

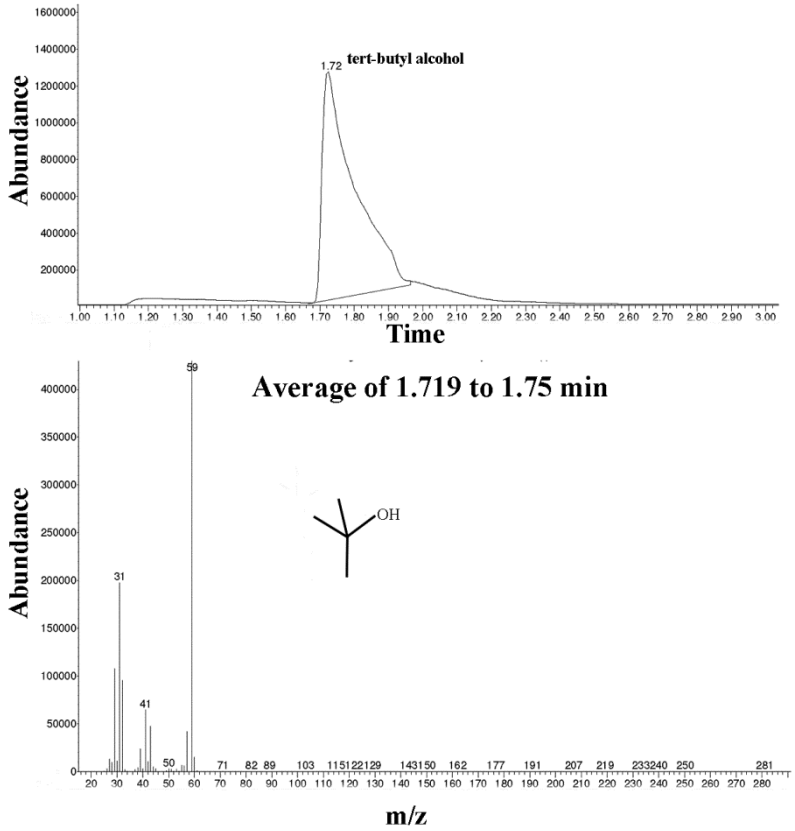
It should be noted that not all bacterial population in the liquid culture can be grown on solid media (plates). But there could be also uncultivable and unidentifiable bacterial population which can be missed with conventional methods such as plating, cloning and 16S rDNA identification causing bias in identifying the right candidate. It should be noted that *Methylophilus* was not found in EB11 due to withdrawal of methanol in the later subcultures. Hence, it can be concluded that *Methylophilus* sp. identified during ETBE degradation in EBE3 were not the true ETBE bio degraders. The absence of methanol did not support the methanol utilizing bacterial population and hence only *Pseudomonas* and *Xanthomonas* were identified in the final subculture used. But the two bacterial genera identified here can be the majority of the bacterial population degrading ETBE and also they have had a long history in bioremediation previously. A consortium consisting of *Pseudomonas* and *Rhodococcus koreensis* was able to grow on MTBE and TBA (Kern et al., 2002). But the capacity to grow on MTBE and TBA was lost when individual isolates were tested. The reason they postulated could be the loss of strains during selective plating that could degrade the chemicals and the other reason could be the presence of fast growing bacteria with non-selective media which could mask the slow growing bacteria in isolating the selective bacteria for degradation. So, analyzing the bacterial population at different and longer incubation periods can give a solution to loss of degrading strains (Lopez-Feirriera et al., 2006). Furthermore, the identified bacterial isolates should be tested individually or synergistically to degrade ETBE and also to elucidate their exact role in the degradation in order to obtain a defined bacterial population for use in bioremediation.

3.4.2. Metabolite identification

One of the primary products of ETBE metabolism is TBA (Barcelo et al, 2007). Hence quantification of TBA can throw light on the mineralization of ETBE (Figure 3.5). Therefore, the amount of TBA was quantified during 6 and 16 days in parallel to the ETBE biodegradation with EB11. The amount of TBA varied in different conditions and was found to be higher in samples with higher concentrations of ETBE. No TBA was detected in abiotic controls which eliminates the possibilities of other modes of ETBE degradation. TBA accumulation can be correlated to partial degradation of ETBE as TBA was getting accumulated in the medium in all the concentrations of ETBE used. But it is difficult to conclude that ETBE was partially biodegraded as there was decrease in TBA concentrations

from 6 to 16 days in samples with lesser amount of ETBE (50 mg/L and 100 mg/L)(Figure 3.6a and 3.6b). On the other hand, concentration of TBA was higher in 500 mg/L and 1000 mg/L of ETBE samples on day 16.

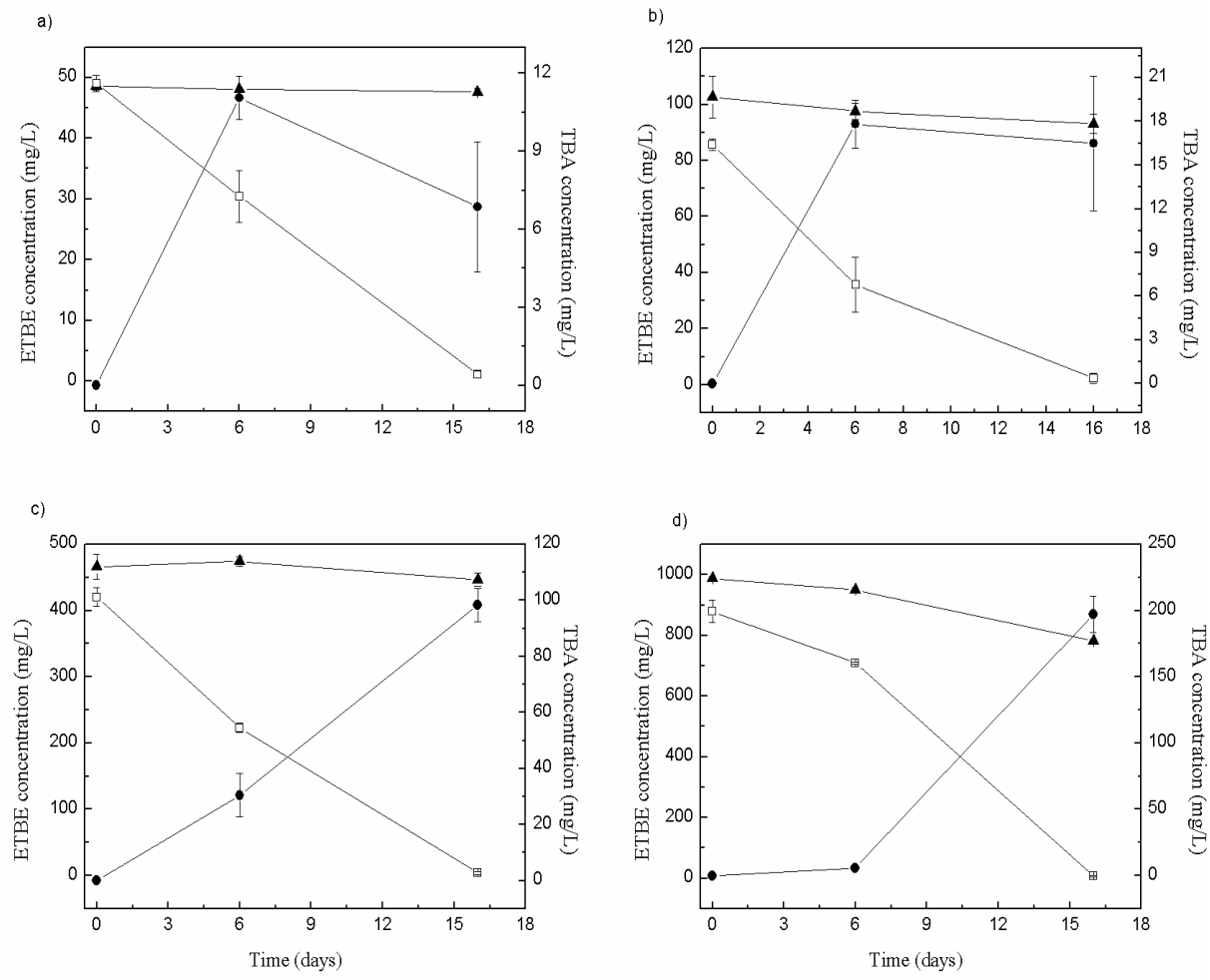
Figure 3.5. GC-MS spectra of the identified *tert*-butyl alcohol



The TBA accumulation was about 15- 20% of the initial concentration of the ETBE added on day 16. Therefore, it can be concluded that TBA was getting accumulated in the media during the time periods monitored here. Further the concentration of TBA should be quantified with longer time points so as to get a conclusive idea about complete mineralization.

Attempts were made to detect HIBA in the biodegradation samples, but HIBA could not be identified by our methods. Hence we could conclude that TBA produced and accumulated in the medium was merely due to bacterial activity which allows us to propose that ETBE degradation pathway follows the previously reported degradation pathway (Figure 3.7).

Figure 3.6. ETBE degradation and TBA formation in the degradation assay with different concentrations of ETBE by EB11



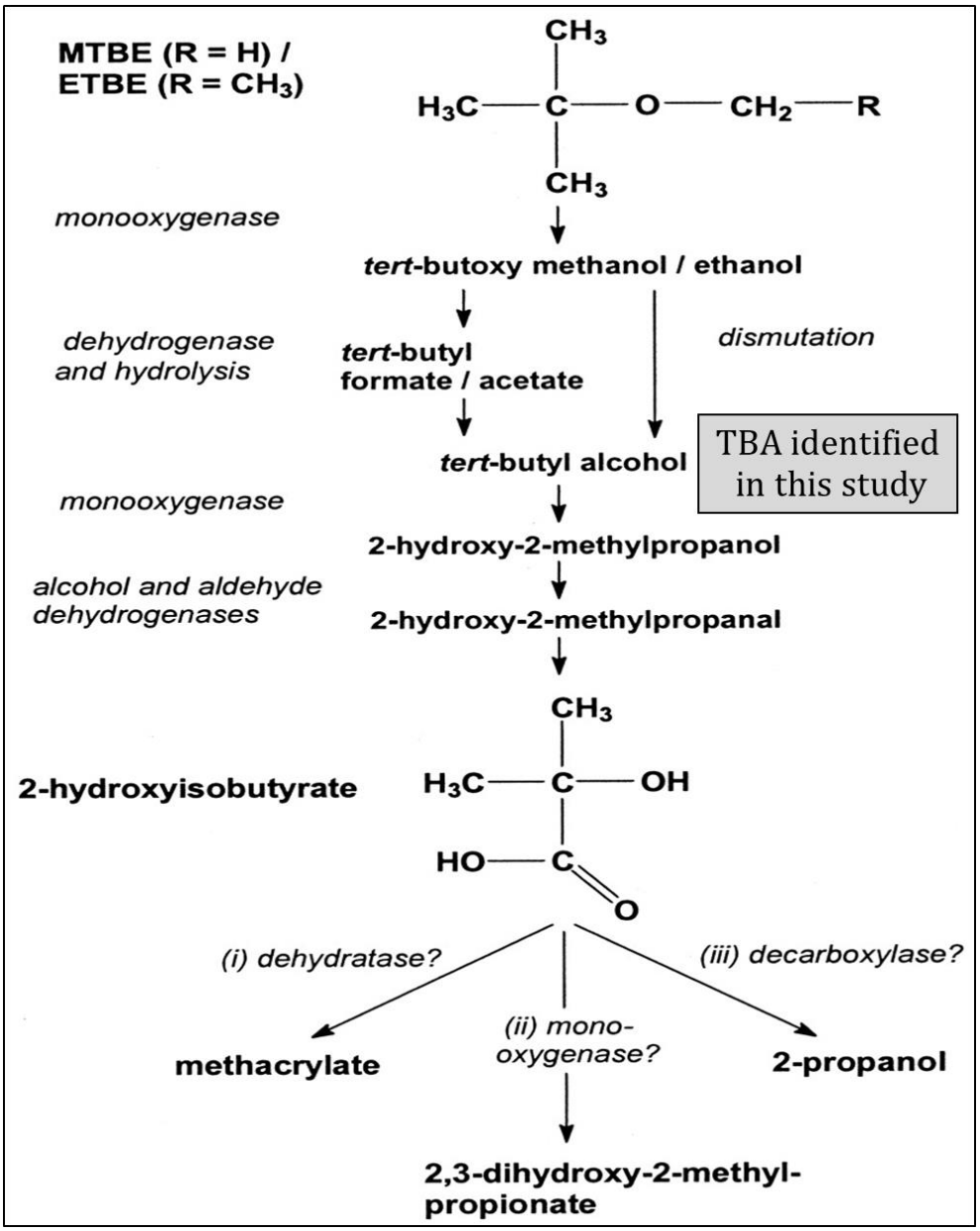
.▲ Control; □ B consortium; ●TBA accumulation. The error bars represent the standard deviation between the replicates

3.4.3. Degradation of MTBE, TBA and BTX

The ability of consortium B to degrade MTBE, TBA, and BTX during its growth was tested independently. It was found that both MTBE and TBA did not support the growth of the bacterial consortium but in the case of TBA, a minimal degradation (less than 10 % degradation) was observed on day 9 after which the concentration remained constant. The initial concentration of the compounds used here was kept at 50 mg/L. However, higher concentrations (100 mg/L and 500 mg/L) of TBA and MTBE were also tried but no

degradation was observed. This implies that these compounds can be toxic to the cells at the concentrations used here.

Figure 3.7. Proposed pathways for the aerobic degradation of the fuel oxygenates MTBE and ETBE



Adapted from Rohwerder et al. Appl. Environ. Microbiol. 2006

None of the BTX compounds supported either degradation or the growth of consortium B. The better susceptibility of bacteria to ETBE may be the result of steric reasons which have to be investigated at the level of the mechanism of initial enzyme attack. Similar behavior was seen with the biodegradation studied with *Rhodococcus ruber* IFP2001

where the strain was able to grow with ETBE as sole carbon source but not with MTBE which require other metabolites to support the growth (Hernandez-Perez et al., 2001). Therefore, it can be concluded that ETBE is necessary for the growth of the bacterial consortium B which might be responsible for inducing the genes which are involved in degradation of ETBE which should be tested in future experiments.

3.5. Conclusion

A bacterial consortium capable of degrading ETBE was enriched and isolated from gasoline contaminated water body. The isolated bacterial consortium was able to grow on ETBE as sole carbon and energy source. This consortium which consisted of *Pseudomonas* sp. and *Xanthomonas* sp. was found to adapt and utilize higher concentrations of ETBE. The present study, for the first time demonstrates the potential of an isolated bacterial consortium to degrade higher concentrations of ETBE which can be useful in bioremediation of sites heavily contaminated with ETBE.

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Chapter 4

Proteomic analysis of the Ethyl *tert*-butyl ether degrading bacterial consortium B

4.1. Abstract

The proteome of the ETBE degrading bacterial consortium B exposed to ETBE at two different concentrations of ETBE (500 mg/L and 1000 mg/L) was analyzed through two dimensional difference in gel electrophoresis (2D-DIGE) technique in an attempt to understand the responses of the complex bacterial consortium at the proteomic level. The proteome of the bacterial consortium B propagated in glucose was used a control to compare the responses against ETBE in the same. A total of 241 proteins were found to respond differently with respect to the bacterial consortium propagated in glucose and ETBE. Aldehyde dehydrogenase which plays an important role in the degradation pathway of ETBE was identified and found to be upregulated in presence of ETBE. Proteins related to amino acid and energy metabolism were found to be up-regulated. We also observed induction of a number of transport proteins, metabolism-related proteins and chaperons. Two different higher concentrations of ETBE were used in the proteomic analysis and it had reflected in the protein expression profiles of the consortium grown in 500 mg/L and 1000 mg/L of ETBE. The expression profiles of five of the identified protein spots differed significantly with an increasing pattern in higher concentration of ETBE used. Aldehyde dehydrogenase which plays a crucial role in ETBE degradation pathway was also found to increase with increase in ETBE concentration. This study gains more significance as it is the first proteomic analysis conducted on a bacterial consortium degrading ETBE.

4.2. Introduction

Ether oxygenates such as ethyl *tert*-butyl ether (ETBE) is added to gasoline to improve fuel combustion and decrease exhaust emissions. ETBE replaced the toxic lead compounds in fuels in Europe and MTBE in US. Soon, MTBE was also phased out in US following severe groundwater contamination (Squillace at al., 1996; Grady & Casey., 2001; Schmidt et al., 2004) switching the choice to ethanol (Rosell et al., 2007). But in Europe, the wide usage of ETBE has been attributed to its production from renewable bio-based ethanol (Inal et al., 2009; Van Wezel et al., 2009). Even though ETBE is of greater use in Europe and in Japan, the distribution of this compound is yet to be studied. ETBE, like MTBE is a water soluble compound and on continued usage or due to accidental spills can pose a severe threat as a groundwater contaminant similar to MTBE, in future. In 2011, the total global consumption of ETBE was about three million metric tons, with about two million metric tons consumed in Western Europe and most of the rest in Japan and Eastern Europe (Digabel et al., 2013). In addition ETBE possess very low threshold (1 µg/L for odour and 2 µg/L for taste) limits than MTBE (7 µg/L for odour and 15 µg/L for taste) for odour and taste according to the Dutch standards (Van Wezel et al., 2009). Adding to it, there are other risks associated with the use of ETBE-blended gasoline, such as the health risks to employees of plants, refineries and service stations who are exposed to ETBE, the impacts on groundwater quality and due to disagreeable smell or taste of water caused by ETBE leakage from underground tanks.

A bacterial consortium capable of degrading ETBE was enriched and isolated from a gasoline contaminated water sample (Chapter 3). This consortium can tolerate and degrade up to 1000 mg/L of ETBE at 30°C. The higher ETBE concentration can possibly trigger significant physiological and proteomic changes in the consortium B. The aim of this study is, therefore, to investigate these responses to ETBE and also its concentration variations in the bacterial consortium B through a proteomic analysis. Proteomics enables the analysis of the bacterial regulation of proteins induced by a particular compound thereby helping us to understand the biodegradation pathway at a molecular level. Difference in gel electrophoresis (DIGE) circumvents many of the issues associated with traditional 2D-PAGE, such as reproducibility and limited dynamic range, and allows for more accurate and sensitive quantitative proteomics studies (Lilley et al., 2004). Proteome analysis is conducted by means of a two-dimensional polyacrylarnide gel electrophoresis (2D-PAGE) with DIGE and matrix-

assisted laser desorption ionization – tandem time of flight mass spectrometry (MALDI-TOF/TOF-MS). The proteomic analysis was also conducted to obtain a deeper insight into the adaptive responses of the bacterial consortium to ETBE. Comparative analysis of the proteome profiles of the bacterial consortium propagated in two different concentration of ETBE (500 mg/L and 1000 mg/L) allowed us to understand the effect on concentration of ETBE.

4.3. Materials and methods

4.3.1. Bacterial consortium and culture conditions

An ETBE degrading bacterial consortium was enriched and isolated as discussed in the previous chapter (Chapter 3). Minimal medium (MM) containing KH_2PO_4 - 0.225 g/L, K_2HPO_4 - 0.225 g/L, $(NH_4)_2SO_4$ – 0.225 g/L, $MgSO_4{\cdot}7H_2O$ – 0.050 g/L, $CaCl_2{\cdot}2H_2O$ - 0.005 g/L, $FeSO_4{\cdot}7H_2O$ - 0.005 g/L along with trace elements was used for culturing bacteria. The trace elements containing ($ZnCl_2$ - 0.1 g/L, $MnCl_2{\cdot}4H_2O$ - 0.03 g/L, H_3BO_3 - 0.3 g/L, $CoCl_2{\cdot}6H_20$ - 0.2 g/L, $CuCl_2{\cdot}2H_2O$ - 0.01 g/L, $NiCl_2{\cdot}6H_2O$ - 0.02 g/L, $NaMoO_4{\cdot}2H_2O$ - 0.03 g/L [9]) were prepared in 1 L and 1 mL was added to every 1000 mL MM. The medium was adjusted to pH 7 with 1N NaOH. The chemical compounds that were used for this study were sterilized using 0.2µm sterile filter before they were added to the medium.

The bacterial consortium was inoculated in a ratio of 1:5 in 130 ml of MM in a 250 ml conical flask and incubated at 30°C at 180 rpm. The bacterial consortium B was grown in two different concentrations, 500 and 1000 mg/L of ETBE for 16 days and was used for analysing the protein profile. The bacterial consortium B propagated in 0.5 g/L of glucose for the same time point (16^{th} day) was included as a control. The growth of the consortium was monitored by measuring the optical density (OD) of cultures (λ = 600) at respective time points. A number of two independent replicates was maintained both for control and test samples.

4.3.2. Sample preparation for proteomic analysis

The bacterial cultures (with normalised OD) were centrifuged (9000 g; 20 min) and the pellets were resuspended and washed three times with phosphate buffered saline (PBS). The washed pellets were resuspended in 1 mL of re-suspension buffer (30 mM Tris, 5 mM EDTA, 5 mM $MgCl_2$, pH 9) along with 2mM of protease inhibitors (Pefabloc SC PLUS,

Roche, Germany). They were then subjected to sonication (five cycles of 30 s at 40% amplitude, 50 watts with 30 s intervals) while maintained on ice. The lysate was centrifuged at 20000 g for 15 min at 4^{o}C. For a DNase treatment, the resulting supernatant was then treated with 300U of Benzonase® Nuclease (Sigma) and incubated for 60 min at 4°C and centrifuged at 20000 g for 15 min. Then a protein precipitation was performed adding 100% ice cold TCA to obtain a final concentration of 10% and incubated on ice for 30 min. The samples were centrifuged at 4^{o}C for 15 min at 20000 g. The protein pellet was resuspended in 800 µL of chilled acetone and incubated overnight at -20^{o}C. The pellet was then repeatedly washed with chilled acetone twice and air dried completely. Finally, the pellet was dissolved in 1 mL of rehydration buffer (RH) [30 mM Tris buffer pH 8.5, 7 M urea, 2 M thiourea, 30 mM DTT and 4% 3-(3-cholamidopropyl) dimethylammoniol- 1-propanesulfonate (CHAPS)] at room temperature (30 min with periodical vortexing). The protein samples were then concentrated and cleaned for any inhibitors with 2-D Clean-Up kit (GE healthcare). The pH of the protein samples were checked and adjusted to an optimum pH of 8.8 and protein concentration was later assessed by RC-DC™ kit (Biorad) following the manufacturer's recommended protocols.

4.3.3. Differential in Gel Electrophoresis

Proteins were labelled using the CyDye DIGE Fluors minimal dyes for Ettan DIGE (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions for DIGE (Difference in Gel Electrophoresis) prior to isoelectric focussing (IEF). Briefly, 400 pmol of each dye (Cy3 or Cy5) was added to each sample containing 50 µg of protein and were incubated for 30 min on ice in dark. The labelling reaction was quenched by adding 1 µL of L-lysine (10 mM) and incubated on ice for 10 min. A sample pool (to serve as an internal standard) was prepared using an equal amount of protein from each sample in the study. The internal standard was labelled with Cy2 in the same fashion that the rest of the samples, and when is included in the gels, provides an internal reference to permit the normalization.

4.3.4. Isoelectric focusing (IEF)

From previous 2D gels (Table 4S.1) the proteome appeared to be very acidic, and in order to improve the resolution, different approaches were performed. Finally the best conditions were fixed which includes usage of IPG strips with pH range 4-7; increased

concentration of IPG buffer up to 3% instead the 0.5-2% as recommend by the manufacturer and a combined passive and active rehydration. Thoroughly, the samples labelled with the different dyes, as well as the Cy2-internal standard, were mixed in 1:1:1 ratio as shown in table 4.1 Finally, labelled and mixed protein samples were diluted to 450 µL with rehydration buffer along with 20 mM DTT and 3% IPG buffer pH 4–7 (GE Healthcare) and trace amount of bromophenol blue for colour.

Table 4.1 DIGE labelling for different samples

CyDye used	Sample	Gel number
Cy2	Internal standard BGI+BGII+BE500I+BE500II+BE1000I+BE1000II	1, 2 and 3
Cy3	BGI	1
Cy5	BGII	2
Cy3	BE500I	3
Cy5	BE500II	1
Cy3	BE1000I	2
Cy5	BE1000II	3

Samples were loaded on a 24 cm IPG strips (Immobiline DryStrip, GE Healthcare) in a linear range of pH 4–7 for better resolution. IEF was carried out at 25°C. Strips were first passively rehydrated at 0 V for 7 hours; followed by an active rehydration at 50 V for 14 h; which accumulated to 288 V/h. IEF was carried out using an IPGphor 3 instrument (GE Healthcare) by the following program: and initial step of 100 V for 5 h, followed by four step gradients of 500 V for 30 min, 500 V for 7 h, 1000 V until 800 V/h and 8000 V until 13500 V/h. At the end, a step of 8000 V until 45000 V/h was reached. A total of 64,076 Vh were accumulated at the end. The IEF was run covered from direct light.

Previously, about 6% of IPG buffer was used instead of the optimum 0.5-2% mistakenly. It was found that the protein was more acidic and accumulated in the pH range 4-7 in the preparative gel where IEF strips with pH range 3-10 was used. And the addition of IPG buffer at 6% improved the separation of proteins in the acidic part. Hence, it was then

optimized to use 3% IPG buffer in all the subsequent IEF experiments which is above the optimum IPG buffer concentration recommend by the manufacturer.

4.3.5. Second dimensional electrophoresis

Following IEF, IPG strips were equilibrated for 15 min in 5mL of reducing equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris HCl pH 8.8, 2% SDS, 10 mg/mL of DTT and traces of bromophenol blue) followed by 15 min incubation in 5mL of alkylating equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris HCl pH 8.8, 2% SDS, 25 mg/mL of iodoacetamide and traces of bromophenol blue). The second dimension SDS-PAGE was performed on an Ettan™ DALTsix Electrophoresis System (GE Healthcare) at 1W/gel at 25ºC, overninght until the bromophenol blue reached the bottom of the gel. The entire electrophoresis unit was covered from direct light during the run.

4.3.6. Image acquisition and image analysis

DIGE gels were immediately scanned after the SDS-PAGE at their respective excitation/emission wavelengths of 488/520 nm for Cy2, 532/580 nm for Cy3 and 633/670 nm for Cy5 using Molecular Imager PharosFX™ Plus (Biorad). Images were analyzed with Progenesis Same Spots (version 3.3, nonlinear Dynamics Ltd, Newcastle upon Tyne, UK). Spots were filtered with ANOVA ($p = \leq 0.05$) and a fold difference of 2 between the control and the conditions was chosen as criterion in the identification of differentially expressed protein candidates. Hierarchical clustering, and principle component analysis (PCA) was performed to assess global changes in responsive proteins in all conditions using the Progenesis Same Spots. PCA allows for grouping of protein samples with overall similar expression characteristics and for identifying proteins, which are responsible for the differences between groups.

4.3.7. Spot picking and In-gel digestion

For spot picking, one preparative gel with 200 µg of protein lysate each was run with same electrophoretic parameters for the first and second dimension. Preparative gel was stained overnight with Coomassie Brilliant blue. Selected spots were excised using EXQuest™ Spot cutter (Biorad, Spain) and digested with trypsin (sequencing grade;

Promega, Madison, WI) overnight at 37 °C according to the manufacturer's instruction. The resulting tryptic peptides were concentrated, purified and desalted using ZipTip® (Millipore, USA).

4.3.8. Mass spectrometry analysis

Tryptic digests of each spots (1 μL) were loaded to the AnchorChip target plate (Brucker-Daltonics) with α-cyano-4-hydroxycinnamic acid (CHCA) matrix. MS and MS/MS spectra were obtained by Ultraflex Xtreme MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics) in auto-mode using Flex Control v3.0 (Bruker-Daltonics) and processed by ProteinScape v3.0 (Bruker- Daltonics). Peptide spectra were acquired in reflectron mode with 1500 laser shots per spectrum. Spectra were externally calibrated using peptide calibration standard (Brucker) and for the MS/MS data 2000 laser shots were accumulated. The combined peptide mass fingerprint and MS/MS search was performed on NCBI and TrEMBL databases for bacteria using the MASCOT 2.0 software (http://www.matrixscience.com) integrated together with BioTools v3.1 and and ProteinScape softwares (Bruker-Daltonics). The parameters used for the search engine were: monoisotopic peptide mass accuracy of 50 ppm, fragment mass accuracy to ±0.5 Da; maximum of only one missed cleavage; carbamidomethylation of cysteine as fixed modification and partial oxidation of methionine as variable modification. There were no restrictions with respect to protein MW and pI. Filtering of peaks was done for known autocatalytic trypsin peaks and keratin peaks; the signal to noise threshold ratio was set to 1:6. The significance threshold was set at pb 0.05, and identification required that each protein contained at least one peptide or an e-value b 0.05.

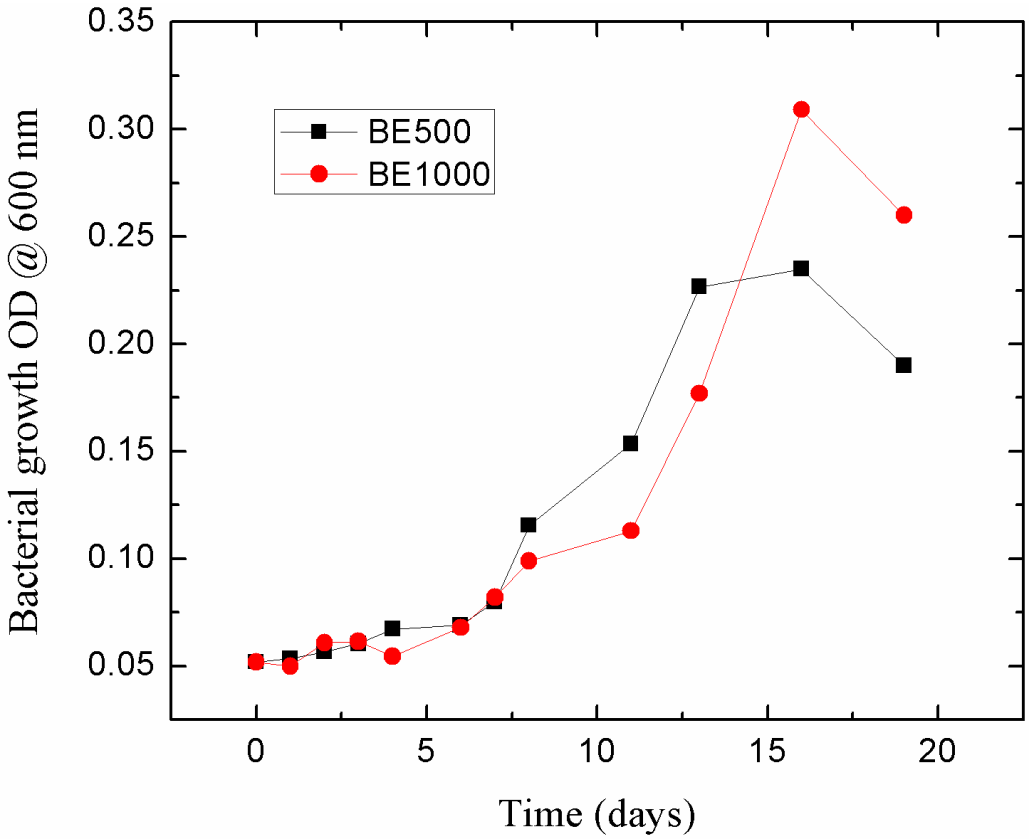
4.4. Results and Discussion

4.4.1. Bacterial growth

In order to gain an insight into the mechanism underlining the biodegradation of ETBE, and TBA accumulation on the general cellular response of the bacterial consortium B, a proteomic analysis was carried out to study the ETBE metabolism and its concomitant metabolic adaptations. The bacterial consortium B was tested on two different concentrations of ETBE (500 and 1000 mg/L) as sole energy and carbon source. Samples were collected for

carrying out the proteomics when significant growth (Figure 4.1) and degradation (Chapter 3, Figure 3.4) was observed on 16th day.

Figure 4.1. Growth of consortium B in two different concentration of ETBE

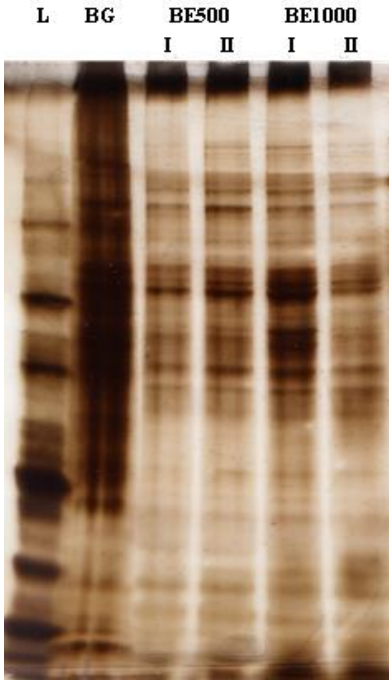


The growth of bacterial consortium B used for proteomic analysis was monitored for 19 days. The maximum growth was obtained during 15 – 16 days.

After 16 days, the consortium B was able to degrade ETBE as sole carbon source to about 98% (Chapter 3, Figure 3.4) approximately at a concentration 500 and 1000 mg/L of ETBE. The ETBE concentration of 1000 mg/L favored the growth of the bacterial consortium B more than the other concentration used (500 mg/L). But an extended lag phase during the growth of the bacterial consortium until 6 days before maximum ETBE degradation was observed. The reason for long lag phase may be due to minimal initial bacterial inoculation. But the previous subcultures as described in chapter 3 (Figure 3.4) were all propagated with same amount of initial bacterial load. It should be noted that the duration of lag phase increases with increase in subcultures. Previously a lag phase of 3-4 days was observed with

subcultures prior to EB11, but with subculture EB11 and the later subcultures had a lag phase of 6 days. The exact reason for this prolonged lag phase is unknown which needs further related investigations but we speculate that this may be due to accumulation of toxic metabolites in the medium. According to Jennings et al., (2009), the extended lag phase observed in bacteria propagated in solvent may be related to gene regulation or due to solvent toxicity when cells were switched from preferred substrates to solvents. Therefore, we expected that the different concentrations of ETBE would most likely affect the protein expression patterns.

Figure 4.2. 1D SDS-PAGE of the samples used for proteomics



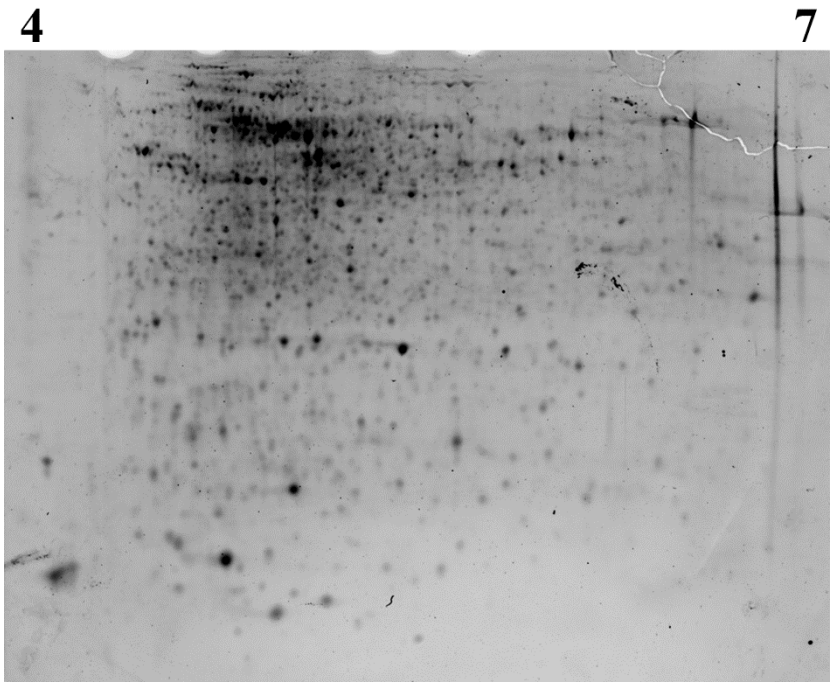
L represent molecular weight ladder used and BE500 corresponds to consortium B grown on ETBE with concentration 500 mg/L. Similarly, BE1000 corresponds to consortium B grown on ETBE with concentration 1000 mg/L

4.4.2. Protein extraction and 2D electrophoresis

The total soluble protein of the consortium B was extracted by mechanical lysis using sonicator and employing excess of protease inhibitors as the protein samples from consortium showed some degradations in the 1D SDS PAGE gel. The extracted proteins of all samples were checked in a 1D SDS-PAGE gel (Figure 4.2). As seen in the figure, the band patterns were similar in all the protein samples of consortium B propagated in ETBE compared to the banding patterns. The samples were then concentrated and quantified. The concentration of the protein samples is illustrated in the table 4S.1. Proteins from all the test samples were more acidic, accumulating the proteins in the pH range of 4 to 7, when analyzed in the 2D gel

with an IEF strip of pH 3-10 (Figure 4S.1). Hence, an IEF strip of pH 4-7 was used for focusing the proteins for more clear separation.

Figure 4.3. Preparative 2D-PAGE gel



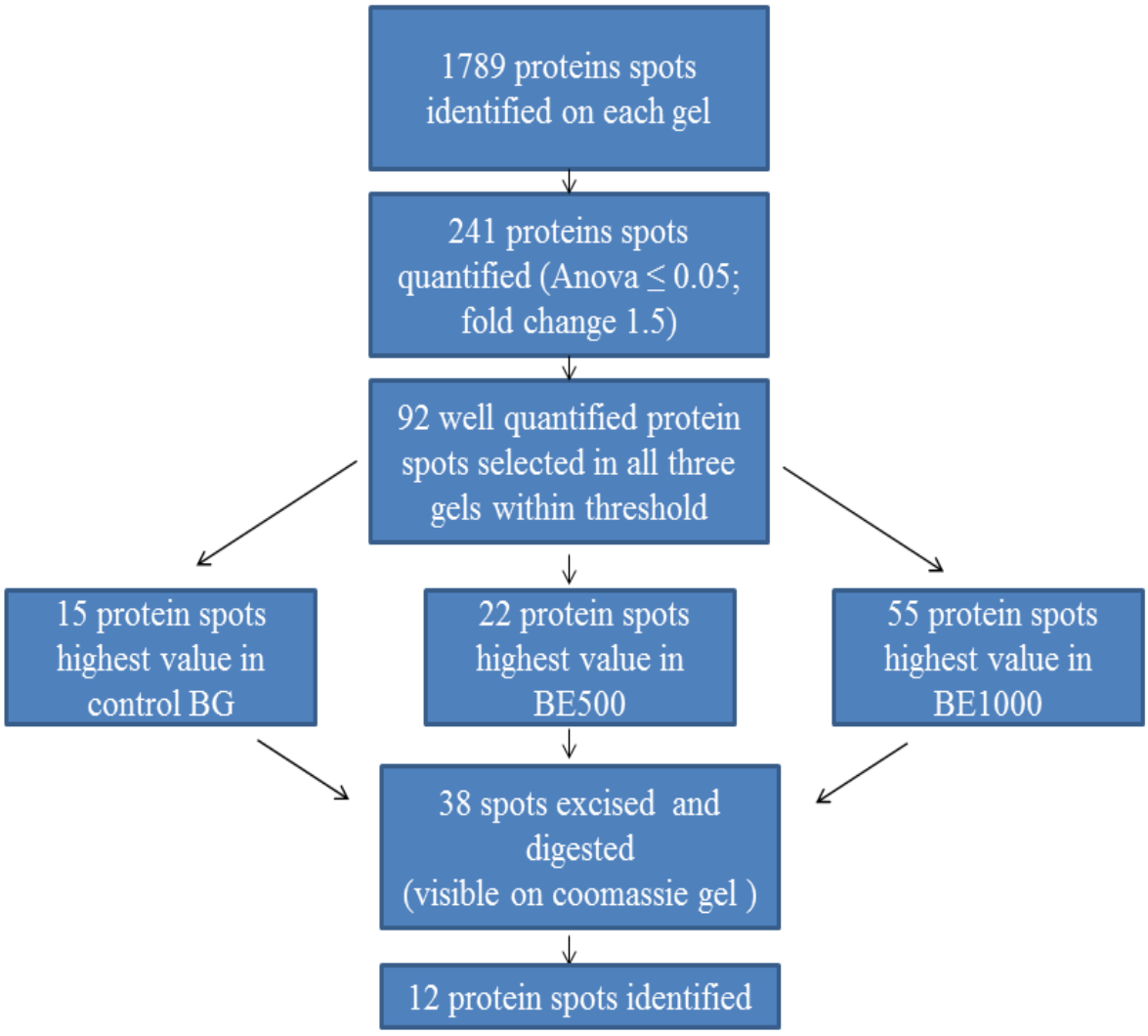
Preparative 2D-PAGE gel stained with Sypro-ruby. IEF was carried out with IPG strip of 24 cm long, and pH 4-7, and 3% ampholytes.

About 1789 protein spots were detected in all the three gels (Figure 4.3). A one-way ANOVA analysis ($p \leq 0.05$) revealed 241 spots as being differentially expressed with an average ratio of 1.5 fold change in the samples with ETBE and glucose (Figure 4.4). All the 241 spots were examined for spots definite patterns by realignment, noise levels and the number of protein spots were reduced to 92. Of them, 77 protein spots were up-regulated in consortium B treated with ETBE in both concentrations of 500 and 1000 mg/L and 15 protein spots were up-regulated in the control. The number of up-regulated protein spots was highest in the sample containing 1000 mg/L of ETBE (55), while 22 spot were higher in expression in the sample containing 500 mg/L of ETBE. Similarly, 44 spots were down-regulated in the control sample with glucose and 48 spots were down-regulated in both test conditions with ETBE (27 very low in BE500 and 21 in BE1000) (Figure 4.4).

The differential protein data sets were also subjected to PCA to investigate inter- and intra-group relationships among the given conditions and to identify protein groups

responsible for the correlated variations. There is a clear difference between the test sample (bacterial consortium with ETBE) and the control (bacterial consortium with glucose), which are grouped together. The protein sample replicates (in different gels) clustered closely, indicating that the biological variation is responsible for the separation of the different treatments (Figure 4.5) which explains the quality of the data presented here. The power analysis was done using Progenesis same spots to predict the number of replicates required among the observed protein spots variation (Figure 4S.2). It revealed that duplicates are enough to obtain more than 80% confidence over the statistical analysis of the protein spots.

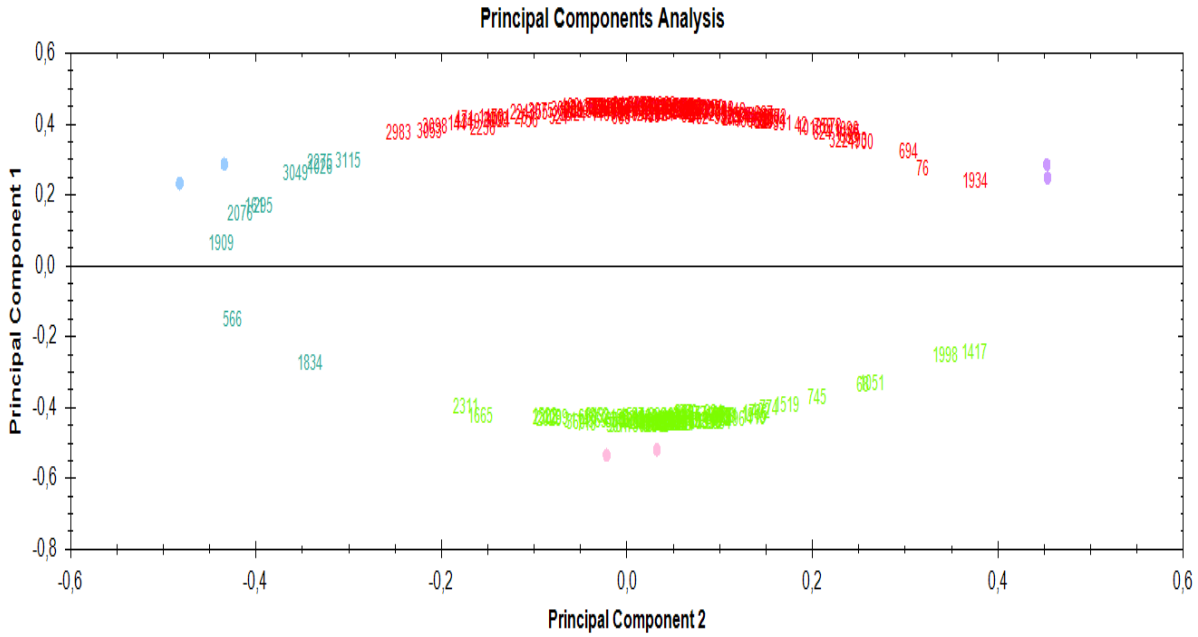
Figure 4.4. Schematic representation of protein spot identified and analyzed



Like expected we were able to get three different clusters from the protein datasets based on similar protein expression patterns (Figure 4.6). The first cluster revealed the up-

regulation of proteins in the controls and the down regulation in ETBE conditions. The other two clusters the showed proteins that were up-regulated in the presence of ETBE (with difference in expression profiles within the two different concentrations of ETBE used) and down-regulated in the controls. So it is clear that the two different concentrations of ETBE used here reflected in the protein profile of the bacterial consortium which was confirmed from the different clusters of protein expression patterns. Of which, five protein spots (316, 552, 727, 1013, 1280) expression profiles, increased with increasing concentrations of ETBE (Figure 4S.3).

Figure 4.5. Principal component analyses of all the samples



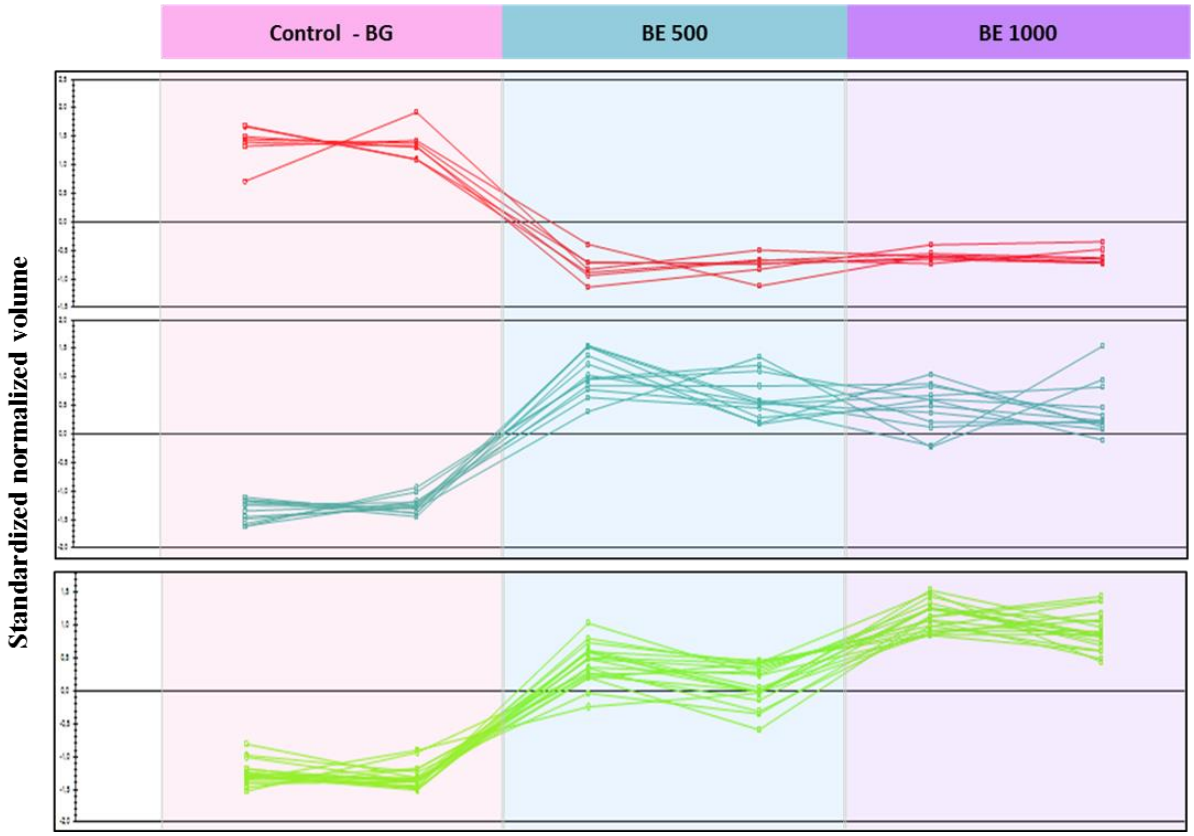
The small round spots colored pink corresponds to control BG, blue to BE500 and violet to BE1000. It should be noted that the protein spots belonging to the respective sample were distributed closely indicating the similarity between the replicates. The protein spot numbers corresponding to different colors refers to the protein clusters they belong to.

4.4.3. Identification of protein spots

Only 38 spots were selected for the analysis in MALDI-TOF/TOF preliminarily (Figure 4.7). All three clusters of the protein expression patterns were observed in the selected spots as described above. Figure 4.8 shows the protein spot belonging to the different

patterns of clusters observed in this study. Table 4.2 represents the protein spots identified with the corresponding identified molecular weight (MW) and isoelectric point (pI).

Figure 4.6. Hierarchical clustering patterns of protein spots



The clustering was able to classify the samples into three main groups, i) up-regulation in BG and down-regulation in treatments BE500 and BE1000, ii) Upregulation in BE500 and BE1000 and down-regulation in control BG and iii) down-regulation in control BG and upregulation in BE500 and BE1000 with increase in induction in both the treatments.

4.4.4. Proteins associated with ETBE metabolism

Two aldehyde dehydrogenases (spot 410 and 552) were identified with different spot locations in the gel. Of them one (spot 552) was up-regulated in both the concentrations of ETBE with a fold change of 4.2 than the control and it corresponded to NAD dependent aldehyde dehydrogenase and ETPC inducible aldehyde dehydrogenase in NCBI and TrEMBL database. Aldehyde dehydrogenase is reported to be involved in the lower pathway of the MTBE degradation in *Mycobacterium austroafricanum* IFP 2012 (Ferreira et al., 2006) encoded by the gene MpdC. This enzyme catalyzes the dehydrogenation of

hydroxyisobutyraldehyde converting into hydroxyiso-butyric acid (HIBA). The identified protein matched with the *Rhodococcus erythropolis* PR4 (Sekine et al., 2006) and *Gordonia bronchialis* or *Rhodococcus bronchialis* in the nearest hits. Both the strains matched here belong to the genera *Rhodococcus* of which, R. *erythropolis* PR4 is an alkane degrading strain. The identification of this protein possibly explains that the consortium consists of bacterial species that can metabolize ETBE and its metabolites. But, another aldehyde dehydrogenase identified (spot 410; 1.6x fold) was up-regulated in glucose but down-regulated in BE500 and BE1000 with a stronger repression in BE500. The possibility of presence of isoforms or probable modifications in the protein while processing can be ruled out as the expression patterns of the two proteins are completely different implying that the two spots belong to two different proteins. Then the identification of two different aldehyde dehydrogenases in this study cannot give any conclusive remarks in identifying the right candidate participating in ETBE degradation. The presence of more than one aldehyde dehydrogenase in the consortium may reflect either a single strain can harbour more than one aldehyde dehydrogenase or they may belong to different bacterial species in the bacterial consortium B.

Figure 4.7 2D-DIGE gel showing the selected protein spots

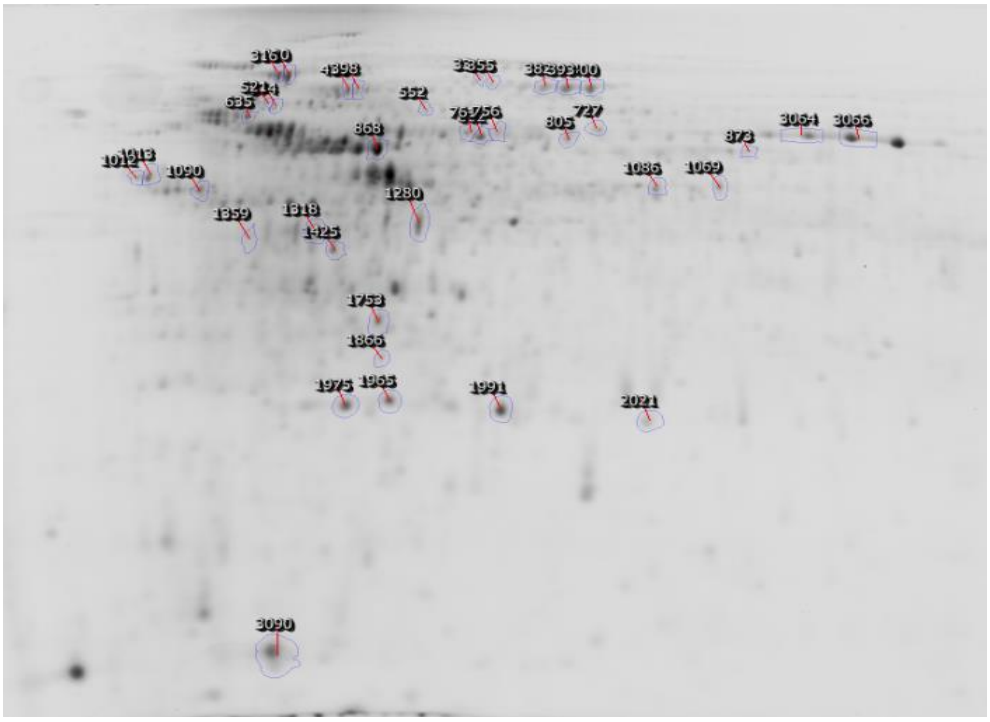


Table 4.2 Protein identification using MALDI-TOF/TOF-MS

SPOT ID	Accession	Protein identified	MW [kDa]	pI	Database Search	Organism	Fold change	Upregulation
320	gi|84494866	nicotine dehydrogenase chain C	88.7	4.7	NCBI	*Janibacter* sp.	4	ETBE 500 ETBE 1000
	A3THC9_9MICO	Nicotine dehydrogenase chain C	88.7	4.7	TREMBL	*Janibacter* sp.		
316	gi|21231605	phosphoenolpyruvate synthase	86.2	5	NCBI	*Xanthomonas campestris*	5.12	ETBE 500 ETBE 1000
	B0RSD3_XANCB	Phosphoenolpyruvate synthase	86.2	5	TREMBL	*Xanthomonas campestris*		
	gi|77165106	fumarate hydratase	50.6	7.1	NCBI	*Nitrosococcus oceani*		
393	H0BRS3_9BURK	Aldehyde oxidase and xanthine dehydrogenase molybdopterin-binding protein	85.1	5.5	TREMBL	*Acidovorax* sp. NO-1	10.7	ETBE 500 ETBE 1000
400	gi|84625297	putative TonB-dependent receptor	84.5	5.4	NCBI	*Xanthomonas oryzae pv. oryzae*	14.89	ETBE 500 ETBE 1000
410	gi|77166367	aldehyde dehydrogenase	56.9	5.2	NCBI	*Nitrosococcus oceani*	1.6	Glucose
552	C0ZVY3_RHOE4	Aldehyde dehydrogenase	55.1	4.8	TREMBL	*Rhodococcus erythropolis*	4.2	ETBE 500 ETBE 1000
	D0LAD0_GORB4	Aldehyde dehydrogenase (NAD(+))	55.3	4.9	TREMBL	*Gordonia bronchialis*		
	gi|1174662	EPTC-inducible aldehyde dehydrogenase	55	4.9	NCBI			
727	gi|152968176	chaperone protein DnaK	66.4	4.7	NCBI	*Kineococcs radiotolerans* SRS30216	4.8	ETBE 500 ETBE 1000
	K1ET82_9MICO	Chaperone protein DnaK	67.1	4.4	TREMBL	*Janibacter hoylei* PVAS-1		
	gi|84497587	molecular chaperone DnaK	67.9	4.5	NCBI	*Janibacter* sp. HTCC2649		
1013	gi|84497843	phosphopyruvate hydratase	45.9	4.4	NCBI	*Janibacter* sp. HTCC2649	2.5	ETBE 500 ETBE 1000
	gi|21221535	phosphopyruvate hydratase	45.5	4.3	NCBI	*Streptomyces coelicolor* A3		
	H0QHC6_ARTGO	Enolase	45	4.3	TREMBL	*Arthrobacter globiformis*		
1086	I0KWX5_9ACTO	Enolase	45	4.5	TREMBL	*Micromonospora lupini* str. Lupac 08	7.2	ETBE 500 ETBE 1000
	C0FPT6_9FIRM	Enolase	50.3	4.8	TREMBL	*Roseburia inulinivorans* DSM 16841		
1280	gi|121998872	UDP-N-acetylmuramoylalanine--D-glutamate ligase	46.1	5.1	NCBI	*Halorhodospira halophila* SL1	6.5	ETBE 500 ETBE 1000
	D0WEA8_9ACTN	Glyceraldehyde-3-phosphate dehydrogenase, type I	35.8	5.2	TREMBL	*Slackia exigua*		

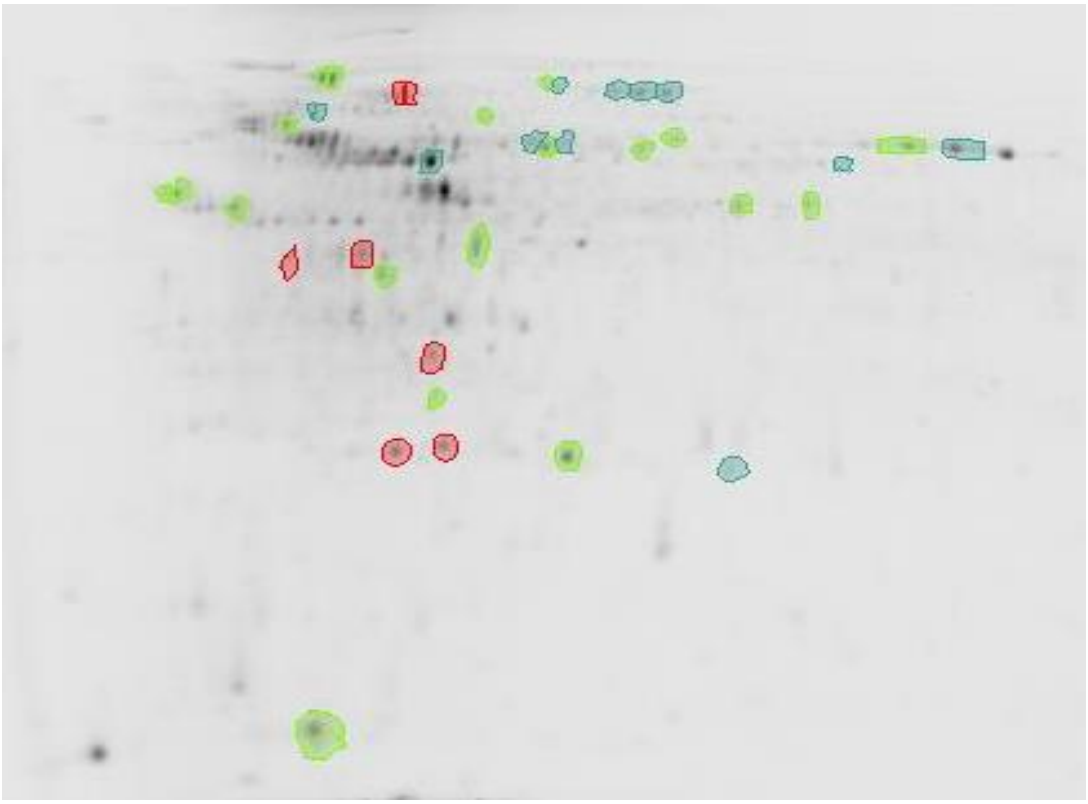
Table 4.2 continued.,

SPOT ID	Accession	Protein identified	MW [kDa]	pI	Database Search	Organism	Fold change	Upregulation
1425	H0QLW5_ARTGO	Ketol-acid reductoisomerase	37.2	4.7	TREMBL	*Arthrobacter globiformis*	2.45	ETBE 500 ETBE 1000
	E3BB64_9MICO	Ketol-acid reductoisomerase	37.2	4.7	TREMBL	*Dermacoccus* sp. Ellin185		
1753	A0JVA2_ARTS2	DivIVA family protein	25	5	TREMBL	*Arthrobacter* sp. (strain FB24)	18.5	Glucose
	gi|116670139	DivIVA family protein	25	5	NCBI	*Arthrobacter* sp. FB24		

** Proteins were identified by MS/MS spectra using Mascot search. Protein spot with two or more identification corresponds to the number of hits obtained from the respective database.*

But not all dehydrogenases can be responsible for ETBE degradation as reported in the study conducted by Hristova et al., 2007. In this study, the identity of the hydroxyisobutyraldehyde (HIBAL) dehydrogenase was difficult as there are 11 genes belonging to the aldehyde dehydrogenase superfamily in the *M. petroleiphilum* PM1 genome when grown on MTBE, but only one was predicted to be the right candidate which showed 33% identity to MpdC of *M. austroafricanum* IFP 2012 with a significant 1.4-fold up-regulation. Therefore, we can predict that the aldehyde dehydrogenase corresponding to the protein spot 552 can be the most likely candidate involved in the ETBE degradation matching perfectly with the theoretical MW (55) and pI (4.9). However other enzymes involved in the initial cleavage of ETBE cannot be identified due to random protein spots selection based on the most abundant fold change.

Figure 4.8. 2D-DIGE gel showing clustering patterns of the selected protein spot



The coloured spots refers to different clustering patterns red coloured spots refers to the cluster where proteins are up-regulated in BG and down-regulated in treatments BE500 and BE1000, blue coloured spots refers to the cluster where proteins are upregulated in BE500 and BE1000 and down-regulated in control BG and green coloured spots refers to the cluster where proteins are down-regulated in control BG and upregulatedn in BE500

4.4.5. Proteins related to amino acid metabolism

Ketol-acid reductoisomerase (KARI) (spot 1425), a key enzyme involved in amino acid biosynthesis was found to be up-regulated with an induction of 2.45 times with ETBE in comparison to the control. According to Dumas et al., 2001, KARI, encoded by ilvC in bacteria, catalyzes the conversion of either acetolatetate to (2R)-2, 3- dihydroxy-3-isovalerate leading to valine and leucine synthesis, or catalyzes the conversion of 2-aceto-2-hydroxybutyrate to (2R, 3R)-2,3-dihydroxy-3-methylvalerate, which is the precursor of isoleucine. An upregulation of this enzyme in our conditions may predict that the presence of ETBE in the medium induces the branched chain amino acid (BCAA) biosynthesis. A transcriptomic study conducted in *Listeria monocytogenes* stated that the low concentrations of BCAA resulted in prolonged lag phase directing the metabolism in to produce more BCAAs ith the upregulation of ilvC genes (Lobel et al., 2012). The observed change in the metabolism in to produce more BCAA gains importance as leucine and valine are involved in host denfense response and/or in synthesis of proteins which are structurally related.

Thus, the increased synthesis of BCAAs in our bacterial consortium during ETBE degradation could be directed to the biosynthesis of BCAAs rich proteins linked to host defence mechanisms.

4.4.6. Proteins involved in cell cycles

Two of the identified proteins, UDP-N-acetylmuramoylalanine--D-glutamate ligase and DivIVA family protein are related to cell cycle, cell wall biogenesis and cell division. UDP-N-acetylmuramoylalanine-D-glutamate ligase (spot 1280; 6.5x fold) was found to be up-regulated in presence of ETBE and found to increase in higher concentrations of ETBE used. This protein is related to cell wall biogenesis and is predicted to maintain the integrity of the cell wall. Hence, this observation implies that the bacterial cells have increased the cell wall biogenesis in order to maintain the cell membrane integrity amidst of harsh environment. On the other hand, the other protein identified namely DivIVA family protein (spot 1753; 18.5x fold) was upregulated in the presence of glucose and was down-regulated in presence of ETBE. This protein plays a main role in cell division as predicted by UniProt and KEGG databases. Therefore, it can be understood that cell division is accelerated in presence of glucose and the presence of ETBE impedes it. The identification of this protein in this proteomic analysis can explain the long lag phase observed with the bacterial consortium B

when grown in ETBE. Furthermore, the down regulation of this protein can play a part in the energy trade-off between growth and acclimatization to external chemical stress wherein the bacterial consortium cedes a part of its energy produced to the metabolism of ETBE rather than cell division and growth. However figure 4.1 shows bacterial growth at 16th day.

4.4.7. Proteins related to energy metabolism and biosynthetis pathways

Phosphoenolpyruvate (PEP) synthase (Spot 316) was found to be increased up to 5 fold in consortium B potentially reflecting the increased metabolic demand imposed by the presence of ETBE. This enzyme is involved in many biological processes such as gluconeogenesis pathway, pyruvate metabolism and phosphorylation reactions. It is a key enzyme in carbon fixation providing substrates for multiple biosynthetic pathways. PEP synthase was reportedly up-regulated (5 fold) in *Alkanivorax* sp in the presence of alkanes. This study postulated that the strain produces phosphoenolpyruvate from pyruvate and by passes the TCA cycle through glyoxylate cycle reducing the carbon dioxide release steps (Sabirova et al., 2006). The spot matched in the second hit corresponds to the enzyme fumarate hydratase (Fumarase) which participates in the important series of reactions known as the citric acid cycle or Krebs cycle, which allows cells to use oxygen and generate energy. So both the relevant identification ended up in a protein that involved in TCA cycle proving participation in increased energy metabolism and biosynthetic processes.

Phosphopyruvate hydratase (spot 1013, 2.5 fold) belongs to the lyases superfamily of bacterial enzymes. This enzyme is involved in the metabolism mainly in the synthesis of phosphoenol pyruvate involved in gluconeogenesis as predicted by KEGG database. Other functions include participation in glycolysis, biosynthesis of secondary metabolites, methane metabolism and in other microbial metabolism in different environments. Similarly, another protein spot 1086 which showed an increase by 7.5 fold with ETBE was identified as enolase which is an ortholog of the protein phosphopyruvate hydratase having similar predicted biological functions. The exact role of the upregulation of this protein in ETBE metabolism is not clear but led to the observation that there is an increased formation of PEP which can be precursor of many molecules as described above.

4.4.8. Other proteins

Aldehyde oxidase and xanthine dehydrogenase molybdopterin-binding protein (spot 393; 10 fold) was identified to be upregulated in presence of ETBE. This protein belongs to the group of oxidoreductase family of proteins catalysing the conversion of an aldehyde (Aldehyde oxidase) in the presence of oxygen and water to an acid and hydrogen peroxide with FAD, molybdenum and two 2FE-2S clusters as cofactors. Xanthine dehydrogenase catalyzes the hydrogenation of xanthine to urate, in presence of cofactors. Xanthine dehydrogenase is often associated in purine metabolism pathway. The protein spot 320 corresponded to nicotine dehydrogenase chain C. This protein exhibited a 4 fold increase when consortium B was propagated in ETBE than in glucose. Nicotine dehydrogenase consists of three subunits (A, B and C) of Mr 30011, 14924 and 87677 of which the chain C was identified in this study. This enzyme belongs to a family of bacterial hydroxylases with molybdopterin dinucleotide, FAD and Fe-S clusters as cofactors (Brandsch et al., 2006).

Spot 400 (14.89x fold) corresponded to putative TonB-dependent receptor in the first hit in MS/MS identification. The biological function of the protein is the uptake of iron from the cellular membrane thereby acting as a receptor (Wan et al., 2004). Another study stated that TonB-dependent receptors control small molecule transport through the outer membrane. They propose that abundant presence of this protein may facilitate imports of metal ion complexes into host environments (Pieper et al., 2009). The exact correlation for the upregulation of this protein in presence of ETBE is unknown. We may speculate there can be increased demand for iron or any other metal ions for the activation of certain enzymes to carry out the biological process in demand in the ETBE environment.

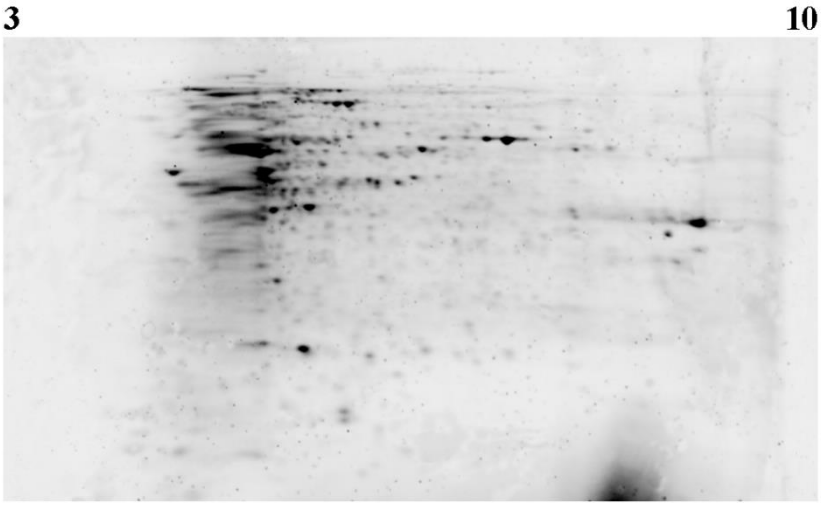
Chaperones DnaK (spot 727) was induced to 4 fold in cells grown in ETBE than in the control with glucose. Chaperones are reported to be involved in a myriad of molecular functions, including nascent protein folding and assembly for protein activation, protein refolding, translocation across membranes, heat-shock responses (housekeeping function), and as a defense strategy against stress (Alix et al., 2006). The upregulation of this protein can reflect either an increased demand for protein folding that are newly synthesized proteins or as stress response of the bacterial cell to the given environment. Similarly molecular chaperon DnaK and GroEL are stimulated in the MTBE grown *M. austroafricanum* IFP 2012 (Ferreira et al., 2006) supporting our findings.

4.5. Conclusion

In summary, the bacterial consortium B possesses several responses when adapting to a higher ETBE concentration. The response of the bacterial consortium with respect to ETBE in comparison to glucose was also discussed. The enzymes identified in a groundwater sample partly mirrored the observed metabolism of ETBE. On the basis of the number of same proteins identified, the autochthonous community established on ETBE appears to contain more than one strain similar to the results obtained in the enrichment. Finally, increasing availability of genome data from environmental samples and improved de novo sequencing strategies will facilitate the analysis of environmental metaproteomes and their functional interpretation.

4.6. Supporting Information

Figure 4S.1 2D-PAGE gel with pH 3-10



Preparative 2D-PAGE gel stained with Syproruby. IPG strip of 3-10, 7 cm length was used during IEF.

Figure 4S.2 Power analysis generated by the Progenesis Same Spot software.

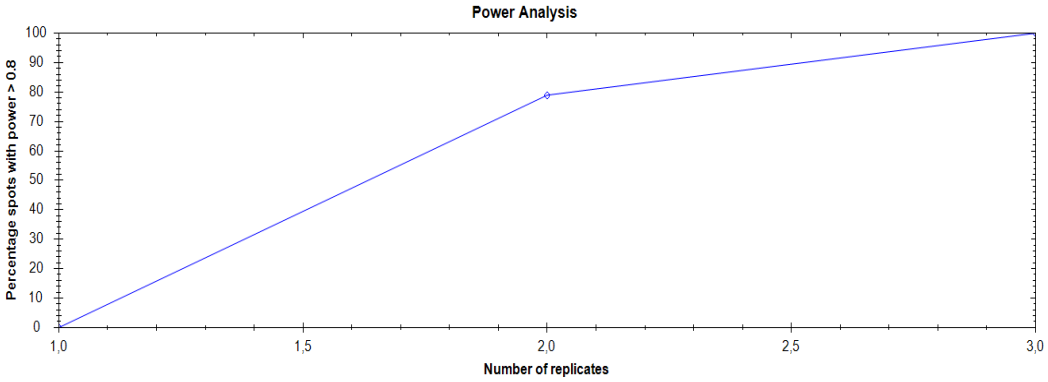


Figure 4S.3 Expression patterns of all identified protein spots

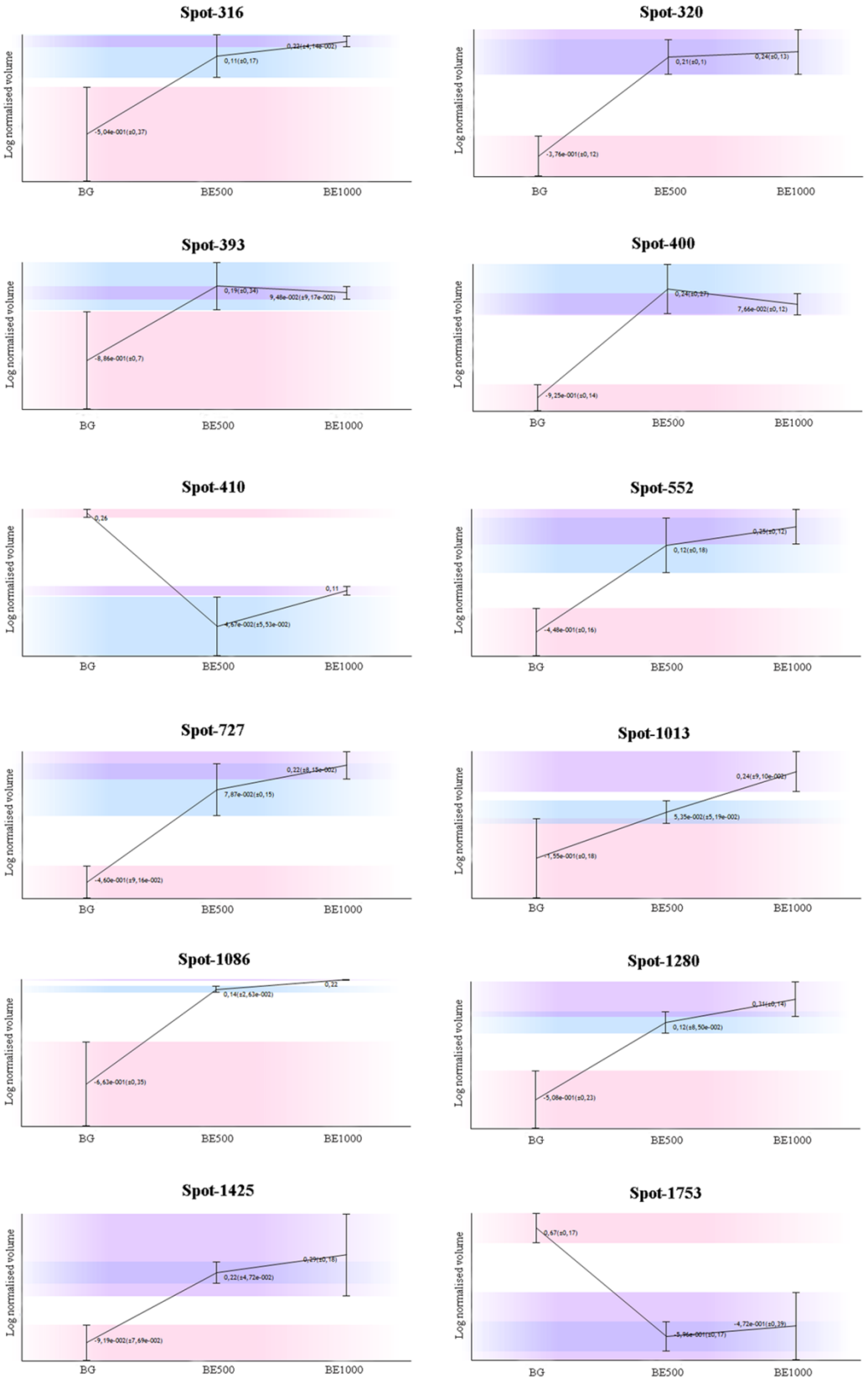


Table 4S.1. Protein quantification by RC-DC

Sample	Protein concentration		Dilution factor	Total protein (µg/µl)
	duplicates	Mean		
BGI-1	0.399	0.4275	10	4.275
BGI-2	0.456			
BGII-1	0.365	0.385		3.85
BGII-2	0.418			
BE500I-1	0.196	0.2275		2.275
BE500I-2	0.259			
BE500II-1	0.347	0.399		3.99
BE500II-2	0.451			
BE1000I-1	0.523	0.5965		5.965
BE1000I-2	0.67			
BE1000II-1	0.695	0.717		7.17
BE1000II-2	0.739			

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Chapter 5

Cloning and expression of a newly identified cytochrome P450 from the ETBE and MTBE degrading *Achromobacter xylosoxidans* MCM2/2/1

5.1. Abstract

The presence of the cytochrome P450 (CYP) gene in an ETBE and MTBE degrading *Achromobacter xylosoxidans* MCM2/2/1 was previously confirmed by amplification of a putative 350bp *CYP* gene fragment. The entire gene sequence which sized 2.3kb was identified by genome walking and DNA-sequencing. The identified *CYP* gene of *A. xylosoxidans* MCM2/2/1 shares a high similarity of about 88% with the *thc*B gene of *A. xylosoxidans* A8. The nucleotide sequence of CYP from *A. xylosoxidans* MCM2/2/1 was submitted to NCBI under the accession number JX454599. The identified CYP was successfully cloned and heterologous expressed in *E.coli*. CYP was analyzed using CO-difference spectra and the concentration was calculated to contain 9.6 µM in the crude cell extracts of *E.coli.*

5.2. Introduction

Fuel oxygenates are compounds used as gasoline additives to boost the octane number and reduce the emission of carbon monoxide, thereby reducing air pollution. Since the late 1970s methyl *tert*-butyl ether (MTBE) has been widely used as a fuel oxygenate in the USA and less extensively in Europe. Continued use of this compound led to it becoming the second most common pollutant found in ground water in the USA during the year 1993-1994 (Squillace et al., 1996). Ethyl *tert*-butyl ether (ETBE), the ethyl analogue of MTBE, is being used as an alternative for MTBE in Spain and other European countries. The probability of this compound contaminating drinking water is significantly high because of its solubility and continued usage. MTBE has been reported to cause renal adenomas and carcinomas in rat (Benson et al., 2011. In addition, *tert*-butyl alcohol (TBA), the primary metabolite of MTBE and ETBE mineralization, has shown some neurotoxicity in male rats at higher doses (McGregor, 2010). These compounds, then, may also be a threat to the health of human beings, and this has generated a considerable number of investigations into how to eliminate them from the environment. MTBE and ETBE that have a stable ether bond and a tertiary carbon structure can be difficult compounds to mineralize. However, bacterial degradation of these compounds has been observed under aerobic (Squillace et al., 1996) and anaerobic (Youngster et al., 2008) conditions since the last decade. Exploring the molecular mechanisms by identifying the genes and enzymes responsible for degradation by microorganisms could be one of the best ways to understand the mechanism of degradation.

Biodegradation of either MTBE or ETBE has been reported to start with the cleavage of the ether bond (o-demethylation) (Youngster et al., 2008; Malandain et al., 2010) mediated by a monooxygenase, which produces respective tertiary alcohols. Cytochrome P450s (CYPs) are one of the most probable candidate enzymes for carrying out these characteristic reactions as it is well known that they perform difficult oxidation reactions (Vlada and Marco, 2012). CYPs are apparently involved in the detoxification of a broad spectrum of environmental chemical pollutants (Karlson et al., 1993; Chauvaux et al., 2001). TBA, the primary measurable compound in MTBE detoxification, may also undergo oxidation by CYP as increased concentrations of acetone were observed in rats exposed to *tert*-butanol (McGregor, 2010). Hatzinger et al. (2001) showed that monooxygenases were involved in TBA oxygenation in *Hydrogenophaga flava* ENV735 which could lead to 2-methyl 1, 2-propanediol (2-M1, 2-PD) (Lopez-Ferreira et la., 2006). And later, a cluster of *mpd* genes

was found to be involved in the conversion of 2-M1, 2-PD to hydroxyisobutyric acid (HIBA) which was isolated from *Mycobacterium austroafricanum* IFP 2012 (Hatzinger et al. 2001).

In a previous study, a number of bacterial isolates were found and identified from gasoline-contaminated soil (Barbera et al., 2011). One isolate, identified as *Achromobacter xylosoxidans*, was chosen for this study. This bacterium was found to degrade was found to degrade ETBE and MTBE by 41.48% and 34.15%, respectively, in 6 days (Gunasekaran et al., 2013) from the initial added concentration of the compound (100 mg/L) previously in our lab. The presence of CYP was confirmed by amplification of the putative CYP sequence of about 350 bp length in the identified strain (Gunasekaran et al., 2013)

In this chapter, we focus on the identification and sequencing of the the full length gene encoding CYP and its redox partners in the ETBE and MTBE degrading *A.xylosoxidans* MCM2/2/1. Further, the heterologous expression of the CYP gene in *E.coli* was accomplished with the aim to study the biotransformation of a number of selected compounds including ETBE and MTBE by the identified CYP in this strain.

5.3. Materials and methods

5.3.1. Materials

ETBE, MTBE and TBA (purity 98%) were purchased from Fluka (Barcelona, Spain). Microbiological reagents were obtained from Scharlab (Barcelona, Spain). Isopropyl-b-D-thiogalactopyranoside (IPTG) was from Takara.Oligonucleotides, and all other chemicals were purchased from Sigma-Aldrich (Barcelona, Spain).

5.3.2. Bacterial strain

A. xylosoxidans MCM2/2/1 was isolated from a contaminated soil (collected from a gasoline-contaminated site in Valencia, Spain) enriched with MTBE and then identified by 16S rDNA amplification and sequenced as described by Barbera et al. (2011). Manipulation and cloning of genes was performed using *Escherichia coli* strain TOP10F0 (Invitrogen) with *E. coli* strain BL21S (DE3) (Invitrogen) used for expression studies. Tryptic soy agar (TSA) plates were used to grow the selected bacterium.

5.3.3. Identification and sequencing of the CYP gene from *A. xylosoxidans* MCM2/2/1

The genomic DNA of *A. xylosoxidans* MCM2/2/1 was extracted using the GenElute™ bacterial genomic DNA kit (Sigma-Aldrich, Barcelona, Spain) according to the manufacturer's instructions. Initially the presence of the *CYP* gene was confirmed by amplifying a small fragment of about 350 bp using the same primers described above (Hyun et al., 1998).

Table 5.1. List of primers used for the amplification of cytochrome P450 in *A. xylosoxidans MCM2/2/1*

Primer	Sequence	Annealing temperature (°C)
AxyCyp1-F	CCCGGAGATTACCCCAATTG	67
AxyCyp1- R	ATGGGTTCGTGGTGCTTGAG	
AxyCyp2-F	CGACAACCACACCTACAG	58
AxyCyp2- R	GGAATGCAGGTACGAGTC	
AxyCyp3-F	CCGAGGAACAAGTTGAGG	56
AxyCyp3-R	GCCATAGCCGAAAGTCAG	
AxyCyp4-F	CAAGCTGCTGATCGTGTC	61
AxyCyp4- R	AGCGATGAGGATCAAACG	
AxyCyp5-F	CAAGCGTTTGATCCTCATC	60
AxyCyp5- R	AGAGTCCTTCCTCGCAAT	
AxyCyp6-F	GTAATTCGGAGCGACACC	58
AxyCyp6-R	TCGTTGAAGCAATCGTAGG	
AxyCyp7-F	CATCGTSGTGCAGGTGCC	56
AxyCyp7-R	CGTGAAGAAGTCGTTGAAGC	

Next, a one-step genome walking strategy involving inverse PCR (Uchiyama and Watanabe et al., 2006) was implemented which yielded a 900 bp fragment. Further, overlapping primers (see Table 5.1) were designed based on the genome sequence of a similar bacterial strain, *A. xylosoxidans* A8 (Strand et al., 2011), covering the entire 2.35 Kb of *thcB*. Each fragment was amplified individually with the Expand High Fidelity PCR System (Roche, Madrid, Spain). The PCR conditions had an initial denaturation for 98°C –

10 min; cycling conditions 98°C – 20 s; annealing temperature (variable; see Table 5. 1) – 30 s; 72°C - 1 min. The amplified products were sequenced using a Beckman Coulter CEQ 8000 automated sequencer. The amplified contigs were aligned using Bioedit v7.0.5.3 software (Clamp et al., 2003) and the entire sequence was generated based on the consensus obtained from the.overlapping fragments. Sequences of DNA and proteins were aligned using ClustalW version 2.0 (Larkin et al., 2007). The signature motifs were confirmed by Conserved Domain Database (CDD) and PROSITE. Phylogenetic trees were generated by MEGA 4.0 software using the Neighbor Joining method with Bootstrap replicates of 1000. The reference sequences were downloaded from the GenBank database and CYP database (Nelson, 2009). The nucleotide sequence of *CYP* from *A. xylosoxidans* MCM2/2/1 was submitted to NCBI under the accession number JX454599.

5.3.4. Cloning and Heterologous Expression of CYP from *A. xylosoxidans* MCM2/2/1 in *E. coli*

The gene encoding for the CYP was amplified from *A. xylosoxidans* MCM2/2/1 genomic DNA by applying the forward primer 5′-GATCggatccATGGCCCAGCCCTCCCC GCAA-3′ with a restriction site for BamHI and reverse primer 5′-GATCTaagcttCTACAGC TCCAGCACGATGCG-3′ with a restriction site for HindIII. PCR was accomplished with KOD Xtreme™ Hot Start DNA Polymerase (Merck KGaA, Darmstadt, Germany) for 30 cycles, including denaturation at 98 °C for 1 min, annealing at 57 °C for 30 sec, and extension at 72 °C for 3 min. The PCR product was ligated with the vector pET28(a) which was digested with the same restriction enzymes and then the ligation reaction was cloned into cloning vector E.coli Topo 10 (Invitrogen) according to the manufacturer's protocol and cells harboring the cloned plasmid were selected by their ability to grow on Luria–Bertani plate containing 100 µg/ml of kanamycin and the insertion was checked with a restriction enzyme analysis. The cloned plasmid was then transformed to expression vector E.coli BL21 (Novagen) and postives clones were selected and sequenced as before. Empty vector without the insert CYP gene was transformed and the strain was used as a control throughout the experiment. Medium-scale cultures were initiated with a single, freshly transformed colony of *E.coli* that served as the inoculum for 1000 mL of Luria-Bertani (LB) medium supplemented with 100 µg/ml of kanamycin as antibiotic, in a 3 L flask. Induction was performed with 0.1 mM isopropyl 1-thio-β-d-galactopyranoside (IPTG) along with 100mM

of FeSO4 for 12 h at 30°C, after growth at 37°C in LB medium containing 100 µg/mL of kanamycin to an OD_{600} of 0.8. Then, cells were centrifuged and suspended in 50 mM phosphate buffer pH 7.5.

5.3.5. Characterization of CYP expression: Co-difference Spectra

Preparation of crude cell lysate: The cells were harvested by centrifugation and the pellets were resuspended in 10 ml of 50 mM Phosphate buffer pH 7.5 along with protease inhibitors (Roche). The cells were disrupted using a sonicator with an output of 40 W, 60% amplitude for 1 min with 1 min break on ice. This process was repeated 5 times. The cell extract was centrifuged at 164000 g for 30 min at 4°C. The cell lysate was collected in a new tube and used as source of CYP in further experiments.

CYP concentrations were determined by the method of Omura and Sato (1964) using the extinction coefficient $\epsilon_{450\text{-}490}$ = 91 mM^{-1} cm^{-1}. SDS–polyacrylamide gel electrophoresis was done according to the basic procedure of Laemmli. The concentration of the expressed CYP was calculated by the formula CYP [µM] = (A450 – A490) *dilution factor*1000/ ε(extinction coefficient) Where $\varepsilon_{450–490}$ = 91 mM-1 cm-1 (Omura and Sato 1964).

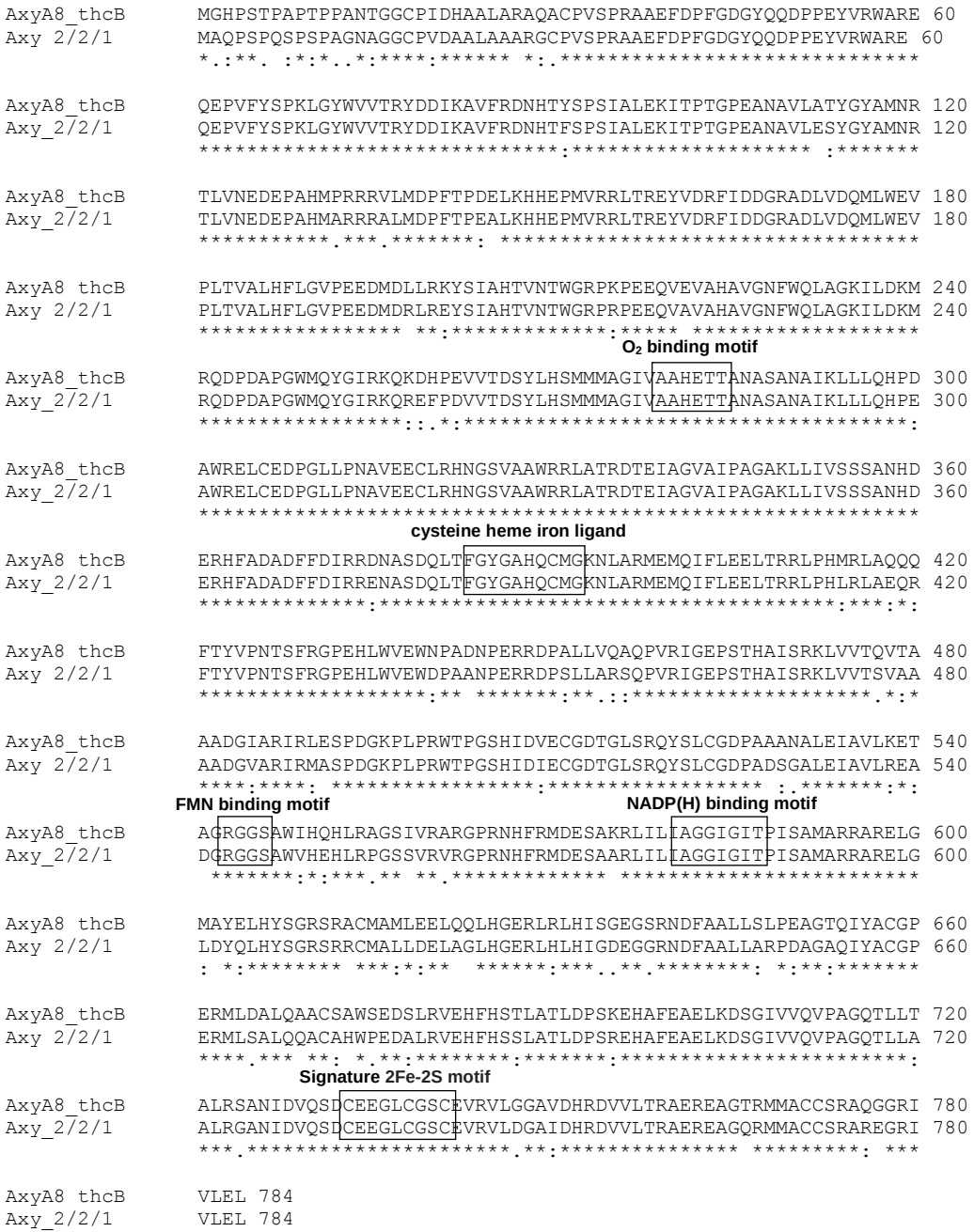
5.4. Results and discussion

A. xylosoxidans MCM2/2/1 used for this study is an ETBE and MTBE degrading strain (Gunasekaran et al., 2013). In general, this bacterial genus appears to be a good xenobiotic degrader since it has frequently been reported to be involved in the degradation of many xenobiotic substrates: for example, malachite green (Wang et al, 2011), toluidine isomers (Hinteregger et al., 2001), endosulfan (Li et al., 2009), chrysene (Ghevariya et al., 2011), PAHs (Andreoni et al., 2004), p-toluenesulfonate (Tralau et al., 2011), chlorocatechol (Jencova et al., 2004), 2,4-dichlorophenol (Quan et al., 2003) and organophosphorous pesticide (Zhang et al., 2005).

5.4.1. Identification of CYP gene in *A. xylosoxidans* MCM2/2/1

The presence of the *CYP* gene was previously confirmed by amplifying a DNA fragment of 350 bp in *A. xylosoxidans* MCM2/2/1 with primers designed based on the conserved oxygen binding and heme binding domain of *CYP* gene (Hyun et al., 1998).

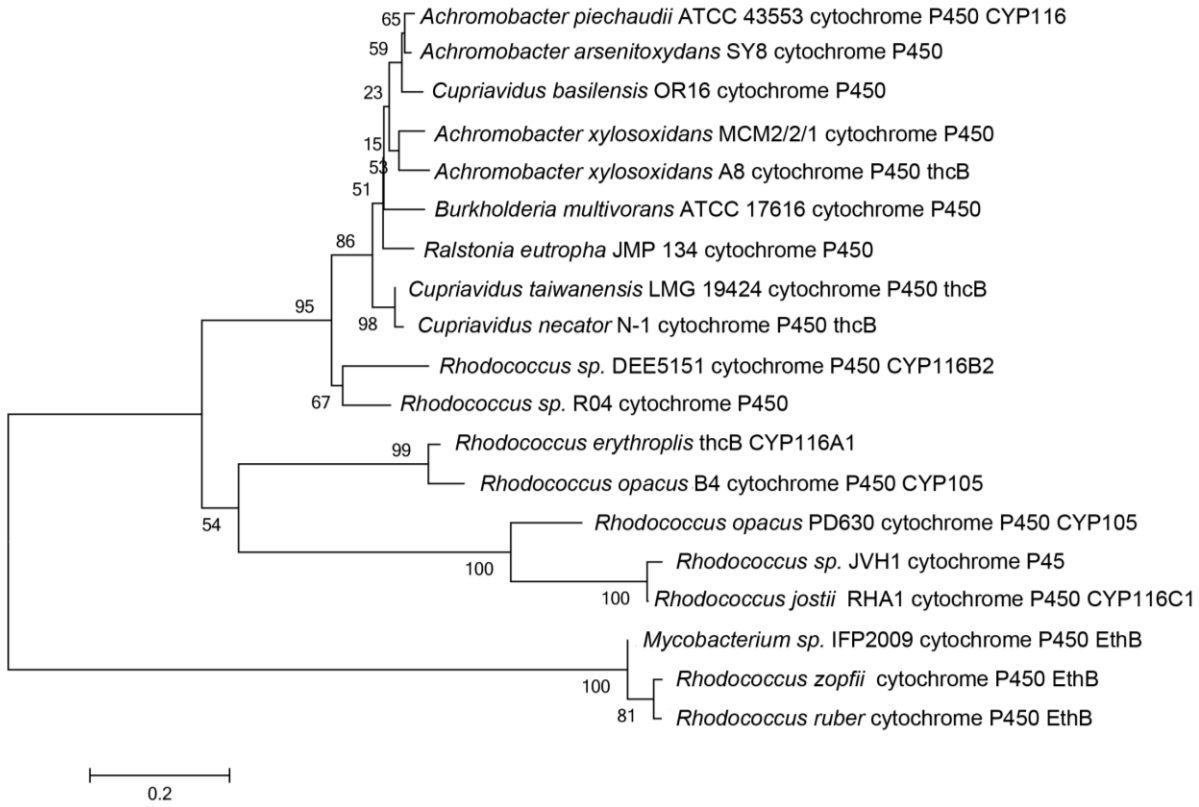
Figure 5.1. Amino acid sequence alignment and signature motif deduction



Amino acid sequence alignment between thcB of A. xylosoxidans A8 (AxyA8_thcB) and A.xylosoxidans MCM2/2/1 (Axy_2/2/1) generated by Clustal W Residues marked with (*) are identical, (:) indicates strong similarity, and (.) indicates weak similarity. The conserved oxygen binding motif, cysteine heme iron ligand, signature FMN binding motif, NAD(P)H binding motif and the 2Fe-2S motif are marked in boxes.

Subsequently our genome walking experiments led to a 900 bp fragment (data not shown). The sequence of this fragment was very similar (90%) to the *thc*B gene of *A. xylosoxidans* A8 coding for a putative *CYP*. This allowed us to use this organism as a reference for designing overlapping primers for PCR amplification. After several runs of PCR amplification and sequencing, the overlapping contigs were aligned using Bioedit to produce the entire sequence. The entire 2.3 kb sequence of the *CYP* gene could thus be identified.

Figure 5.2. Phylogenetic tree of the closely related cytochrome P450 amino acid sequences



Phylogenetic tree was constructed by MEGA 4.0 using neighbor joining method with boostrap replicates of 1000. The amino acid sequences were collected from Genbank and cytochrome P450 database.

The presence of CYP signature motifs, as well as the FMN containing phthalate dioxygenase reductase and iron sulphur protein (ferredoxin), was confirmed in the deduced amino acid sequence using PROSITE (Figure 5.1). Phthalate dioxygenase reductase (PDR) is

Figure 5.3. Amino acid sequence alignment between CYP116B3 of *Rhodococcus ruber* and *A. xylosoxidans* MCM2/2/1 (Axy_2/2/1) generated by Clustal W

```
CYP116B3        MSASVP--------ASACPVDHAALAG--GCPVSTNAAAFDPFGPAYQADPAESLRWSRD 50
Axy_2/2/1       MAQPSPQSPSPAGNAGGCPVDHAALAAARGCPVSPRAAEFDPFGDGYQQDPPEYVRWARE 60
                *: . *        *..*********.  *****..** ***** .** **.* :**:*:

CYP116B3        EEPVFYSPELGYWVVTRYEDVKAVFRDNLVFSPAIALEKITPVSEEATATLARYDYAMAR 110
Axy_2/2/1       QEPVFYSPKLGYWVVTRYDDIKAVFRDNHTFSPSIALEKITPTGPEANAVLESYGYAMNR 120
                :*******:*********:*:******* .***:********.. **.*.*  *.*** *

CYP116B3        TLVNEDEPAHMPRRRALMDPFTPKELAHHEAMVRRLTREYVDRFVESGKADLVDEMLWEV 170
Axy_2/2/1       TLVNEDEPAHMARRRALMDPFTPEALKHHEPMVRRLTREYVDRFIDDGRADLVDQMLWEV 180
                ***********.**********: * ***.*************::.*:*****:*****

CYP116B3        PLTVALHFLGVPEEDMATMRKYSIAHTVNTWGRPAPEEQVAVAEAVGRFWQYAGTVLEKM 230
Axy_2/2/1       PLTVALHFLGVPEEDMDRLREYSIAHTVNTWGRPRPEEQVAVAHAVGNFWQLAGKILDKM 240
                ****************  :*:************* ********.***.*** **.:*:**

CYP116B3        RQDPSGHGWMPYGIRMQQQMPDVVTDSYLHSMMMAGIVAAHETTANASANAFKLLLENRP 290
Axy_2/2/1       RQDPDAPGWMQYGIRKQREFPDVVTDSYLHSMMMAGIVAAHETTANASANAIKLLLQHPE 300
                ****.. *** **** *:::*********************************:****::

CYP116B3        VWEEICADPSLIPNAVEECLRHSGSVAAWRRVATTDTRIGDVDIPAGAKLLVVNASANHD 350
Axy_2/2/1       AWRELCEDPGLLPNAVEECLRHNGSVAAWRRLATRDTEIAGVAIPAGAKLLIVSSSANHD 360
                .*.*:* **.*:**********.********:** **.*..* ********:*.:*****

CYP116B3        ERHFDRPDEFDIRRPNSSDHLTFGYGSHQCMGKNLARMEMQIFLEELTTRLPHMELVPDQ 410
Axy_2/2/1       ERHFADADFFDIRRENASDQLTFGYGAHQCMGKNLARMEMQIFLEELTRRLPHLRLA-EQ 419
                ****  .* ***** *:**:******:********************** ****:.*. :*

CYP116B3        EFTYLPNTSFRGPDHVWVQWDPQANPERTDPAVLQRQHPVTIGEPSTRSVSRTVTVERLD 470
Axy_2/2/1       RFTYVPNTSFRGPEHLWVEWDPAANPERRDPSLLARSQPVRIGEPSTHAISRKLVVTSVA 479
                .***:********:*:**:*** ***** **::* *.:** ******:::**.:.*  :

CYP116B3        RIVDDVLRVVLRAPAGNALPAWTPGAHIDVDLG--ALSRQYSLCGAP-DAPTYEIAVLLD 527
Axy_2/2/1       AAADGVARIRMASPDGKPLPRWTPGSHIDIECGDTGLSRQYSLCGDPADSGALEIAVLRE 539
                  .*.* *: : :* *:.** ****:***:: *  .********* * *: : ***** :

CYP116B3        PESRGGSRYVHEQLRVGGSLRIRGPRNHFALDPDAEHYVFVAGGIGITPVLAMADHARAR 587
Axy_2/2/1       ADGRGGSAWVHEHLRPGSSVRVRGPRNHFRMDESAARLILIAGGIGITPISAMARRAREL 599
                .:.**** :***:** *.*:*:******* :* .* : :::*********: *** :**

CYP116B3        GWSYELHYCGRNRSGMAYLERVAG-HGDRAALHVSAEGTRVDLAALLATPVSGTQIYACG 646
Axy_2/2/1       GLDYQLHYSGRSRRCMALLDELAGLHGERLHLHIGDEGGRNDFAALLARPDAGAQIYACG 659
                * .*:***.**.*  ** *:.:** **:*  **:. ** * *:***** * :*:******

CYP116B3        PGRLLAGLEDASRHWPDGALHVEHFTSSLTALDPDVEHAFDLDLRDSGLTVRVEPTQTVL 706
Axy_2/2/1       PERMLSALQQACAHWPEDALRVEHFHSSLATLDPSREHAFEAELKDSGIVVQVPAGQTLL 719
                * *:*:.*::*. ***:.**:**** ***::***. ****: :*:***:.*:* . **:*

CYP116B3        DALRANNIDVPSDCEEGLCGSCEVTVLEGEVDHRDTVLTKAERAANRQMMTCCSRACGDR 766
Axy_2/2/1       AALRGANIDVQSDCEEGLCGSCEVRVLDGAIDHRDVVLTRAEREAGQRMMACCSRAREGR 779
                 ***. **** ************* **:* :****.***:*** *.::**:*****  .*

CYP116B3        LTLRL 771
Axy_2/2/1       IVLEL 784
                :.*.*
```

Residues marked with (*) are identical, (:) indicates strong similarity, and (.) indicates weak similarity

an FMN-dependent reductase that mediates electron transfer from NADH to FMN to an iron sulfur cluster. PDR has an an N-terminal ferrredoxin reductase (FNR)-like NAD(H) binding domain and a C-terminal iron-sulfur [2Fe-2S] cluster domain similar ro CYP116. It should be noted that the gene identified and amplified is similar to a CYP fusion protein reported in the thiocarbamate degradation pathway in *A. xylosoxidans* A8 (Strnad et al., 2011). Even though the *CYP* gene from our strain initially showed high similarity to the *CYP* gene from *A. xylosoxidans* A8 in the conserved regions, the full length nucleotide and the amino acid sequence shared only 88% and 90% identity, respectively, which is still high enough to conclude they belong to the same CYP family. This was confirmed by the fact that the amino acid sequence of this gene was clustered together with the *thc*B family of genes in a phylogenetic tree (Figure 5.2).

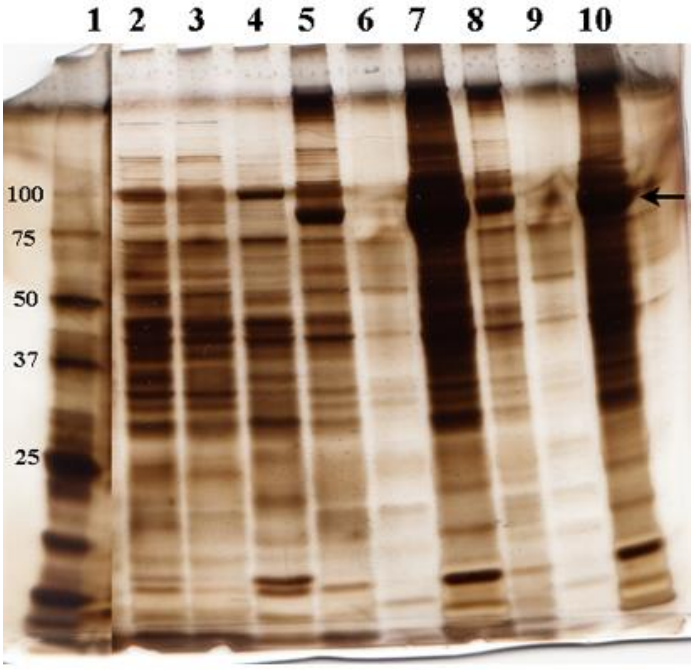
ClustalW alignment with other closely related CYP families (Figure 5.3) denoted that the amino acid sequence of CYP shared about 67% similarity with CYP116B3 from *Rhodococcus ruber*. On the other hand the P450 amino acid sequence from *A. xylosoxidans* MCM2/2/1 shared only 23% similarity with the *eth*B gene encoding the P450 from *R. zopfii* (Malandain et al., 2010), which has been reported to be involved in ETBE degradation. The *thc*B gene has been reported in numerous bacteria, notably in *Rhodococcus* sp. which have been reported to be good xenobiotic degraders that use a broad spectrum of substrates. One such strain, namely *Rhodococcus* sp. strain NI86/21, which can degrade structurally different herbicides, is believed to have a CYP with broad substrate specificity (Nagy et al., 1995). Having said that, the presence of a similar CYP system in *A.xylosoxidans* MCM2/2/1 could be expected to be one of the key enzymes for metabolizing structurally and functionally.

5.4.2. Expression and characterization of CYP

Full length CYP DNA was constructed by PCR suitable for expression in E. coli, was cloned into the vector pET28(a). E. coli cells were transformed and a variety of bacterial growth condition was optimized.

Optimal condition for expression of CYP includes the addition of 100 µM $FeSO_4$ to the growth medium and incubation at 30°C with shaking at 180 rpm for 12 h. From Figure 5.4 it can be observed that CYP was detected in the cell lysate after induced by IPTG and in all fractions (soluble, insoluble, and whole cell) of crude cell lysate indicating that the expressed CYP was present in both the membrane-bound fraction and the cell debris.

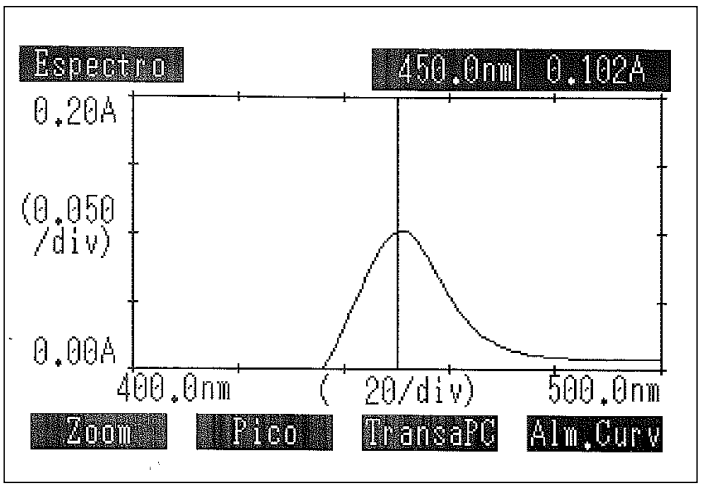
Figure 5.4. Characterization the expression of cytochrome P450 by SDS–PAGE



Lane 1, molecular size markers; lanes 2–4, the soluble, insoluble and whole cell lysate of E. coli with the pET28(a) vector with a non-expressed insert; lanes 5–7, the soluble, insoluble and whole cell lysate of E. coli with the pET28(a) vector containing the 86KDa expressed CYP with IPTG 0.1 mM; lanes 5–7, the soluble, insoluble and whole cell lysate of E. coli with the pET28(a) vector containing the 86KDa expressed CYP with IPTG 0.5 mM lanes 8–10.

To characterize and determine the CYP content, spectral studies were applied. We found that the spectrum showed the characteristic 450 nm peak, indicating that the crude extract preparation contained P450 in the form of reduced P450-CO (Figure 5.5). The CO-difference spectra of the soluble crude cell extracts showed no appearance of P420 peak. The small peak at 420 nm may represent the presence of contaminating hemoglobin, or the breakdown inactive product of P450 (Waterman, 1978; Schenkman and Jansson, 1998). It indicated that CYP has the general properties characteristic of an active cytochrome P450 enzyme. Figure 5.5 shows the typical CO-difference spectra of CYP in the fractions. The CO-difference spectra of the crude cell extracts of the control *E.coli* transformants with empty plasmid (without the P450) showed no appearance of P450 peak. The experiment was carried out twice and the concentration of CYP in the cell lysate was calculated to be 9.6 µM.

Figure 5.5. CO-difference spectra of the expressed cytochrome P450



Absolute spectra of the expressed cytochrome P450. Ferrous–CO protein (peak) is shown in the figure. Typical CO-difference spectra of P450 was examined using the soluble fractions of E.coli crude cell extracts.

5.5. Conclusion

The entire gene sequence of CYP in MTBE and ETBE degrading *A. xylosoxidans* MCM2/2/1 was identified and sequenced. The identified CYP gene sequence presented here is found to be fusion protein with it redox partners belonging to the *thc*B gene family. Further the heterologous expression of the identified gene encoding the *CYP* from *A. xylosoxidans* MCM2/2/1 in *E.coli* was successfully accomplished. This study shows that *A. xylosoxidans* MCM2/2/1 affect the activity of the expressed CYP enzyme with ETBE, MTBE and TBA through NADPH depletion assay. The role of this enzyme in transforming fuel oxygenates through *in-vitro* biotransformation will be studied as part of our effort to understand its catalytic role in *A. xylosoxidans* MCM2/2/1 and also the identified CYP will be exploited for developing a biosensor to study insecticide metabolism or to monitor ETBE pollution in environment in future.

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Chapter 6

Overall discussion and future outlook

Fuel oxygenates are oxygen-rich compounds that are added to reformulated gasoline (RFG) to enhance the octane number, and also to increase the combustibility of the gasoline so as to reduce toxic emissions. Ethyl *tert*-butyl ether (ETBE), is now used as an alternative fuel oxygenate, to Methyl *tert*-ethyl ether (MTBE), which contaminated the ground water largely in Europe and US since last decade. The aim of the PhD thesis is to study the degradation of ETBE by the enriched two bacterial consortia.

Contaminated water samples from two different zones in Spain were used to enrich two new bacterial consortia. These bacterial consortia were able to degrade ETBE and were named as bacterial consortia A and B. The growth of initial inoculated subculture (ECA1 and EB1) in both the consortium was higher than the later subcultures which were used for degradation studies. This may be due to the presence or absence of other compounds in the initial gasoline-contaminated water sample which was used for inoculation. However, the subsequent cultures contained only ETBE as a sole carbon source in consortium B and ETBE and methanol in consortium A. In case of bacterial consortium A, it is evident that the growth of bacterial population needs additional substrates which might be present in the initial contaminated water sample (for example, BTEX compounds) and hence the growth can be impeded as these compounds were not present in the later subcultures. Hence, the enriched bacterial consortium A was tested to degrade ETBE in presence and absence of BTX compounds. Surprisingly, ETBE degradation was negatively regulated in presence of BTX by consortium A. Further, the presence of BTX compounds together with ETBE did not help in increasing the biomass, instead consortium A degraded better BTX compounds in presence of ETBE in an order of preference in the following: toluene > xylene > benzene > ETBE.

On the other hand, bacterial consortium B was able to grow with ETBE as sole carbon source even after withdrawal of methanol in the later subcultures which was used in the initial subcultures as a diluent factor whereas in consortium A methanol was needed for better growth of the same. But still, the consortium B showed a decrease in growth in the later subcultures from the initial subcultures. The initial subculture EB3 was able to remove 50% of 50 mg/L of added ETBE in 6 days.

The bacterial consortium B was also tested for its ability to degrade different and higher concentrations of ETBE in the later subculture EB11. The degradation assay was monitored on day 6 where 50% of ETBE was achieved from previous experiment and on 16 day where maximum growth was found. The bacterial consortium EB11 was able to degrade 500 mg/L and 1000 mg/L of ETBE by about 98% in 16 days. The ETBE concentration of 10000 mg/L

was found to be inhibitory which impeded the growth of the consortium as measured on 16th day. But, a minimal biodegradation in the ETBE concentration was observed on day 6 and day 16 which was difficult to be calculated by our methods as the fiber got saturated at this concentration. *Tert*-butyl alcohol, the primary measurable metabolite of ETBE degradation was identified and also quantified in consortium B which was given in Figure 3.5 and 3.6. In case of consortium A, TBA has not been detected all the subcultures.

Further, the bacterial consortium B was tested for its ability to degrade MTBE and TBA. MTBE is fuel oxygenate used in less proportion in Spain and most widely in other parts of the world. The consortium B cannot grow in presence of either MTBE or TBE or degrade them.

Regarding the bacterial identification, *Xanthomonas* sp. and *Methylophilus* sp. were found in common in both of the presented bacterial consortia In case of consortium A, *Methylophilus*, *Methylovorous*, and *Xanthomonas* plays an important role in ETBE degradation as identified by DGGE. But, 16S rDNA identification in the initial subculture yielded another genus belonging to the *Microbacterium* which was not present in the DGGE profiles. This deletion of certain bacterial population may be as a result of selection through the subcultures. Similarly, the 16S rDNA identification yielded *Xanthomonas* sp., *Herbaspirillum* sp., *Pseudoacidovorax* sp., *Arthrobacter* sp., and *Pseudomonas* sp. with bacterial consortium B in the initial subculture EB1. But, the DGGE profiles contradicted with the 16S rDNA identification which resulted in identifying *Methylophilus* sp in subculture EB3. Presence of methanol during the subculture EB3 could increase the population of methanol utilising bacteria rather than ETBE degrading population or these methanol untilising population are also capable of utilising ETBE are the possible explanations that can be drawn from these results. But finally, in the last subculture used, only two distinct colonies were found on the plates, which were identified as *Xanthomonas* and *Pseudomonas* sp by 16S rDNA identification. This implies that the methnol utitilising *Methylophilus* population did not get selected in further subcultures where methanol was not present Further, these two bacterial isolates would be tested independently and synergistically to prove its ability to degrade ETBE as sole carbon source and also to degrade other fuel oxygenates and the associated contaminants of fuel oxygenates as a continuation of the project.

Of the two consortia enriched and tested here, consortium B gave promising results as it can withhold and grow in higher concentrations of ETBE upto 1000 mg/L than the

consortium A. The consortium B was studied at a proteomic level to understand the basic cellular response to the external stimuli i.e., ETBE. Gel based proteomic analysis gives an insight into all participating enzymes and proteins avoiding the disadvantages in a genomic analysis such as the gap caused by post translational modification. For comparison, consortium B in presence of glucose was utilized as control. As expected, the presence of ETBE generated a vast response in about 1789 proteins. About 38 protein spots were selected and the proteins were identified. Aldehyde dehydrogenase is reported to play an important role in metabolising TBA to HIBA i.e., in the lower pathway of the MTBE degradation in *Mycobacterium austroafricanum* IFP 2012. The overexpression of this protein explains that the consortium B consists of bacterial species that can metabolize ETBE metabolites which is in controversy with the degradation experiments in chapter 3. Consortium B failed to grow and degrade TBA with the time period tested. In such case, we may assume that TBA degradation can be inhibited by presence of other metabolites which may suppress the metabolism of TBA and its associated enzymes.

The other proteins which were identified related to cell cycle, amino acid metabolism, chaperons etc. These proteins when taken together, give an impression that there is increased amino acid synthesis probably, branched chain amino acids (BCAA) which were involved in host defense mechanisms. The failure in identification of other protein spots may be due to the fact that metaproteomics is still in its infancy and large amount of data is required to make the identification. Further, identification will be improved by including translated genome databases of the suspected bacterial isolates. The other protein spots will be identified and quantified in future to get a global idea of the molecular response of the consortium B to ETBE.

The other part of the PhD thesis concentrates on a single bacterial ETBE degrading strain known as *Achromobacter xyloxosidans* strain MCM2/2/1 in which cytochrome P450 gene was previously identified. Full length sequence identification of the CYP gene was achieved through genome walking and sequencing. Further, the identified CYP gene was isolated and successfully cloned heterogeneously to *E.coli.* The enzyme activity of expressed CYP gene was tested by co-difference spectra. Further, the expressed CYP will be tested for its ability to transform ETBE and other fuel oxygenates in-vitro as a preliminary effort in the way to develop a biosensor.

Chapter 7

Conclusions

The following conclusions are drawn form the work conducted in the thesis.

- Enrichment of two bacterial consortia namely consortium A and consortium B against the fuel oxygenate ethyl *tert*-butyl ether was accomplished.
- Bacterial consortium A was found to degrade the added ETBE co-metabolically in presence of methanol up to 51% in 9 days. *Xanthomonas* sp., *Methylibium* sp., *Methylobacillus* sp., and *Methylovorus* sp. were identified as the participating bacteria during ETBE degradation. In addition to ETBE, this consortium degraded benzene, toluene and xylene isomers (BTX) when they were present as the sole carbon source. It was also identified that ETBE degradation is slowed down or inhibited when BTX compounds are present which is a crucial observation for ETBE degradation in the natural environment.
- Bacterial consortium B was found to utilize ETBE as sole carbon and energy source and degrade it to 95-98% with higher concentrations of ETBE up to 100, 500, 1000mg/L without any inhibition. This study, for the first time demonstrates the potential of an isolated bacterial consortium (B) to degrade higher concentrations of ETBE which can be useful in bioremediation of sites heavily contaminated with ETBE. *Pseudomonas* sp. and *Xanthomonas* sp. were found to be the major populations of the bacterial consortium B.
- The global protein expression profiles were studied using proteomic studies. A total of 241 proteins were found to respond differently with respect to the bacteria propagated in ETBE. Aldehyde dehydrogenase which plays an important role in the degradation pathway of ETBE was identified and found to be upregulated 4.2 fold in presence of ETBE. Proteins related to amino acid and energy metabolism were found to be up-regulated. We also observed induction of a number of transport proteins, metabolism-related proteins and chaperons. The use of two different higher concentrations of ETBE in the proteomic study also reflected in the protein profiles of the bacterium grown in 500 mg/L and 1000 mg/L of ETBE. This study gains more significance as it is the first proteomic analysis conducted on a bacterial consortium degrading ETBE.
- The full lenghth gene sequence of cytochrome P450 (CYP) gene was identified in an ETBE and methyl *tert*-butyl ether (MTBE) degrading bacterial isolate *Achromobacter*

xyosoxidans MCM2/2/1. Further, the identified gene was successfully cloned and heterogenously expressed in *E.coli.* The expressed CYP protein was enzymatically active which was confirmed with the CYP-CO assay. The expressed CYP enzyme can be used for invitro biotransformation studies of xenobiotic compounds including ETBE, MTBE and can possibly serve as a biomarker of ETBE degradation.