INVESTIGATION ON INDIGENOUS BACTERIA FOR INDIVIDUAL BTEX DEGRADATION POTENTIALS AND RELATIVE PATHWAYS USED

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ABSTRACT

INVESTIGATION ON INDIGENOUS BACTERIA FOR INDIVIDUAL BTEX DEGRADATION POTENTIALS AND RELATIVE PATHWAYS USED

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Monoaromatic hydrocarbons including benzene, toluene, ethylbenzene and xylene collectively called as BTEX are found in the composition of crude oil and gasoline as an additive and thought to be the most serious contaminants of soil and groundwater. It is expected that indigenous bacteria isolated from petroleum hydrocarbon contaminated sites probably have degradation potential for the BTEX compounds. In this study, out of 22, 19 bacterial strains were selected as potential degraders for at least one of the BTEX compounds. The degradation abilities of the bacterial strains were determined by using HS-GC/MS and 9 bacterial strains namely *R. plancticola* Ag11, *S. aureus* Ba01, *S. nematodiphila* Ba11, *A. calcoaceticus* Fe10, *P. koreensis* Hg10, *P. koreensis* Hg11, *S. nematodiphila* Mn11, *A. johnsonii* Sb01 and *M. luteus* Sr11 were chosen as efficient degraders for pathway analyses. Polymerase Chain Reaction (PCR) was first performed for the detection of catabolic genes. PCR results revealed that most of the bacterial strains harbored the *todC1* gene and only two of them carried the *tbmD* gene. To evaluate biodegradation pathways, BTEX intermediates produced during degradation were also analyzed by using GC/MS. The results showed that monooxygenation

pathway was more common within bacterial strains although dioxygenation genotype (*todC1*) was more prevalent. Intermediate analyses and PCR-detection of catabolic genes revealed that benzene was degraded by *R. plancticola* Ag11 and *M. luteus* Sr11 through monooxygenation pathway. Toluene was metabolized through side chain monooxygenation pathway by the strain *A. calcoaceticus* Fe10 and ring monooxygenation pathway by the strain *M. luteus* Sr11. During ethylbenzene degradation by *R. plancticola* Ag11, *P. koreensis* Hg11 and *S. nematodiphila* Mn11 styrene pathway was mainly used. Comparison of PCR-detection and intermediate analysis results led to conclude that new primer sets were required to detect all possible subfamilies of the ring hydroxylating monooxygenase and side chain monooxygenase genes. The study revealed five efficient bacterial strains namely *R. planticola* Ag11, *A. calcoaceticus* Fe10, *P. koreensis* Hg11, *S. nematodiphila* Mn11 and *M. luteus* Sr11 with their corresponding pathways for aerobic degradation of the individual BTEX compounds.

Keywords: Monoaromatic hydrocarbons, BTEX, Polymerase chain reaction, catabolic gene

ÖZ

LOKAL BAKTERİ İZOLATLARINDA BTEX DEGRADASYON POTANSİYELİ VE YOLAKLARININ ARAŞTIRILMASI

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BTEX olarak adlandırılan benzen, toluen, etilbenzen ve ksileni kapsayan monoaromatik hidrokarbonlar, ham petrolün yapısında ve ilave madde olarak akaryakıt ürünlerinde mevcut olup toprak ve yer altı sularının en ciddi kirletici maddeleri olarak kabul edilirler. Petrol hidrokarbonlarıyla kirlenmiş bölgelerden izole edilen yerel bakterilerin BTEX bileşikleri üzerinde bozunma potansiyeline sahip olması beklenmektedir. Bu çalışmada, 22 bakteri suşu arasından 19' u en az bir BTEX bileşiği için potansiyel parçalayıcı olarak seçilmiştir. Daha sonra bakteri suşlarının BTEX degradasyon yetenekleri HS-GC/MS ile ölçülmüş ve içlerinden 9 bakteri türü, sırasıyla *R. plancticola* Agll, *S. aureus* Ba01, *S. nematodiphila* Ba11, *A. calcoaceticus* Fe10, *P. koreensis* Hg10, *P. koreensis* Hgll, *S. nematodiphila* Mn11, *A. johnsonii* Sb01 ve *M. luteus* Sr11 bakterileri etkin BTEX parçalayıcısı olup bozulma yolağı analizi için seçilmiştir. Bu nedenle Polimeraz Zincir Reaksiyonu (PZR) ile katabolik genlerin saptanması gerçekleştirildi. PZR sonuçları, bakteri kökenlerinin çoğunun *todC1* genini barındırdığını ve sadece ikisinde *tbmD* geninin bulunduğunu ortaya koymuştur. Bakterileirn bozunmada kullandıkları yolakları belirlemek için BTEX degradasyonu sırasında oluşan ara ürünler GC/MS kullanılarak analiz edilmiştir. Sonuçlara göre,

dioksijenasyon genotipi (*todC1)* daha yaygın olduğu halde monooksijenasyon yolağının bakteri suşlarında daha sık görüldüğünü ortaya koymuştur. Ara ürün analizleri ve katabolik genlerin PZR ile saptanması ile birlikte benzenin *R. plancticola* Ag11 ve *M. luteus* Sr11 tarafından monooksijenasyon yolağı ile bozunduğunu ortaya koymuştur. Tolüen ise *A. calcoaceticus* Fe10' da metil monooksijenasyonu yolağı ile ve M. luteus Sr11'de halka mono oksijenasyon yolağı ile metabolize edilmiştir. *R. plancticola* Ag11, *P. koreensis* Hgll ve *S. nematodiphila* Mn11 etilbenzen degradasyonunda stiren yolağını kullanmıştır. Bu çalışma, beş etkin parçalayıcı olan *R. planticola* Ag11, *A. calcoaceticus* Fe10, *P. koreensis* Hg11, *S. nematodiphila* Mn11 ve *M. luteus* Sr11 bakterilerinin her bir BTEX bileşeni için aerobik bozunma yolaklarını ortaya koymuştur.

Anahtar kelimeler: Monoaromatik hidrokarbonlar, BTEX, Polymerase chain reaction, katabolik gen

yeğenim Ömer'e

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ABBREVIATIONS

ATSDR	Agency for Toxic Substances and Diseases Registry
BHB	Bushnell Haas Broth
BLAST	Basic Local Alignment Search Tool
PCR	Polymerase Chain Reaction
BSTFA	Bis (trimethylsilyl) trifluoroacetamide
C12O	Catechol 1,2 dioxygenase
C23O	Catechol 2,3 dioxygenase
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPA	Environmental Protection Agency
GC/MS	Gas Chromatography/Mass Spectrometry
HS-GC/MS	Headspace Gas Chromatography/Mass Spectrometry
IACR	International Agency for Cancer Research
MEGA	Molecular Evolutionary Genetics Analysis
NA	Nutrient Agar
NB	Nutrient Broth
PAHs	Polyaromatic Hydrocarbons
RNA	Ribonucleic Acid
TMO	Toluene Monooxygenation
TOD	Toluene Dioxygenation
TPH	Total Petroleum Hydrocarbon

CHAPTER 1

INTRODUCTION

1.1 Petroleum hydrocarbons, their derivatives and ecological importance

Petroleum-based hydrocarbons are main source of energy in daily life and raw materials for various industries. The usage of petroleum and petroleum products are increasing day by day since world's population continues to grow. World annual petroleum production is predicted to reach twelve million metric tonnes (Koshlaf & Ball, 2017). British Petroleum stated that global oil consumption growth averaged 1.6 million barrels per day (Mb/d), above its 10-year average (1.2%) in 2016 (British Petroleum, 2017). However, it has been estimated that, due to natural and anthropogenic effects between 1.7 to 8.8 million metric tonnes of petroleum are released into the environment annually (Sihag *et al*., 2014). Petroleum hydrocarbon contamination may happen due to industrial effects, municipal runoffs, effluent leakages, spills from underground gasoline storage tanks, during transportation of petroleum, offshore petroleum drilling activity, and accidental spills (Figure 1.1) (Das *et al*., 2011).

Figure 1.1 An aerial view of the oil spilled from Deepwater Horizon platform into the Gulf of Mexico in 2010 (from REUTERS) on the left and a deliberately opened pipeline from Kuwait to slow the assault of American forces during the first Gulf War (by Anne Casselman) on the right side

Many accidental spills and blowouts have occurred since the discovery of petroleum to date as shown in Figure 1.2 As an example to tanker spills Exxon Valdez oil spills in 1989 and large accidental ocean blowout in the BP/Deepwater Horizon in 2010, known as the largest marine hydrocarbon discharge to date due to human activity can be given (Kleindienst *et al*., 2016). Figure 1.2 shows the historical map of worst oil disasters since 1978. The most recent is an explosion on the Deepwater Horizon platform in the Gulf of Mexico on April 20, 2010 led to the largest offshore oil spill in history. Gulf War oil spill, also known as Persian Gulf oil spill is the largest oil spill of all times caused by the deliberate act of man rather than accidental spillage to hamper American forces during the assault and 300 million gallons of oil were released into Persian Gulf (Vallero & Letcher, 2013).

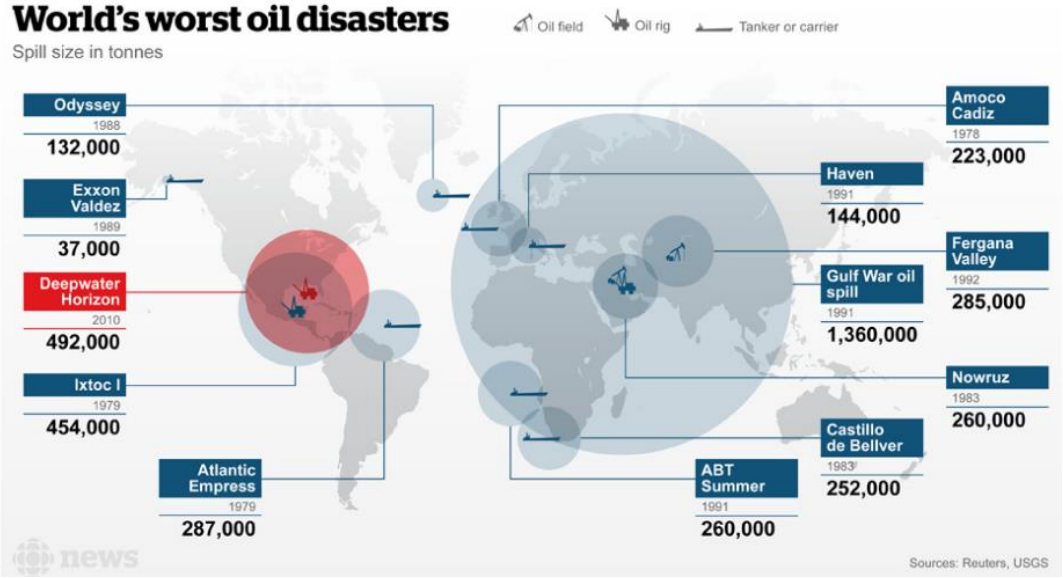


Figure 1.2 The history of the worst petroleum oil disasters in the world (from REUTERS)

Petroleum hydrocarbons are defined as serious pollutants because they are recalcitrant (Costa *et al*., 2012). On earth, all life forms and natural habitats are affected by this pollution (Sajna *et al*., 2015). Once crude oil and other petroleum related products leaked to environment, primary biological effect occurs by blocking the availability of water, oxygen, nutrients and light, affecting soil fertility, plant growth and germination (Martine *et al*., 2010).

1.1.1 Composition of crude petroleum oil

Petroleum is formed over millions of years by thermal decaying of any kind of organic material. Once extracted, petroleum is dark, sticky and viscous liquid (Varjani *et al*., 2007). Crude oil is a mixture containing varying proportion of carbon and hydrogen atoms (Seidel *et al*., 2015). It can be categorized in four broad fractions Figure 1.3; saturates (aliphatic, branched, and cycloalkanes), aromatics (monoaromatics and polyaromatics), resins and asphaltenes (Bagi *et al*., 2013). Saturates with the lack of double bond, represent the highest percentage of petroleum oil. They are divided depending on chemical structures into alkanes and cycloalkanes (Abbasian *et al*., 2015).

Aromatic hydrocarbons have one or more benzene ring coupled with different alkyl side chains (Meckenstock *et al*., 2016). When compared to saturates and aromatics, resins and asphaltenes contain non-hydrocarbon polar compounds like nitrogen and sulfur (Chandra *et al*., 2013).

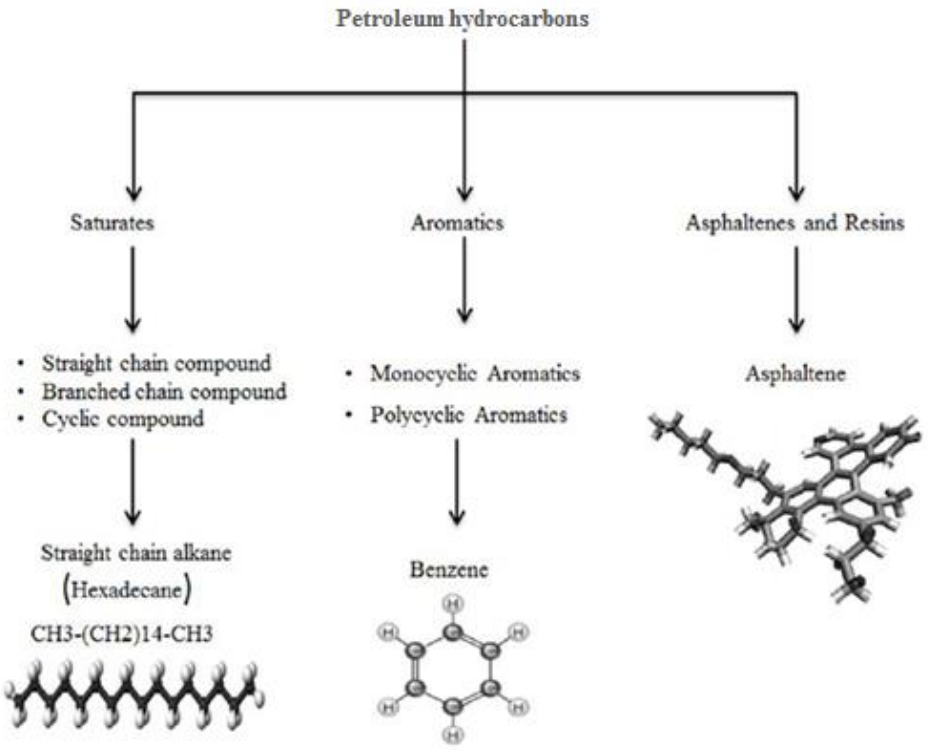


Figure 1.3 The various fractions of hydrocarbons comprising crude oil

Monoaromatic hydrocarbons including benzene, toluene, ethylbenzene and xylene, also called as BTEX are belong to the group of aromatics in the fraction of crude oil (El-Naas *et al*., 2014). Since BTEX are highly volatile, flammable and many hazardous effects to human health an environment, they are thought as primary contaminants in soil, air, groundwater and marine environment (Stasik *et al*., 2015).

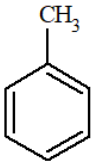
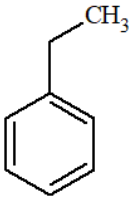
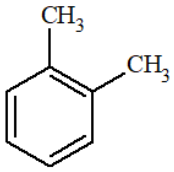
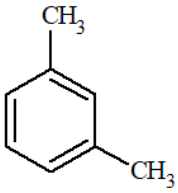
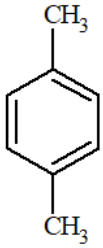
1.1.2 Importance of BTEX

Aromatic hydrocarbons belong to the group of top chemicals by production volume because of their broad range of usage in world's industry (Atlas *et al*., 2015). In particular, monoaromatic hydrocarbons including benzene, toluene, ethylbenzene and *o*-, *p*-, *m*-xylenes commonly called as BTEX are one of the components of crude oil and gasoline and thought to be most significant contaminants for soil, marine environment and groundwater (Abbasian *et al*., 2015). Apart from accidental leakages of crude oil into the environment, BTEX are found in the environment because of the fact that they are in association with anthropogenic emission of combustion processes as well as vehicle exhausts (El-Naas *et al*., 2014). BTEX are also consumed as industrial solvents during the synthesis of many organic compounds such as plastics, synthetic fibers and pesticides (Farhadian *et al*., 2008). Moreover, monoaromatic hydrocarbons are highly volatile, flammable, toxic and carcinogenic (Olajire & Essien, 2014).

1.1.2.1 Benzene

Benzene, also known as benzol, is a colorless liquid with sweet odor and it is highly inflammable (Brugnone *et al*., 1998). It has basic aromatic hydrocarbon ring made up of six carbon atoms bonded to each other and twofold hydrogen atoms bonded to carbon atoms with equal number (Table 1.1) (Fruscella, 2000). Benzene, coming from both natural and environmental sources is found in air, water and soil depending on its intended use (Luttrell & Conley, 2011). Benzene can vaporize into air and relatively water soluble (U.S. Department of Health and Human Services, 2011). Nowadays it is mostly produced from crude oil and classes in the top 20 chemicals by production volume in the US (Agency for Toxic Substances and Disease Registry (ATSDR), 2007). Many industrial companies make use of benzene as precursor molecule to synthesize other hydrocarbon based chemicals such as styrene (for plastics) and cumene (for different resins) and cyclohexane (for nylon and synthetic fibers) (Snyder *et al*., 1993).

Table 1.1 Properties of BTEX compounds (Mitra & Roy, 2011)

Name	Molecular formula	Molecular weight (g/mol)	Density (kg/l)	T_m (˚C)	T_b (˚C)	Vapor pressure (kPa)	Aqueous solubility (mg/L)
Benzene		78.1	0.878	5.5	80.1	10.13	1,780
Toluene		92.1	0.867	-95	110.8	2.93	515
Ethylbenzene		106.2	0.867	-95	136.2	0.93	152
o-xylene		106.2	0.880	-25	144.4	0.67	175
m-xylene		106.2	0.864	-48	139.0	0.80	200
p-xylene		106.2	0.860	13	138.4	0.87	198

Density, vapor pressure, aqueous solubility measured at 20˚C. T_m, melting point; T_b, boiling point

Additionally, it is used in the production of various types of rubbers, lubricants, detergents, pesticides, drugs and dyes (Weisel, 2010). The presence of benzene in the environment is also arisen from natural sources including gas emissions from volcanoes and forest fires. Most people expose to benzene through inhalation, food, beverages and

contaminated drinking water. The exposure to benzene through food or beverages is not as much as through air (ATSDR, 2007). Exposure to very high level of benzene in air (10.000-20.000 ppm) even in short time (5-10 minutes) can cause death. Lower levels can lead to unconsciousness, drowsiness, dizziness, rapid hearth rate, headaches, tremors, confusion. When eating food or drinking liquids contaminated with high amount of benzene, death, coma, tachycardia, sleepiness, dizziness, convulsions, vomiting, irritation of the stomach (Environmental Protection Agency (EPA), 2012). Long-term exposure to benzene may have harmful effects on tissues that produce blood cells, mainly in bone marrow (Zhang *et al*., 2010). It damages the blood production and decreases the crucial blood components. Reduction of red blood cells can cause anemia. Decrease in other constituents in blood may lead to excessive bleeding (Arnold *et al*., 2013). Moreover, exposure to benzene has been linked to a particular type of leukemia known as acute myeloid leukemia (Finkelstein, 2000). It can also cause cancer through attenuating the immune system (Snyder, 2012). Benzene was already determined as carcinogenic to humans by both the International Agency for Cancer Research (IACR) and EPA.

1.1.2.2 Toluene

Toluene, also known as toluol or methylbenzene, is a colorless, clear liquid with its characteristic odor (Haley, 1987). Unlike benzene, toluene contains one methyl group bonded to benzene ring (Table 1.1), (Fabri *et al*., 2000) It is naturally found in the composition of crude oil and in the tolu balsam tree (ATSDR, 2015). Since toluene has good solvent properties, it is widely used in various industrial process such as making paints, leather tanning, rubber, printing, fingernail polish, adhesives, lacquers and paint thinners (EPA, 2016). It is also used during the production of some aromatic hydrocarbon-based industrial products including trinitrotoluene, polyurethane, toluene diisocyanate, benzoic acid, benzoyl chloride (Fishbein, 1985). To increase the octane rate, toluene is added to gasoline along with benzene and xylene (ATSDR, 2015). Depending on where it is used for or produced from, toluene can be found in air, water and soil. Due to its relative solubility in water, toluene, leaked from underground

gasoline storage tanks, can transfer into groundwater and surface waters (Wilbur *et al*., 2004). People are exposed to toluene through breathing, drinking and contacting with toluene containing staffs (Pierce *et al*., 2002). It has been concluded that toluene may have serious effects on nervous system leading to temporary action such as unconsciousness, dizziness and headaches. If a continuous exposure to toluene occurs, that may result in dizziness, headaches and even death by interfering the breath and heart rate (Clough, 2014).

1.1.2.3 Ethylbenzene

Ethylbenzene is a colorless liquid, with an odor similar to that of gasoline (ATSDR, 2015). Compared to benzene, ethylbenzene has ethyl group as side chain linked to basic aromatic ring (Table 1.1), (Banton, 2014). Ethylbenzene is found in the air as 2 parts of ethylbenzene per million parts of air (ATSDR, 2010). It evaporates at room temperatures and it is highly flammable (Cannella, 2007). Crude oil naturally contains ethylbenzene (Henderson *et al*., 2007). In the US, large amount of ethylbenzene is produced and used in many industrial processes like production of styrene and gasoline, paints, pesticides, glues, tobacco products and automotive products (ATSDR, 2015). Ethylbenzene in soil and surface waters can contaminate groundwater because it is relatively soluble in water (EPA, 2015). People are exposed to ethylbenzene by inhalation and drinking (Tang *et al*., 2000). For humans, eye and throat irritation can be seen in the short-term exposure to high concentration of ethylbenzene and this may result in vertigo and dizziness (Saillenfait *et al*., 2003). It was stated by the IARC that long-term exposure to ethylbenzene may lead to cancer in humans (IARC, 2011).

1.1.2.4 Xylenes

The terms xylene, xylenes, and total xylenes can be used interchangeably since there are three forms of xylene depending on two methyl groups binding to different positions on benzene ring; *ortho*-xylene, *meta*-xylene and *para*-xylene (Table 1.1). The term total xylenes encompass all tree forms of xylene and mix xylene also contains 6-15% ethylbenzene (Denayer *et al*., 2011). Xylene, also known as xylol or dimethylbenzene, is

mainly produced synthetically but is it also found naturally in crude oil (Kandyala *et al*., 2010). Xylene is a colorless, flammable liquid with sweet odor. By production volume, xylene is among the top 30 chemicals in the US and it is primarily used as solvent in the leather, cleaning, printing and rubber industry (ATSDR, 2005). It is also used in the production of chemical compounds such as plastics (Jiang *et al*., 2015). People are exposed to xylenes at 3.7 ppm in air and 0.53-1.1 ppm in water (Niaz *et al*., 2015). Usually, xylene is taken from air by inhalation. In some cases, it can be taken from drinking waters or food if there is a serious oil spills accidentally around the place people live in (Rajan & Malathi, 2014). It has been previously stated that three forms of xylene have similar effect on human health (Jacobson & McLean, 2003). Short-term exposure to xylenes may cause irritation of eyes, throat, nose, skin, disrupting breathe capacity, damaged function of lungs, slow response to a visual stimulus, impaired memory and stomach aches (Chatterjee *et al*., 2005). Long-term exposure to xylene may have number of effects on nervous system including headaches, deficiency in muscle coordination, confusion, dizziness and death (Buesa & Peshkov, 2009). Studies, carried out in animals demonstrate that high level of xylene can cause disruption in the liver and hazardous effects on the nervous system, heart, lungs and kidneys (Schupp *et al*., 2005). According to both the IACR and EPA, there is no clear evidence and acceptable information to say that whether xylene is a carcinogenic or not.

Thus, contamination of air, soil and water with petroleum or petroleum-based products accidentally and by human effects, exposure to BTEX is inevitable. For this reason, development of new technologies for removal of BTEX from environment and attenuation the risk of these compounds, have gained great interest over the last decades.

1.2 Remediation strategies for hydrocarbon contaminated sites

BTEX can migrate tens to thousands kilometer far away from contaminated site by passing through groundwater from air and soil since they are highly soluble in water relative to other petroleum hydrocarbons (Mazzeo *et al*., 2010). These monoaromatic hydrocarbons are very stable molecules due to the lack of an activating group such as

oxygen or nitrogen substituent renders them recalcitrant to degradation (Mitra & Roy, 2011). Alternative methods including physical, chemical and biological treatments have been developed to remediate environment contaminated with BTEX.

1.2.1 Physical-chemical vs. biological methods

Physical and chemical treatments of hydrocarbons are conventional and engineering based methods. They are expensive due to the transportation of large amount of contaminated materials for *ex situ* remediation and cost of soil washing, excavation, chemical inactivation and incineration (Farhadian *et al*., 2008). Alternative physico-chemical remediation strategies are dilution, dispersion, volatilization, sorption and abiotic transformations etc. (Chandra *et al*., 2013). The high costs and inefficient yield of these conventional physical and chemical techniques have promoted the development of alternative strategies for *in situ* applications, especially based on biological remediation capabilities of plants (Singh & Jain, 2003) and microorganisms (Farhadian *et al*., 2008). Bioremediation, a green technology can be defined as using of living organisms to degrade or remove pollutants from contaminated sites. It is an environmentally friendly, efficient, economic and versatile technology.

1.2.2 Bioremediation of hydrocarbons by microorganisms

Bioremediation is a cutting-edge technology in which microorganisms detoxify, reduce or degrade dangerous organic pollutants to harmless compounds like H_2O, CH_4 and CO_2 and biomass that has no adverse effect on environment (Ron & Rosenberg, 2014). Terrestrial and aquatic systems contaminated with petroleum hydrocarbons can be remediated by microbial bioremediation application (Varjani *et al*., 2015). Different microorganisms play a crucial role in biogeochemical reactions and in many cases they are the only living organisms having ability in the production of modified forms of molecules needed for other organisms in nature. There is a high probability that microorganisms isolated from petroleum hydrocarbon contaminated soil, sediments or water have ability to degrade hydrocarbons (Ron & Rosenberg, 2014). Biodegradation of hydrocarbons is energetically favorable reaction because microorganisms use

hydrocarbons as a carbon sources that is essential for the synthesis of all cellular components (Abbasian *et al*., 2015). Various research studies focusing on hydrocarbon biodegradation have been performed in last decade (Farhadian *et al*., 2008; Sajna *et al*., 2015; Sunita *et al*., 2016).

1.2.3 Microbial degradation of BTEX

Biodegradation of hydrocarbons naturally found in petroleum or produced by synthetically is a complex process (Kim *et al*., 2014). Generally, microorganisms have a huge metabolic diversity making them pervasive in different environmental conditions. Since they are very common in nature, biotechnological applications including petroleum bioremediation can be managed by the utilization of hydrocarbon degrading microorganisms (Atlas & Bragg, 2009). Microorganisms such as bacteria, fungi and algae are capable of BTEX biodegradation in oil contaminated sites (Fritsche & Hofrichter, 2008). Their reported activity in degradation ranged from 0.003% to 100% for marine bacteria (Hollaway *et al*., 1980), 0.13% to 50% for soil bacteria (Mulkins & Stewart, 1974) and 0.6% to 82% for soil fungi (Kostka *et al*., 2011). In BTEX biodegradation, bacteria are known as most active agent and primary degraders (Meckenstock *et al.*, 2016). In several studies, it is reported that combined populations with broad enzymatic capacity are advantageous for the complete degradation of complex mixtures of hydrocarbons such as crude oil in soil or marine environment (Singh & Celin, 2010)

1.2.4 BTEX-degrading bacteria

Bacteria isolated from different environment or found indigenously, are the most prominent agent in hydrocarbon biodegradation in the place contaminated by oil spills or industrial factors (Wackett, 2003). Until today, several bacteria belong to different genera including *Burkholderia, Acinetobacter* (Hendrickx *et al*., 2006), *Pseudomonas, Comamonas, Rhodococcus, Mycobacterium* (Lee *et al*., 2012), *Gordonia, Brevibacterium, Micrococcus* (Jin *et al*., 2013), *Arthobacter, Sphingomonas, Alcaligens, Acidovorax, Bacillus, Marinobacter* (Guzik *et al*., 2013), *Agrobacterium, Ralstonia,*

Cladophialopora, Aquaspirillum, Variovorax, Stenotrophomonas, Bordetella, Bradyrhizobium and *Nocardia* (Jiang *et al*., 2015) have been isolated from soil and marine environments and determined as active petroleum degraders.

Biodegradation of BTEX can be performed under both aerobic and anaerobic conditions. Although comprehensive genetic and metabolic information are available for aerobic BTEX degradation, our knowledge on anaerobic BTEX degradation is still scarce.

1.2.4.1 Anaerobic BTEX degradation

With the increasing number of studies under nitrate-, iron-, or sulphide-reducing conditions the presence of anaerobic bacteria capable of BTEX degradation have also been shown (Weelink *et al*., 2010). Under anaerobic conditions, the first step of degradation mediated by the addition of fumarate to the methyl group to form benzylsuccinate (Widdel *et al*., 2010). The reaction is catalyzed by benzylsuccinate synthase (Figure 1.4). Then in subsequent oxidation reaction, benzyl succinate is converted to benzoyl-CoA an identical intermediate product in anaerobic BTEX biodegradation (Dou *et al*., 2008).

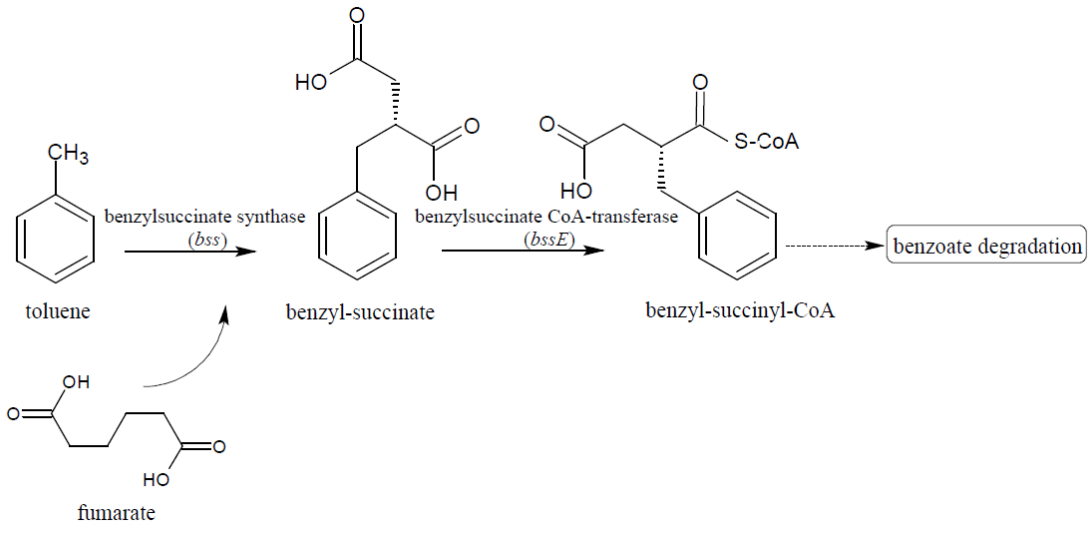


Figure 1.4 Bacterial anaerobic toluene degradation (modified from KEGG)

1.2.4.2 Aerobic BTEX degradation

The most efficient and complete degradation of hydrocarbons is catalyzed under aerobic conditions (Jindrová *et al*., 2002). Figure 1.5 shows the main principles of aerobic hydrocarbon degradation. In the aerobic BTEX degradation, the initial attack of hydrocarbons is an oxidative process that is started by the incorporation of oxygen into hydrocarbon. All these enzymatic reactions are catalyzed by oxygenases (Bombach *et al*., 2010). Step by step, hydrocarbons are converted to intermediate products of central metabolism like tricarboxylic acid cycle (TCA) and further transformed to precursor molecules like pyruvate and acetyl-CoA *(Cao et al*., 2009).

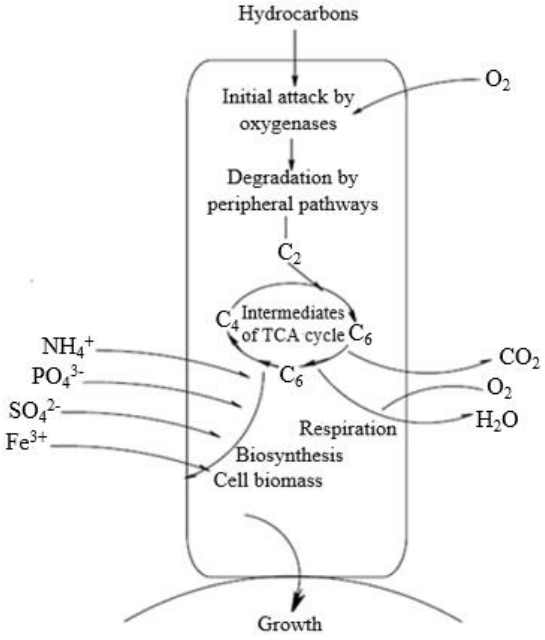


Figure 1.5 Main principles of aerobic hydrocarbon degradation by microorganisms (Olajire & Essien, 2014)

As shown in Figure 1.6, in aerobic BTEX degradation studies, toluene is generally used as a model hydrocarbon since it represents basic characteristics of all monoaromatic hydrocarbons, having benzene ring and methyl group as an alkyl side chain (Harayama *et al*., 1999). As in the case of toluene, the aerobic BTEX degradation is initiated by incorporation of oxygen atoms into aromatic ring or alkyl group by mono or

dioxygenase enzymes (Nicholson & Fathepure, 2005). Catechol or catechol derivatives are produced as identical intermediates in aerobic aromatic hydrocarbon degradation reactions. That is followed by aromatic ring fission through the activity of ring cleavage dioxygenases (Dalvi *et al*., 2012).

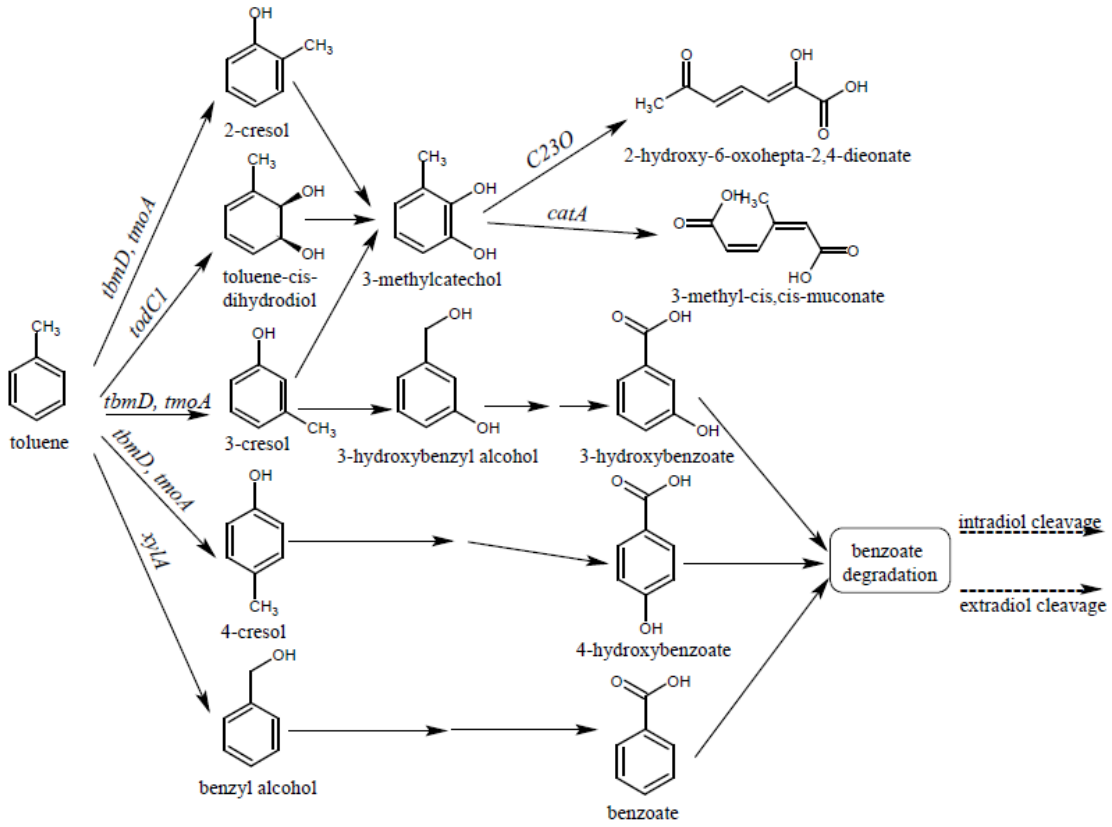


Figure 1.6 Aerobic routes in toluene degradation as a model (modified from KEGG)

Many aerobic bacteria capable of BTEX degradation have been isolated and characterized from different environments such as activated sludge (Deng *et al*., 2017), hydrocarbon contaminated site (Baquiran *et al*., 2012), groundwater from hot springs (Chen & Taylor, 1995) and bioreactor (Li & Goel, 2012).

Table 1.2 Aerobic monoaromatic hydrocarbon-degrading bacteria and biodegradation characteristics (Jeon & Madsen, 2013)

Microorganisms	Substrates	References
Pseudomonas sp. strain CFS-215 *Arthrobacter* sp. strain HCB	Benzene, toluene, and *p*-xylene	Alvarez & Vogel, 1991
Pseudomonas sp. B1 *Pseudomonas* sp. X1	Benzene, toluene, and *p*-xylene	Chang *et al*., 1997
Pseudomonas strain PP01	Benzene, toluene, *p*-xylene	Oh *et al*., 1994
Thermus aquaticus ATCC 25104 *Thermus* sp. ATCC 27978	Benzene, toluene, ethyl benzene, *o*-, *m*-, and *p*-xylene	Chen & Taylor, 1995
Pseudomonas sp. D8	Benzene, toluene, and phenols	Chang *et al.*, 1997
Rhodococcus rhodochrous	Benzene, toluene, ethyl benzene, *o*-, *m*-, and *p*-xylene	Deeb & Cohen, 1999
Pseudomonas putida F1 *Ralstonia pickettii* PKO1 *Burkholderia cepacia* *Pseudomonas mendocina* KR1 *Pseudomonas putida* PaW15	Toluene	Parales *et al*., 2000
Pseudomonas putida F1	Benzene, toluene and phenol	Reardon *et al*., 2000
Burkholderia JS150	Toluene and phenol	Rogers & Reardon, 2000
Bacillus sp. *Pseudomonas* sp.	Toluene, ethyl benzene, and *o*-xylene	Amor *et al*., 2001
Pseudomonas putida F1	Benzene and toluene	Alagappan & Cowan, 2004
Ralstonia pickettii PKO1	Toluene	Demir, 2004
Rhodococcus pyrindinovorans PYJ1	Benzene, toluene, and *m*-xylene	Jung & Park, 2004
Planococcus sp. strain ZD22	Benzene	Li *et al*., 2006

1.3 Factors affecting biodegradation of BTEX

Microorganisms are highly sensitive to changes in their surrounding environment. Many factors including type of pollutant, type of microorganisms, availability, volatilization and dispersion into aqueous phase affect biodegradation rate of hydrocarbons (Chandra *et al*., 2013). Furthermore, rate of biodegradation is also influenced by environmental conditions such as pH, temperature, water content, salinity, oxygen availability, carbon and nitrogen source, and other nutrients (Varjani *et al*., 2016). One of the factor influencing the degradation is the type of hydrocarbon (Barathi & Vasudevan, 2001).

The easiness of hydrocarbon degradation can be ordered as follow: straight chain alkanes > branched alkanes > monoaromatic hydrocarbons > cyclic alkanes > polyaromatic hydrocarbons (Das *et al*., 2011). In most cases, complete degradation of polyaromatic hydrocarbons (PAHs) is infeasible (Li *et al*., 2008; Ghosal *et al*., 2016). For successful bioremediation, physico-chemical conditions and bioavailability of hydrocarbons play very significant role. Hydrocarbons have oil-like structure and their hydrophobic behavior makes them unavailable for microorganisms in aqueous environment (Saeki *et al*., 2009). To enhance the availability of hydrocarbons, wide variety of hydrocarbon degraders produce a dispersing agent called as biosurfactant (Table 1.3) (Sotirova *et al*., 2009).

Table 1.3 Biosurfactatns produced by different microorganisms

Biosurfactants	**Microorganisms**	**References**
Sophorolipids	*Candida bombicola*	Daverey & Pakshirajan, 2009
Rhamnolipids	*Pseudomonas aeruginosa*	Kumar *et al*., 2008
Lipomannan	*Candida tropicalis*	Muthusamy *et al*., 2008
Rhamnolipids	*Pseudomonas fluorescens*	Youssef *et al*., 2007
Surfactin	*Bacillus subtilis*	Youssef *et al*., 2007
Glycolipid	*Aeromonas* sp.	Adebusoye *et al*., 2007
Glycolipid	*Bacillus* sp.	Tabatabaee & Assadi, 2005

Biosurfactants renders the hydrocarbons available for microorganisms to utilize it by increasing the surface area. Once the surface tension is reduced by the emulsifying activity of biosurfactans, hydrocarbons are formed in a micelles and these encapsulated microdroplets in the microbial cell surface, can be taken into cell and degraded enzymatically (Rosenberg & Ron, 2013) (Figure 1.7).

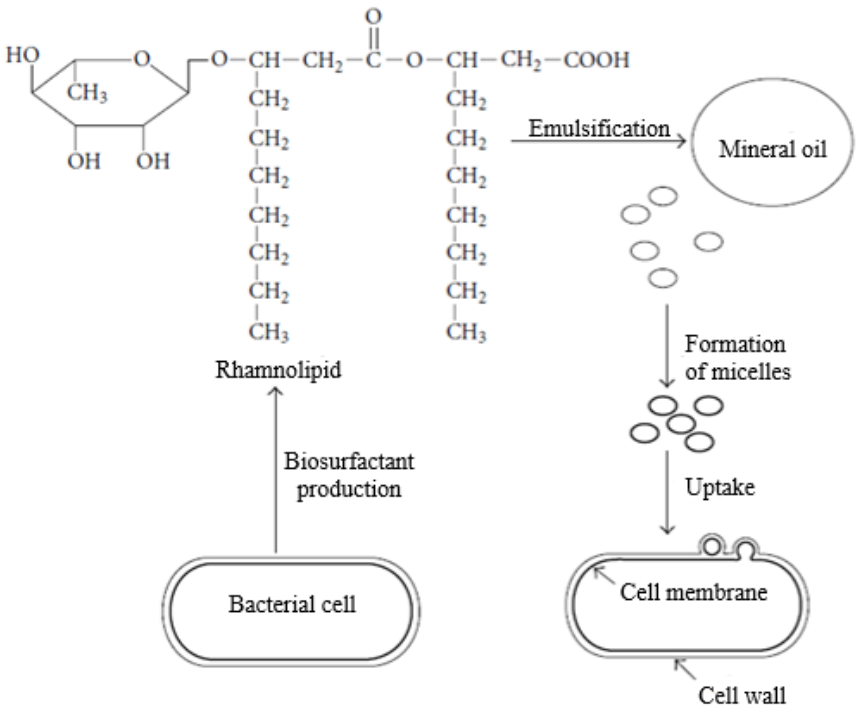


Figure 1.7 The role of biosurfactant (rhamnolipid) produced by *Pseudomonas* sp. in the uptake of hydrocarbons (Rosenberg & Ron, 2013)

1.4 Pathways for aerobic BTEX degradation

Five different initial attack pathways are named depending on the activity of enzyme catalyzing the incorporation of oxygen into different positions (Baldwin *et al*., 2003). All initial attack reactions are achieved by mono or dioxygenase enzyme systems. Aromatic ring can be directly oxidized by monooxygenation; toluene monooxygenation pathway (TMO) (Parales *et al*., 2008) or dioxgenation; toluene dioxygenation pathway (TOD) reactions (Shinoda *et al*., 2004). Depending on the position of incorporated oxygen atom on aromatic ring, TMO pathways are divided into three group as T2MO, T3MO and T4MO (Choi *et al*., 2013). Unlike direct ring oxidation, monoaromatic hydrocarbon degradation can be initiated by the oxidation of alkyl-side chain (TOL-pathway) found in toluene and xylenes (Figure 1.6) (Ikuma & Gunsch, 2013)

1.5 Monitoring catabolic genes and enzymes involved in aerobic BTEX degradation

The genes encoding the aerobic BTEX degradation enzymes are commonly found in operons (TMO and TOD pathways) and often located on catabolic plasmids (TOL plasmid) *(*Nebe *et al*., 2009). After initial oxidation reactions, intermediates are further converted to form catechol (from benzyl alcohol), 3-methylcatechol (from toluene *cis*-dihydrodiol and *m*-cresol) and protocatechuate (from *p*-cresol) which are directed to *ortho* or *meta* cleavage and then metabolized to TCA intermediates (Cao *et al*., 2009). Genes encoding these key enzymes catalyzing both initial oxidation reactions and subsequent ring cleavage reactions can be used as marker genes to detect BTEX-degrading bacteria from BTEX-contaminated sites (Table 1.4) (Lünsmann *et al*., 2016).

Table 1.4 Genes encoding the initial step enzymes of bacterial aerobic toluene degradation

Gene	Enzyme	Reference organism	Pathway	Reference
tom/tbm/tbc	toluene-2-monooxygenase	*Burkholderia cepacia* G4, *Pseudomonas* sp. JS150	TOM/T2MO	Zylstra & Gibson, 1989
tbu/tbh	toluene-3-monooxgenase	*Burkholderia cepacia* G4, *Pseudomonas* sp. JS150	TBU/T3MO	Johnson & Olsen, 1995
tmo	toluene-4-monooxygenase	*Ralstonia pickettii* PKO1, *Burkholderia cepacia* AA1	T4MO	Yen *et al*., 1991
tod	toluene dioxygenase	*Pseudomonas putida* F1	TOD	Zylstra & Gibson, 1989
xyl	toluene/xylene monooxygenase	*Pseudomonas putida* mt-2	TOL	Suzuki *et al*., 1991

In addition to bacterial oxygenases, hydrocarbon degradation can be mediated by heme-containing cytochrome P450 enzymes found in all living organisms (Van Beilen & Funhoff, 2007). Although there are several studies demonstrating the activity of cytochrome P450 enzymes on degradation of straight-chain alkanes in bacteria and detoxification of polyaromatic-based drug metabolites in human, mechanism of possible aerobic BTEX degradation catalyzed by cytochrome P450 enzymes has not been well established (Wang & Shao, 2013).

1.5.1 Initial oxidation enzymes in the aerobic degradation of BTEX

The first common step of aerobic BTEX degradation is the activation of benzene ring by oxidation reactions. As indicated in previous section, there are two distinct oxidation mechanisms namely monooxygenation and dioxygenation. In monooxygenation system, oxygen is incorporated into aromatic ring or alkyl side chain depending on the key enzymes whereas in dioxygenation, only aromatic ring is oxidized by introducing two oxygen atoms (Kovacs, 2003).

1.5.1.1 Ring hydroxylating monooxygenases

Direct oxidation of aromatic ring can be initiated by ring hydroxylating monooxygenases (RHMO) including toluene monooxygenase and phenol hydroxylase oxidizing the *o*-, *-m* and *–p* positions (Leahy *et al*., 2003). Different operons have been described in bacteria having ring monooxygenation ability (Figure 1.8) (Armando & Díaz, 2004). In such cases, bacteria harbor toluene monooxygenase to activate toluene or benzene and phenol hydroxylase to transform phenolic metabolites into catechols (Arenghi *et al*., 2001; Cafaro *et al*., 2004).

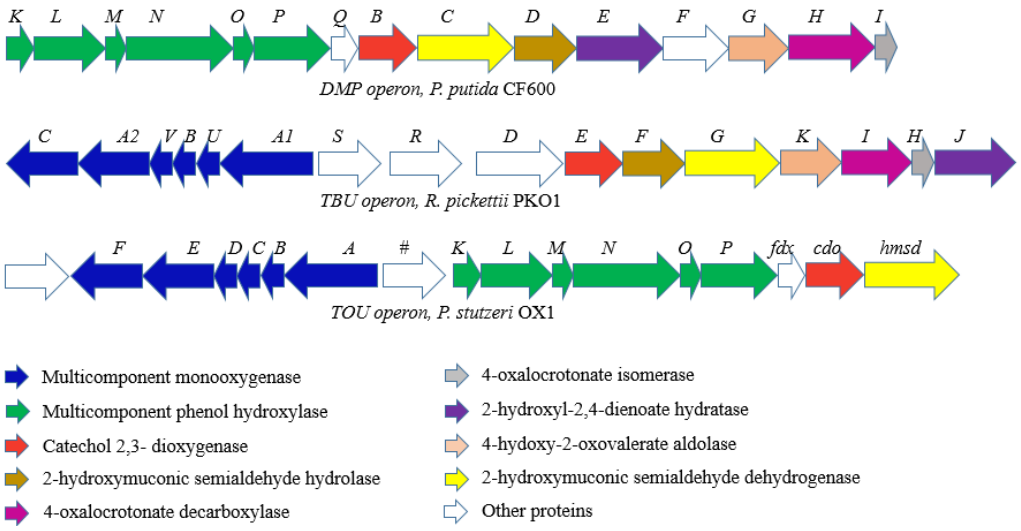


Figure 1.8 Genetic orientation and structure of *dmpKLMNOPQBCDEFGHI, tbuCA2VBUA1SRDEFGKIHJ* and *touFEDCBAKLMNOP-fdx-cdo-hmsd* operons in *P. putida* CF600, *R. pickettii* PK01 and *P. stutzeri* OX1 respectively (Armando & Díaz, 2004)

Various monooxygenases including phenol hydroxylase (Kasak *et al.*, 1993), alkene/aromatic monooxygenase (Coufal *et al*., 2000), salicylate hydroxylase (Bosch *et al.*, 1999) etc., have been studied in different microorganisms (Figure 1.10). In the phylogenetic analysis done by Baldwin *et al*. (2003), catabolic genes encoding the large subunits of toluene monooxygenases from different organisms were aligned (Figure 1.9). According to this alignment, two types were determined differed in their mode of action; ring hydroxylating monooxygenases (Type R) including toluene/benzene monooxygenase and phenol hydroxylase and side-chain monooxygenases (Type T) harbored by TOL plasmid. With some exceptions, ring hydroxylating monooxygenases can be divided into three families depending on the substrate specificity; two families of toluene monooxygenases (R.2 and R.3) and one family of phenol hydroxylases (R.1).

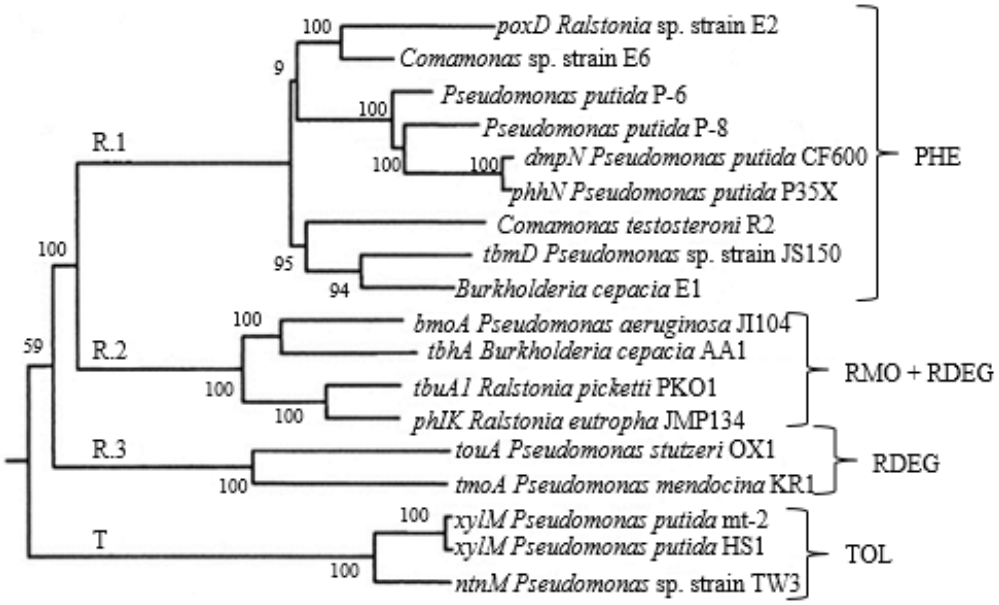


Figure 1.9 Phylogenetic analysis of catabolic genes encoding the large α-subunit of aromatic ring hydroxylating monooxygenases among different bacterial species. R, indicates the type and numbers shows the families. T indicates TOL type.

Multicomponent monooxygenases catalyze the initial oxidation reaction of various monoaromatic hydrocarbons by NAD(P)H-dependent hydroxylation (Leahy *et al.*, 2003). They are composed of three or four components including a hydroxylase protein consisting two or three subunits (αβ or αβγ), a catalytic effector protein, a NADH-dependent oxidoreductase and a ferredoxin with a Rieske-iron center (Powlowski & Shingler, 1994).

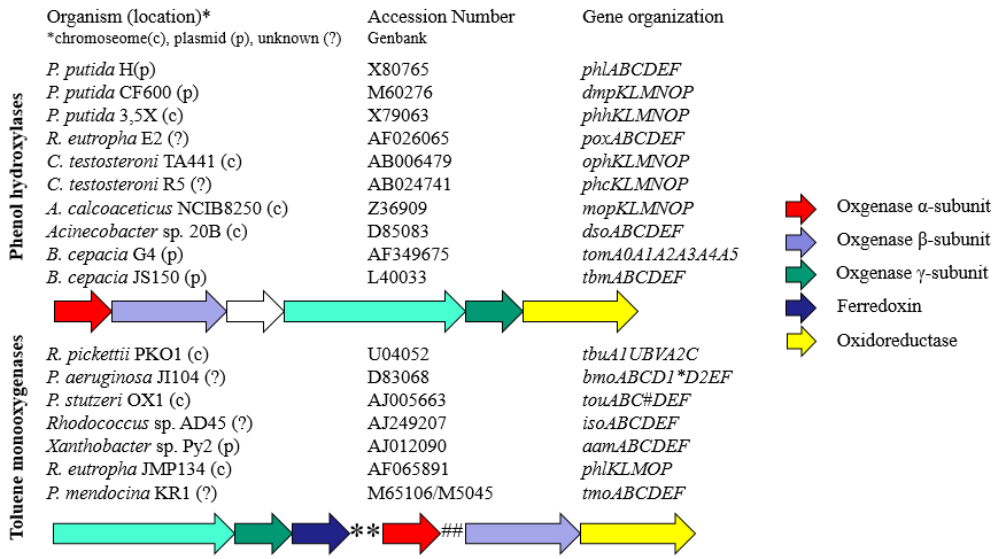


Figure 1.10 Schematic representation and genetic orientation of aromatic RHMO found in different bacteria. Intergenic regions longer than 200 bp in TOU and BMO operons are shown with, * and #, respectively (Armando & Díaz, 2004).

1.5.1.2 Alkyl side chain monooxygenases

The enzymes catalyzing the archetypal toluene degradation pathway (TOL) were shown firstly on plasmid pWW0 of *Pseudomonas putida* mt-2 (Williams & Murray, 1974). Bacteria harboring this ~110-130 kb plasmid, gain ability to grow on *m*-, *p*-xylene, and toluene. Toluene and xylenes can be used as only source of carbon by the activity of genes located on catabolic and regulatory part of plasmid, comprised in 39 kb sequence length (Figure 1.11) (Yano *et al*., 2010).

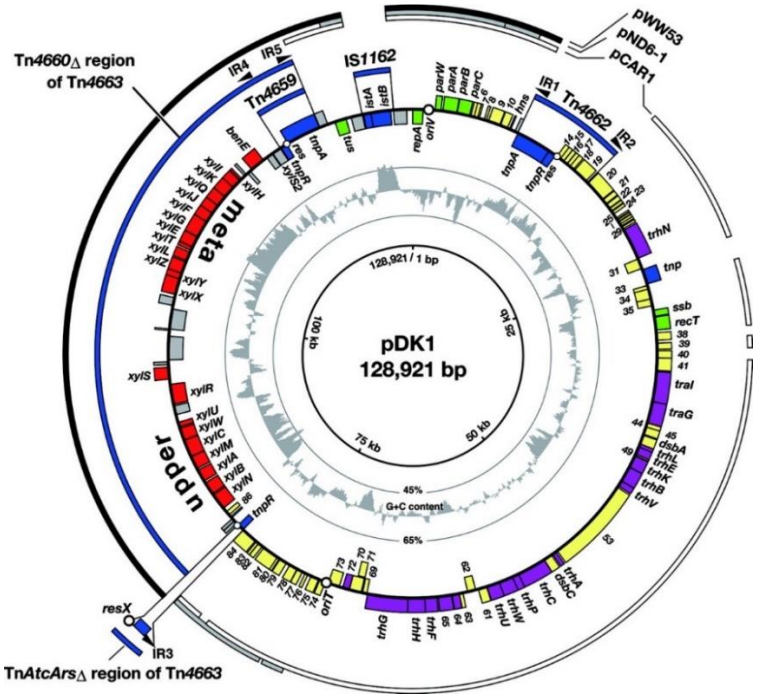


Figure 1.11 Circular map of TOL plasmid pDK1 (Yano *et al*., 2010)

In TOL plasmid, catabolic genes responsible for subsequent degradation of hydrocarbons, are encoded on two distinct operons (Benjamin *et al*., 1991). In the upper pathway, oxidation enzymes are produced by *xylUWCMABN* operon, forming benzoates derivatives while in the lower pathway (Venkata *et al*., 2009), the enzymes catalyzing the oxidation and decarboxylation of benzoates to catechols, extradiol cleavage of catechol derivatives and further conversion to TCA intermediates are encoded on *xylXYZLTEGFJQKIH* operon (Figure 1.12) (Armando & Díaz, 2004).

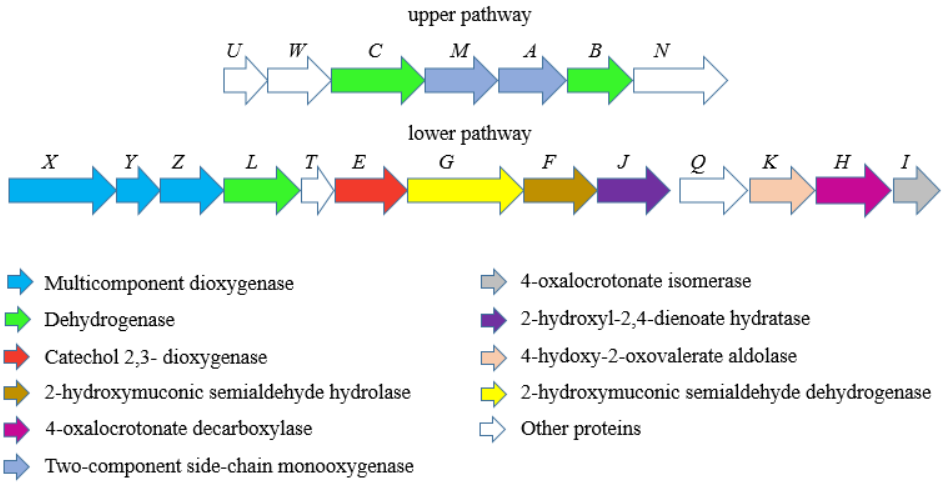


Figure 1.12 Genetic orientation and structure of *xylUWCMABN* (upper pathway) operon encoding the initial attack enzymes, and *xylXYZLTEGFJQKHI* (lower pathway) operon encoding the catechol *meta*-cleavage enzymes in *P. putida* mt-2 (Armando & Díaz, 2004)

1.5.1.3 Ring hydroxylating dioxygenases

Ring hydroxylating dioxygenases (RHDO) oxidizing aromatic compounds produce *cis*-dihydrodiols that are then transformed by NAD-dependent *cis*-dihydrodiol dehydrogenase into catechol derivatives (Friemann *et al*., 2009). Ring hydroxylating dioxygenases need ferrous iron (Fe^{++}), oxygen and reduced pyridine nucleotide from NAD to catalyze the reaction (Gibson & Parales, 2000). They are soluble and multicomponent enzymes consisting two or three distinct proteins including terminal oxygenase and electron transport chains (Figure 1.13).

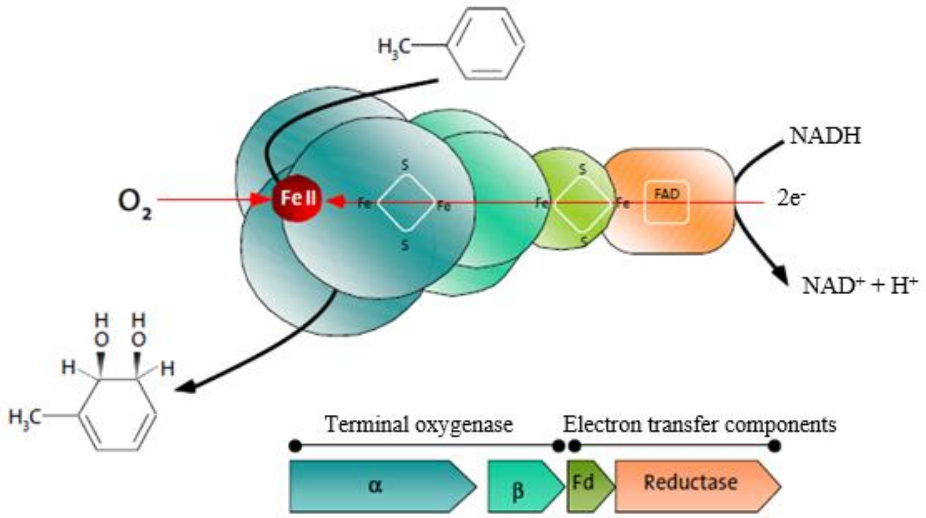


Figure 1.13 Structural organization of toluene 2,3-dioxygenase enzyme (Armando & Díaz, 2004)

In electron transport chain, the electrons are carried from NADPH to the catalytic terminal oxygenase component where the conversion of aromatics into cis-dihydrodiols takes place. Depending on the type of organisms, electron transport chains comprise either two distinct proteins; an iron-sulphur ferredoxin and a flavoprotein reductase or an entire iron-sulphur flavoprotein reductase (Figure 1.13). The terminal catalytic component is an iron-sulphur heteromer protein complex consisting large α-subunit (50 kDa) and a small β-subunit (20 kDa). Substrate binding site and non-heme mononuclear Fe^{++} center constitute α-subunit. There enzymes are also called as Rieske-type non-heme iron oxygenases because all monomers found in the structure of α-subunit and flavoprotein reductase contains Rieske-type [2Fe-2S] clusters (Parales & Ju, 2011).

In RHDO enzyme system, toluene 2,3-dioxygenase, catechol 2,3-dioxygenase and other enzymes for further reactions, are encoded in the TOD operon *todFC1C2BADEGIH* (Figure 1.14) (Armando & Díaz, 2004).

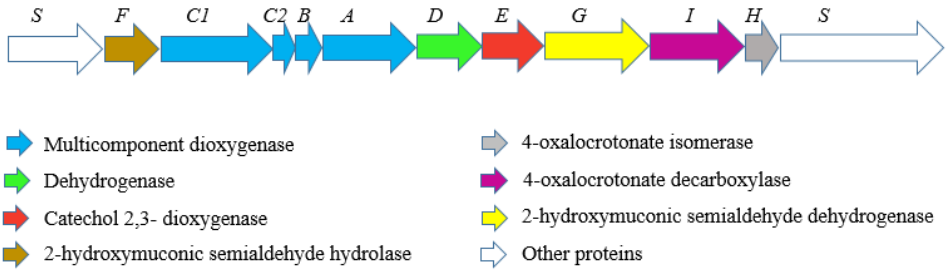


Figure 1.14 Genetic orientation and structure of *todFC1C2BADEGIH* operon in *P. putida* F1 (Armando & Díaz, 2004)

Ring hydroxylating dioxygenases have been classified several times depending on their subunit composition, function and sequence similarity of large α-subunit of terminal oxygenase component. Substrate specificity and evolutionary history of α-subunits are closely related but distinct types are evident. In the first type N, naphthalene dioxygenases are found further divided into two subfamilies (N.1 and N.2) each with various further subfamilies (Figure 1.15) (Baldwin *et al.*, 2003). Biphenyl dioxygenases and simple aromatic hydrocarbon dioxygenases constitute the second type of aromatic dioxygenases including two families designated as D.1 and D.2. Similarity between the sequences of alkyl-benzene dioxygenases and biphenyl dioxygenases have been shown in previously (Furukawa *et al.*, 1993). D.1.B and D.1.C subfamilies of biphenyl dioxygenase from gram-negative organisms and D.1.A subfamily of alkyl-benzene dioxygenases found in the D.1 family. In the second family of D type aromatic dioxygenases, there are two subfamilies of biphenyl dioxygenases from gram-negative organisms (D.2.A and D.2.B) and toluene dioxygenase subfamily (D.2.C) (Figure 1.15).

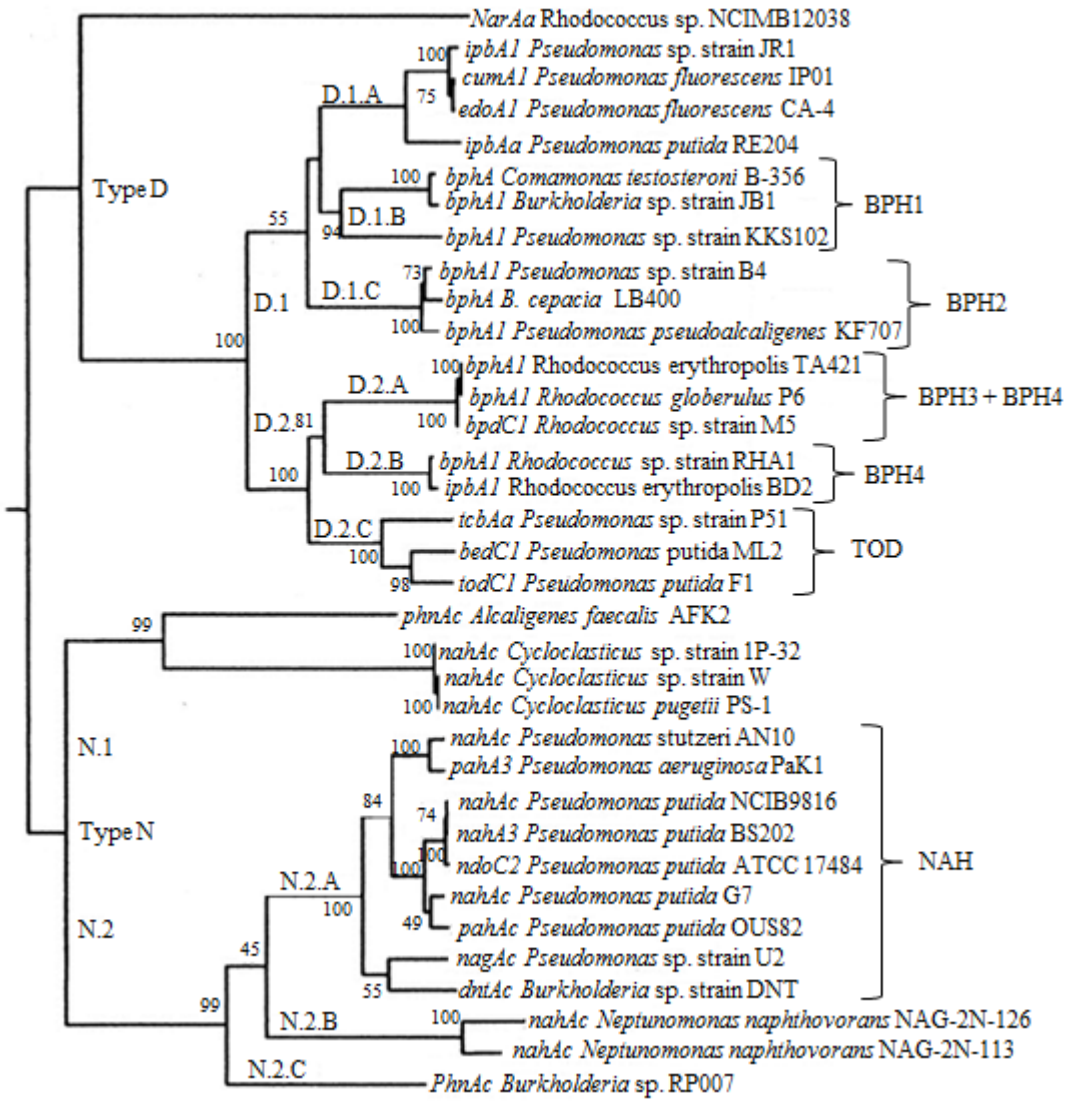


Figure 1.15 Phylogenetic analysis of catabolic genes encoding the large α-subunit of aromatic ring hydroxylating dioxygenases among different bacterial species. N and D indicate types. Numbers show families. Subfamilies are designated as A, B and C. (Baldwin *et al*., 2003).

1.5.2 Aromatic ring cleavage enzymes in the aerobic BTEX degradation

In aerobic degradation of aromatic compounds, catechol or catechol-derived products are formed by the activity of monooxygenases or dioxygenases. These central metabolites are then further metabolized into TCA cycle by ring cleavage catechol dioxygenases (Alfreider *et al.,* 2003). Ring cleavage dioxygenases can be divided into two major group depending on the type of ring fission as intradiol (*ortho*) and extradiol (*meta*) dioxygenases (Fetzner, 2012). Both classes typically utilize non-heme iron as a cofactor. Intradiol catechol dioxygenases contain ferric iron and extradiol dioxygenases harbor ferrous iron (Vetting & Ohlendorf, 2000). While intradiol cleavage enzymes cleaves the aromatic ring between two hydroxyl group, extradiol cleavage breaks the aromatic ring next to one of the hydroxyl group (Vaillancourt *et al*., 2006) (Figure 1.16).

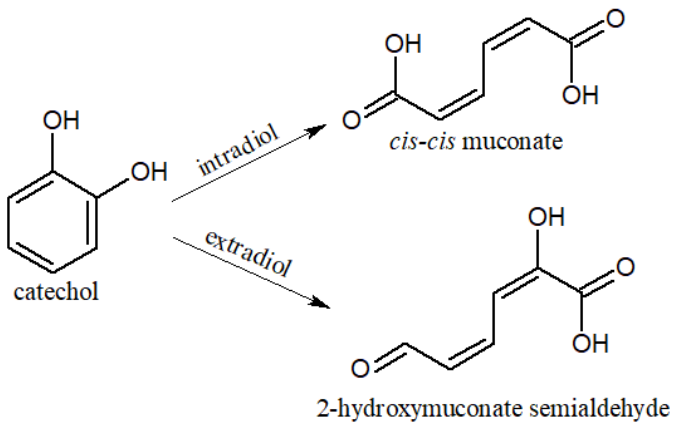


Figure 1.16 Modes of aerobic aromatic ring cleavage (modified from KEGG)

Since catechol and its derivatives are key intermediates in the aerobic degradation of all aromatic hydrocarbons, catabolic genes encoding the catechol dioxygenase enzymes are called as marker genes (Marcos *et al*., 2009). By using marker genes, the presence of aromatic hydrocarbon degrading bacteria can be investigated in contaminated sites (Guo *et al*., 2015). Amplification of these functional genes is generally carried out by PCR by using specific primers and various molecular techniques have been developed to

elucidate the genetic diversity among species (Hendrickx *et al*., 2006; Kasuga *et al*., 2007)

1.5.2.1 Intradiol ring cleavage dioxygenases: *ortho* cleavage

In aerobic BTEX degradation, intradiol cleavage is catalyzed by catechol dioxygenases or protocatechuate 3,4-dioxygenases (Borowski & Siegbahn, 2006). The reactions are ended up with *cis-cis* muconic acid or its derivatives (Whiteley & Lee, 2006). Catechol 1,2 dioxygenases (C12O) contain non-heme non-iron sulfur Fe^{3+} as a prosthetic group. In *Pseudomonas, cat* and *pca* operons are shown on (Figure 1.17). The genes *catA* encoding the C12O convert catechol into *cis-cis* muconic acid (Harwood & Parales, 1996) and further transformation to TCA cycle is catalyzed by different enzymes encoded by *pca* operon, also involved in protocatechuate degradation (Jiménez *et al*., 2002).

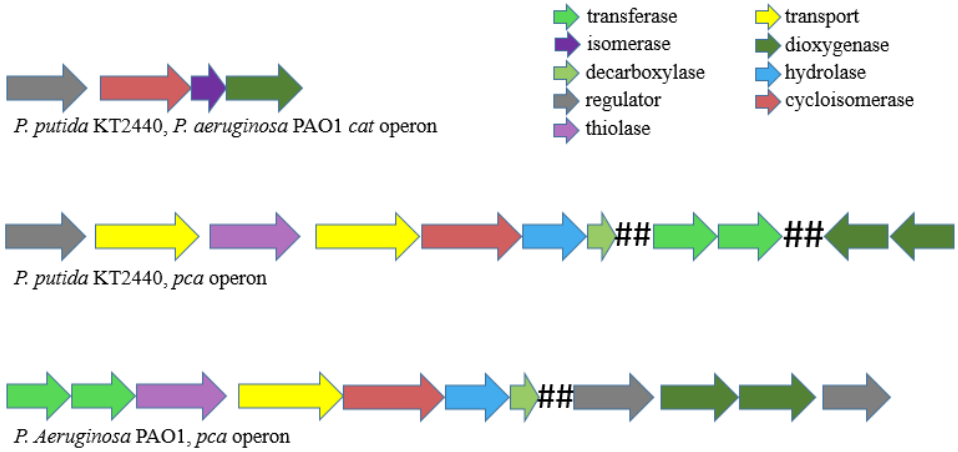


Figure 1.17 Genetic orientation of *catA* and *pca* operons in *P. putida* and *P. aeruginosa* (Jiménez *et al*., 2002)

Depending on their substrate specifity, intradiol catechol dioxygenases can be divided into several subfamilies (Whiteley & Lee, 2006). Structure and function of various catechol 1,2 dioxygenases have been identified so far from different microorganisms

including *Sphingomonas* (Bruijnincx *et al*., 2008*), Rhodococcus, Arthobacter* (Miller, 2008) and *Geobacillus* (Giedraityte & Kalėdienė, 2009), *Pseudomonas* (Guzik *et al*., 2011).

1.5.2.2 Extradiol ring cleavage dioxygenases: *meta* cleavage

Extradiol cleavage reactions are initiated by catechol 2,3-dioxygenases (*C23O*) and protocatechuate 2,3 or 4,5-dioxygenases and resulting in muconic semialdehydes (Wojcieszyńska *et al*., 2012). Genetic organization of the lower pathways including the *C230* related genes were previously shown in Figure 1.12-Figure 1.8. Catechol 2,3 dioxygenases can be divided into several subfamilies; I.2.A, I.2.B and I.2.C within family I.2 and I.3.A and I.3.B within family I.3 (Eltis & Bolin, 1996). Subfamily 1.2.A commonly consists *C23O* sequences from fluorescent *Pseudomonas* bacteria while subfamily I.2.B mainly contains *C23O* sequences from *Sphingomonas* bacteria. Subfamily 1.2.C includes *C23O* genes mainly involved in phenol degradation derived from a wide variety of bacterial genus including *Ralstonia, Comamonas, Pseudomonas* and *Burkholderia.* It was previously reported that *C23O* genes belong to the subfamily I.2.A, are widely distributed in hypoxic, petroleum hydrocarbon contaminated groundwater, making them crucial in the degradation of BTEX under oxygen-limited environments. It was also observed that *Betaproteobacteria* are very common in these environments (Táncsics *et al*., 2010). PCR primer sets for the detection of *C23O* genes belong to the subfamilies I.2.A and I.2.B have been reported in several studies (Mesarch *et al.*, 2000; Hendrickx *et al*., 2006). Family I.3 with its subfamilies I.3.A and I.3.B includes *C23O* sequences from different *Rhodococcus* and *Pseudomonas* species mainly responsible for biphenyl degradation (Hendrickx *et al*., 2006).

1.6 Intermediates produced during aerobic BTEX degradation

Mineralization of BTEX has been reported several times under aerobic conditions (Jindrova *et al*., 2002). However, variety of pathways are considered to be responsible for an effective degradation of BTEX compounds directed by two enzymatic systems known as monooxygenases and dioxygenases (Farhadian *et al*., 2008). Therefore,

microbial consortium is involved in many cases for complete mineralization of BTEX (Daghio *et al*., 2015). For these reasons, deciphering the exact degradation pathways by detecting the key metabolites is needed for productive BTEX bioremediation. Different intermediates are produced during the aerobic degradation of benzene, toluene, ethylbenzene and xylenes and each pathway has identical intermediate.

Intermediates in BTEX degradation pathways can be detected by analytical techniques like capillary electrophoresis-UV array detector (Wang *et al*., 2003), solid-phase microextraction followed by high performance liquid chromatography (González *et al*., 2017) and gas chromatography/mass spectrometry (Choi *et al*., 2013).

1.6.1 Intermediates produced during aerobic benzene degradation

Aerobic benzene degradation can be catalyzed by monooxygenases and dioxygenases (Aburto *et al*., 2009). In monooxygenation, one oxygen atom is incorporated into aromatic ring and phenol is produced as intermediate. As for dioxygenation, two oxygen atom is added to benzene ring and cis-dihydrobenzenediol is formed (Hendrickx, *et al*., 2006). Regardless of type of first oxidation reactions, catechol is produced in all degradation pathways for benzene degradation (Marcos *et al*., 2009). Catechol is then further mineralized into TCA cycle by the activity of catechol dioxygenases (Figure 1.18) (Arenghi *et al*., 2001).

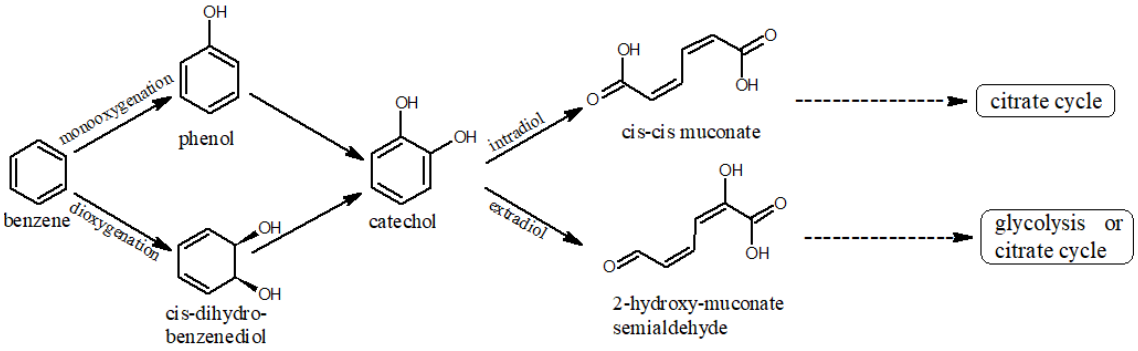


Figure 1.18 Routes of aerobic benzene degradation (modified from KEGG)

1.6.2 Intermediates produced during aerobic toluene degradation

In aerobic toluene degradation three different enzyme systems take place (Parales *et al*., 2008). Direct oxidation of the ring is catalyzed by monooxygenases and dioxygenases. While 2-, 3-, 4-cresol are produced by monooxygenase activity, toluene dihydrodiol is formed by dioxygenase activity (Baldwin *et al*., 2003). Catechol or catechol derivatives are produced as central intermediates and ring fission is occurred as intradiol or extradiol cleavage (Figure 1.19) (Tuan *et al*., 2011)

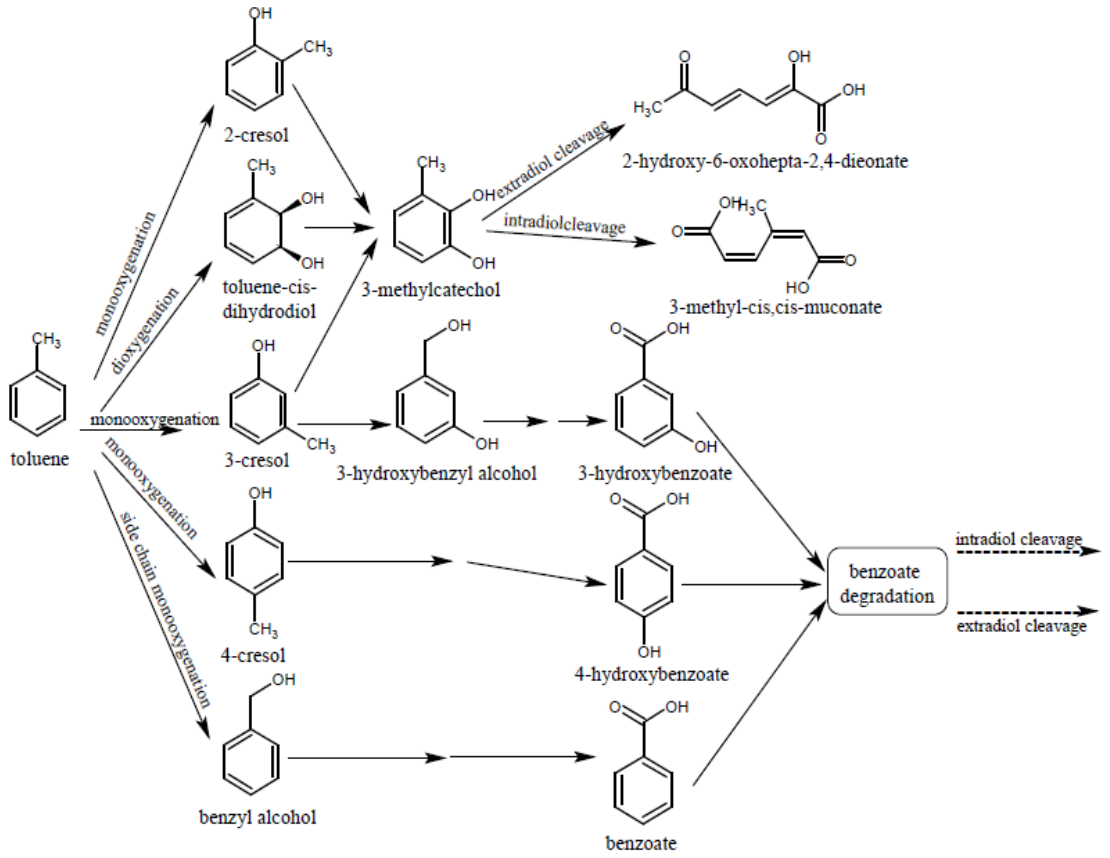


Figure 1.19 Routes of aerobic toluene degradation (modified from KEGG)

1.6.3 Intermediates produced during aerobic ethylbenzene degradation

In the initial step of aerobic ethylbenzene degradation, there are two routes catalyzed by monooxygenases and dioxygenases (Ma *et al*., 2007). In the monooxygenation system, styrene is formed as first intermediate. After that reaction is divided into two

pathways(Choi *et al*., 2013). On the other hand, as a product of dioxygenase activity, cis-1,2-dihydroxy-2,3-dihydroethylbenzene is formed (Figure 1.20). Catechol derivatives are cleaved as intradiol or extradiol by the activity of catechol dioxygenases (Sei *et al*., 1999).

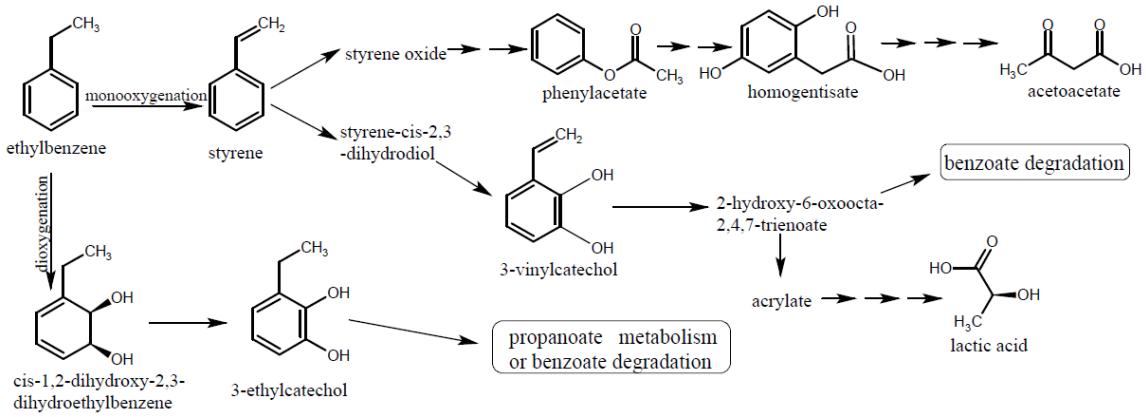


Figure 1.20 Routes of aerobic ethylbenzene degradation (modified from KEGG)

1.6.4 Intermediates produced during aerobic xylene degradation

Unlike benzene, toluene and ethylbenzene degradation, xylene degradation is catalyzed by different enzyme system (Barbieri *et al*., 1993). Toluene/xylene monooxygenase enzyme system incorporate oxygen atom into one of the methyl group bound to aromatic ring (Shim & Wood, 2000). Firstly, methyl benzyl alcohols are formed. Then they are transformed to benzoates and benzaldehydes subsequently (Siani *et al*., 2006). Methylcatechols are formed as central intermediate (Figure 1.21).

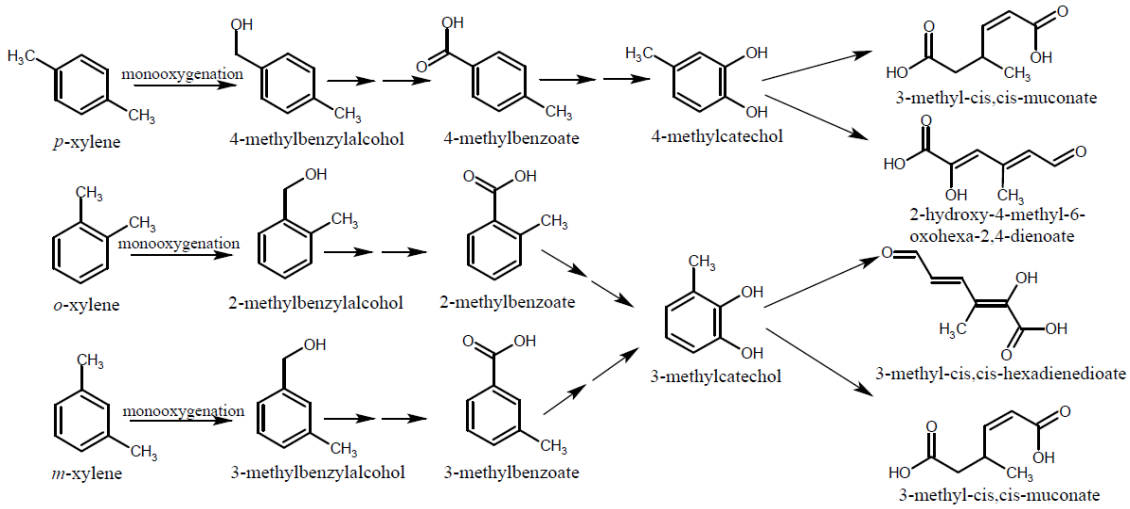


Figure 1.21 Routes of aerobic xylene degradation (modified from KEGG)

1.7 Aim of the study

Aromatic hydrocarbons are common groundwater and soil contaminants due to their frequent usage in industrial processes and leakages of petroleum during drilling, transportation and storage. Among them, monoaromatic hydrocarbons also known as BTEX are relatively water soluble compared to other hydrocarbons allowing them to transfer far away from contaminated sites. Bioremediation seems to be as more advantageous for removing of BTEX from environment due to its cost effectiveness. Biodegradation of BTEX has been well studied and many bacteria have been found as BTEX degraders. However, distribution of the catabolic genes and exact metabolic pathways used during the aerobic degradation of individual BTEX compounds are still unclear. Therefore, the main objective of this study was to evaluate individual BTEX compounds degradation potential of the indigenous bacteria of petroleum hydrocarbon contaminated surface water and to predict the pathways used during degradation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture media

Culture mediums used in the study are described below:

2.1.1 Nutrient broth

Nutrient Broth (NB) (Merck, Germany) was prepared by weighing 8 g for 1 L distilled water. The suspension was mixed and dissolved in appropriate flasks. The solution was sterilized in autoclave at 121°C for 15 min. Sterilized medium was stored at 4°C and routinely used for bacterial growth for further experiments.

2.1.2 Nutrient agar

Nutrient Agar (NA) (Merck, Germany) was prepared by weighing 20 g of medium in 1 L distilled water. NA powder was dissolved completely by boiling and sterilized in autoclave at 121°C for 15 min. After autoclaving and cooling down, NA medium was poured onto petri dishes.

2.1.3 Bushnell Haas broth

Bushnell Haas Broth (BHB) was prepared according to the protocol as follows; 0.2 g magnesium sulfate, 0.02 g calcium chloride, 1 g monopotassium phosphate, 1 g dipotassium phosphate, 1 g ammonium nitrate, 0.05 g ferric chloride in 1 L distilled water (Hendrickx *et al*., 2006). Ingredients were dissolved completely by heating and pH was adjusted to 7.0. The medium then was sterilized by autoclaving at 121°C for 15 min. The BHB medium was routinely used for degradation tests containing BTEX as an only source of carbon.

2.2 Study area and sample collection

Bacterial isolates used in the study were previously isolated from Kızılırmak River in Kırıkkale province from following coordinates; 39°22'16.39"N, 33°26'49.26"E, 890 m to 39°57'22.98"N, 33°25'04.35"E, 679 m in close vicinity to a petrol refinery. 22 bacteria were identified by 16S rRNA sequencing method (Table 2.1) and their hydrocarbon degradation abilities were demonstrated by Onur *et al*. 2015.

Table 2.1 Bacterial isolates used in the study

Bacterial strains	EMBL accession number	References
Pseudomonas plecoglossicida Ag10	KJ395363	Icgen & Yilmaz, 2014
Raoultella planticola Ag11	KJ395359	Koc *et al*., 2013
Staphylococcus aureus Al11	KJ395360	Yilmaz *et al*., 2013
Staphylococcus aureus Ba01	KJ395371	Yilmaz *et al*., 2013
Stenotrophomons rhizophila Ba11	KJ395362	Yilmaz *et al*., 2013
Delftia acidovorans Cd11	KJ209817	Icgen & Yilmaz, 2014
Staphylococcus warneri Co11	KJ395373	Yilmaz *et al*., 2013
Enterococcus faecalis Cr07	KJ395365	Icgen & Yilmaz, 2014
Pseudomonas koreensis Cu12	KJ395364	Icgen & Yilmaz, 2014
Acinetobacter calcoaceticus Fe10	KJ395366	Akbulut *et al*., 2014
Pseudomonas koreensis Hg10	KJ395377	Icgen & Yilmaz, 2014
Pseudomonas koreensis Hg11	KJ395378	Icgen & Yilmaz, 2014
Staphylococcus aureus Li12	KJ395370	Yilmaz *et al*., 2013
Serratia nematodiphila Mn11	KJ395369	Icgen & Yilmaz, 2014
Acinetobacter haemolyticus Mn12	KJ395367	Akbulut *et al*., 2014
Comamonas testosteroni Ni11	KJ395372	Icgen & Yilmaz, 2014
Enterococcus faecalis Pb06	KJ395380	Aktan *et al*., 2013
Acinetobacter johnsonii Sb01	KJ395376	Akbulut *et al*., 2014
Pantoea agglomerans Sn11	KJ395361	Cerit *et al*., 2014
Micrococcus luteus Sr02	KJ395374	Koc *et al*., 2013
Micrococcus luteus Sr11	KJ395375	Koc *et al*., 2013
Acinetobacter haemolyticus Zn01	KJ395368	Akbulut *et al*., 2014

Total petroleum hydrocarbon (TPH) contamination of Kırıkkale-Kızılırmak river were determined previously by Gas Chromatography in Petroleum Research Center at Middle East Technical University (Onur *et al*., 2015).

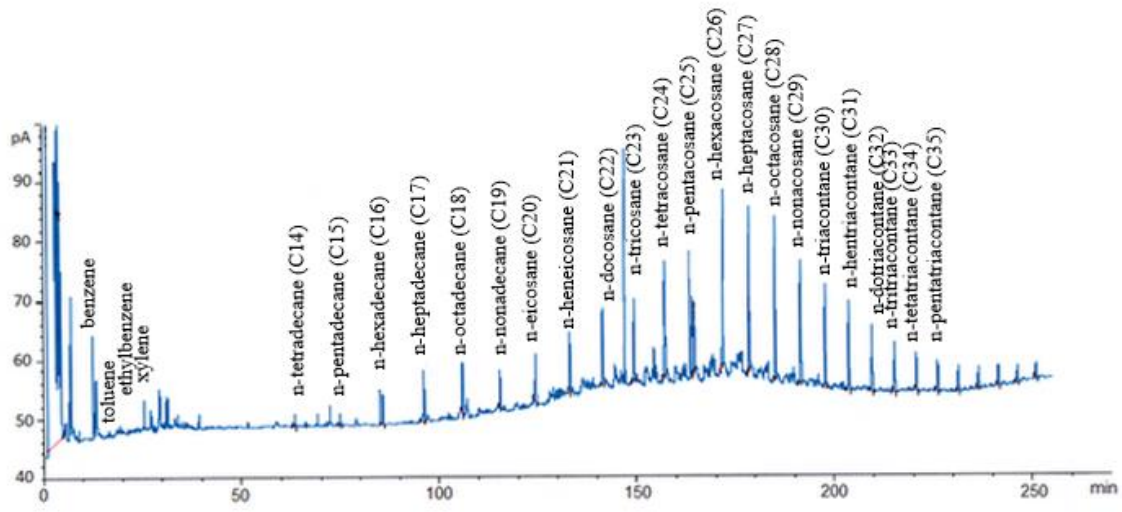


Figure 2.1 GC based total hydrocarbon profiles of Kırıkkale-Kızılırmak river water (Onur *et al*., 2015)

2.3 Selection of BTEX-degrading bacteria

Pre-selection of BTEX degraders was carried out as described in the following procedure (Singh & Celin, 2010) Overnight grown bacterial cells were harvested by centrifugation at 10.000 rpm for 5 min and cell pellets were washed two times with BHB medium to remove NB medium completely. Washed cell pellets were resuspended in BHB medium to obtain approximately 10^8 cell/mL 100 µL resuspended cells were inoculated into 100 mL flasks that contain 20 mL BHB medium and 1 % (v/v) benzene, toluene, ethylbenzene and 0.5 % xylene (Sigma Aldrich, Germany) separately as a sole source of carbon. Three weeks incubation was performed in aerobic conditions at 25°C in orbital shaker at 150 rpm. At the end of incubation, by observing the turbidity of medium bacterial growth was investigated (Figure 2.2).

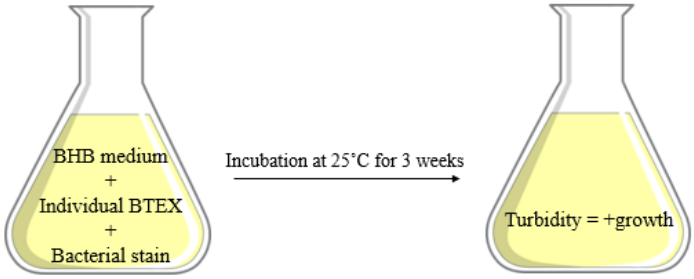


Figure 2.2 Experimental setup for selection of BTEX degraders

2.4 Determination of emulsification indices of bacterial isolates

The emulsification index of each strain was determined by following the protocol developed by Desai & Banat (1997). Each strain was grown in 15 ml eppendorf tubes containing NB medium, for 7 days at 30-37℃. After that the cells were separated by centrifugation at 6000 rpm for 20 min. Supernatants including the biosurfactants were taken. For each strain, 1 ml supernatant suspension and 1 mL benzene, toluene, ethylbenzene and xylene were added separately as carbon source into one cylindrical tube and mixed by vortexing vigorously for 2 min and incubated 24 hours at room temperature. After incubation was completed, the emulsification index was measured by the formula as indicated below.

$$\mathrm{E24}(\%) = \frac{\text{Height of emulsified layer}}{\text{Total height of solution}} \mathrm{x100}$$

2.5 Analysis of aerobic BTEX degradation rates of bacterial isolates

Head Space Gas Chromatography and Mass Spectrometry (HS-GC/MS) system were used at Petroleum Research Center in Middle East Technical University to precisely determine the BTEX degradation rates of bacterial strains which are previously determined as potential BTEX degraders. Following protocol was used with some modifications (Choi *et al*., 2013) Prior to analysis, bacteria, were grown in NB medium for overnight. After incubation bacterial cells were harvested by centrifugation. Cell pellets were washed twice by BHB medium and cell concentration were adjusted to 10^8 cell/mL and inoculated as 100 µL into 40 mL serum bottles containing BHB medium

and 1 % (v/v) benzene, toluene, ethylbenzene and 0.5 % xylene (Sigma Aldrich, Germany) separately as only source of carbon in 5 mL total volume. Samples were incubated at 25˚C, 150 rpm for three weeks in aerobic conditions. After incubation, samples were transferred to headspace vials and residual BTEX were analyzed by using a QP2020 GCMS HS-20 (Shimadzu Corporation, Japan) rtx-624 column with 60 m x 0.25 mm x 1.4 µm dimensions (Figure 2.3). Helium was used as carrier gas. The temperature of the GC oven was adjusted to rise from 45˚C to 90˚C at 8˚C/min after which it was increased to 220˚C at 6˚C/min and held 5 min. Temperature of the sample line and transfer line was 110˚C. Equilibration time was 30 min.

Figure 2.3 Experimental setup for the measurement of BTEX degradation

2.6 Analysis of the aerobic BTEX degradation pathways of the bacterial isolates

In order to investigate degradation pathways used by bacterial isolates, some molecular biological and biochemical tests were performed.

2.6.1 Total DNA extraction

Total DNA extraction for 22 bacterial isolates was done by following protocol developed by (Eltis & Bolin, 1996) with some modifications. Buffers and solutions used in the total DNA extraction are given below:

- Resuspension buffer; 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, (pH:8)
- Lysis solution; 0.2 N NaOH, 1% (mg mL^{-1}) sodium dodecyl sulfate (SDS)
- Neutralization solution; 3 M potassium acetate (pH:4.8)
- 60 µL of Proteinase K (20 mg mL^{-1}) (Sigma-Aldrich, Germany)
- Phenol (saturated with 10 mM Tris-HCl, pH:8), chloroform (equal volume)
- Lysozyme (10 mg mL^{-1} in 10 mM Tris-Cl, pH:8
- RNase (Thermo Fisher, USA)
- 70% Ethanol (Sigma-Aldrich, Germany)
- Absolute isopropanol (Sigma-Aldrich, Germany)

1 mL overnight grown cultures in NB were transferred into 1.5 mL eppendorf tubes and cells were harvested by centrifugation at 8.000 rpm for 5 min. Supernatants were removed and cell pellets were resuspended in 200 µL resuspension buffer by pipetting gently. After that 400 µL lysis solution were added. For Gram-positive bacteria, 5 µL lysozyme (10 mg mL^{-1}) were added into suspension. Eppendorf tubes were incubated at 37°C for 1-3 h until complete lysis was observed. RNase were added with a final concentration of 1 mg mL^{-1} to remove total RNA before DNA separation. 150 µL neutralization buffer were added onto solution and incubated 5 min at room temperature. After centrifugation at 13.000 rpm for 10 min at 4˚C the supernatant containing the genomic and plasmid DNA was transferred to fresh eppendorf tubes. An equal volume of phenol/chloroform was added into suspension and mixed well but gently by pipetting. Centrifugation was applied at 13.000 rpm for 10 min. Two distinct layer was observed separated by thin white layer that contain proteins. Upper aqueous phase containing DNA was harvested carefully by micropipette and transferred to fresh eppendorf tubes. Phenol/chloroform step was repeated until white interphase was disappeared. To remove remaining phenol in solution, an equal volume of chloroform was added and centrifuged at 13.000 rpm for 10 min. Upper layer was collected by micropipette and transferred to fresh eppendorf tubes. In order to precipitate DNA 6:10 volume of pure isopropanol was added into solution and mixed very gently until white precipitate was visible. In some cases, tubes were incubated at -20°C to improve the precipitation yield. After that tubes were centrifuged at 13.000 rpm for 15 min at 4°C. Supernatants were discarded carefully by using micropipette. DNA pellets were washed by 300 µL 70% ethanol and

centrifuged at 13.000 rpm for 10 min at 15°C. Supernatants were removed and tubes were exposed to air drying at 37°C to remove ethanol completely. After air dry, DNA was resuspended in 100-200 µL TE buffer. Total DNA extracted from 22 bacterial isolates was run on 1% agarose gel for 1 hour at 80V. The concentration and purity of isolated DNA was measured by using Colibri Microvolume Spectrophotometer (Titertek Berthold, Germany). The samples were stored at -20˚C and used routinely as template in the PCR-detection of catabolic genes (Figure 2.4).

2.6.2 PCR analysis of catabolic genes in aerobic BTEX degradation

PCR analysis was carried out to amplify the genes encoding the aerobic BTEX degradation enzymes in bacterial isolates. The primers used in the study and their target genes are represented in Table 2.2. Primers were synthesized by Alpha DNA, Canada. First of all, optimization was done for each primer by changing the reference annealing temperature and $MgCl_2$ concentration (Table 2.2). After that optimized conditions were applied to all bacterial isolates. PCR was performed in a 50 µL reaction mixture containing 10 ng template DNA, 10 pmol of forward and reverse primers 200 µM of each dNTPs and 5 mL of 10x PCR buffer and $MgCl_2$ and 0.2 µL *Taq* DNA polymerase (NEB, USA). Amplifications were performed in Thermal Cycler (Bio-Rad T-100, USA) and PCR temperature/time profile used for all primers was an initial denaturation of 5 min at 95 ˚C followed by 30 cycles of denaturation for 1 min at 94 ˚C, annealing for 1 min at the optimized temperature and elongation for 1 min at 68 ˚C. The last step was final extension for 5 min at 68 ˚C. The PCR products were run on 1% agarose gel for 1 hour at 80 V and then stained with ethidium bromide and visualized under UV light. 100 bp DNA ladder (NEB, USA) was used as size marker and standard curves for each agarose gel picture were drawn to estimate the molecular weight of PCR amplicons.

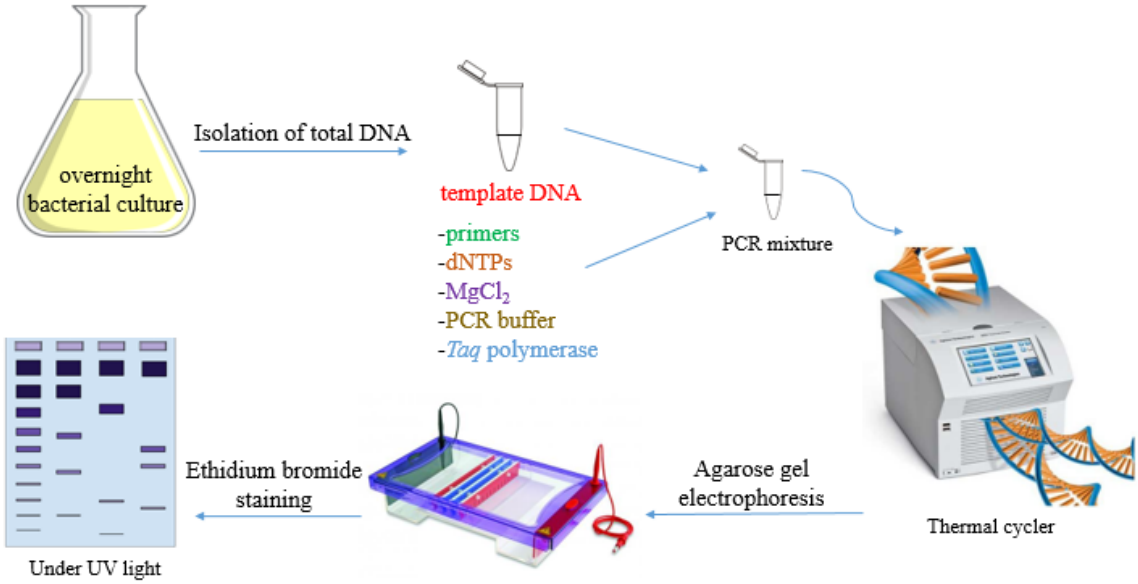


Figure 2.4 Experimental flow in PCR-detection of catabolic genes

Table 2.2 PCR primers and the conditions used in the study

	Target sequence	Substrates	Primer sequence (F-R) (5'→ 3')	Amplicon size (bp)	Annealing temperature (℃)	$MgCl_2$ (mM)	References
tbmD	Subfamily 1 of a-subunits of hydroxylase component of monooxygenases	B, T, E	GCCTGACCATGGATGC(C/G)TACTGG CGCCAGAACCACTTGTC(A/G)(A/G)TCCA	640	65.5	2	Hendrickx *et al*., 2006
tmoA	Subfamily 2 of a-subunits of hydroxylase component of monooxygenases	B, T	CGAAACCGGCTT(C/T)ACCAA(C/T)ATG ACCGGGATATTT(C/T)TCTTC(C/G)AGCCA	505	61.5	2	Hendrickx *et al*., 2006
todC1	Subfamilies D.1.B+D.1.C+ D.2.A+D.2.B+D.2.C of a-subunits of Type D iron–sulfur aromatic dioxygenases	B, T, E	CAGTGCCGCCA(C/T)CGTGG(C/T)ATG GCCACTTCCATG(C/T)CC(A/G)CCCCA	510	66	2	Hendrickx *et al*., 2006
xylA	Electron transfer component of two-component side chain monooxygenases	T, X	CCAGGTGGAATTTTCAGTGGTTGG AATTAACTCGAAGCGCCCACCCCA	291	64	2	Hendrickx *et al*., 2006
tol	Subfamily 5 of hydroxylase component of two-component side chain monooxygenases	T, X	TGAGGCTGAAACTTTACGTAGA CTCACCTGGAGTTGCGTAC	475	55	2	Baldwin *et al*., 2003
nahAc	Naphthalene dioxygenase	T, E	CAAAA(A/G)CACCTGATT(C/T)ATGG A(C/T)(A/G)CG(A/G)G(C/G)GACTTCTTTCAA	377	47	2.5	Baldwin *et al*., 2003
phhN	Subfamily 1 of a-subunits of hydroxylase component of monooxygenases	B, T	GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC CGCCAGAACCA(C/T)TT(A/G)TC	206	49	4	Baldwin *et al*., 2003
C23O-I	Subfamily I.2.C of catechol extradiol dioxygenases	BTEX	CTGGATCATGCCCTGTTGATG CCACAGCTTGTCTTCACTCCA	216	55	2	Mesarch *et al*., 2000
C23O-II	Subfamily I.2.A of catechol extradiol dioxygenases	BTEX	CCGCCGACCTGATC(A/T)(C/G)CATG TCAGGTCA(G/T)CACGGTCA(G/T)GA	242	61.5	2	Hendrickx *et al*., 2006
catA	Catechol 1,2 dioxygenase	BTEX	GCCAACGTCGACGTCTGGCA CGCCTTCAAAGTTGATCTGCGTGGT	282	57	2	Mesarch *et al*., 2000

B, benzene; T, toluene; E, ethylbenzene; X, xylene; F, forward; R, reverse

2.6.3 Sequencing of PCR amplicons

The PCR amplicons were sequenced in one way with corresponding primers by using 3730x1 DNA synthesizer (Applied Biosystems, USA). Then sequences were validated and multiple sequence alignment was performed by using the NCBI Basic Local Alignment Search Tool BLASTn software. The phylogenetic tree with 1000 bootstrap replicates was constructed by Neighbor-Joining method in Molecular Evolutionary Genetics Analysis (MEGA) program version 7.

2.6.4 Detection of intermediates during BTEX degradation

GC/MS analysis was carried out at Petroleum Research Center in Middle East Technical University to detect the key intermediates produced during BTEX degradation. The following protocol developed by Choi *et al*. (2013) was applied with some modifications. Bacterial isolates showing high degradation rates were selected for this analysis. These bacterial isolates were grown in NB for overnight. After incubation, bacterial cells were harvested by centrifugation. Cell pellets were washed twice by BHB medium and cell concentration were adjusted to 10^8 cell mL^{-1} and inoculated as 100 µL into 40 mL serum bottles containing BHB medium and 1% (v/v) benzene, toluene, ethylbenzene and 0.5% xylene separately as only source of carbon in 5 mL total volume. Samples were incubated at 25˚C, 100 rpm for two weeks in aerobic conditions. After incubation, prior to GC/MS analysis intermediate products were extracted. For this purpose, the pH of the medium was adjusted to 3 and hydrocarbons were transferred to organic phase by using 5 mL ethyl acetate repeating two times and then dried over with anhydrous Na_2SO_4. Anhydrous samples were concentrated with evaporator to a volume of 500 µL. After that, extracts were derivatized with 25 µL bis (trimethylsilyl) trifluoroacetimide, trimethylchlorosilane (BSTFA + TMCS) (Sigma-Aldrich, Germany) and incubated at 70˚C in water bath for 20 min. Samples were then analyzed using QP2020 GC/MS (Shimadzu Corporation, Japan) rtx-5MS column with 30 m x 0.25 mm x 0.25 µL dimensions (Figure 2.5). Helium was used as carrier gas. The temperature of the GC oven was programmed to increase from 70 ˚C to 260˚C at 8 ˚C/min and held 5 min; increased to 300 ˚C at 10˚C/min and held 5 min. Total analysis time was 37 min.

Flow rate was 1.2 mL/min. Injection temperature and volume was 250˚C and 1 µL, respectively. Chromatograms were interpreted by using NIST 17 mass spectral library and intermediates were determined.

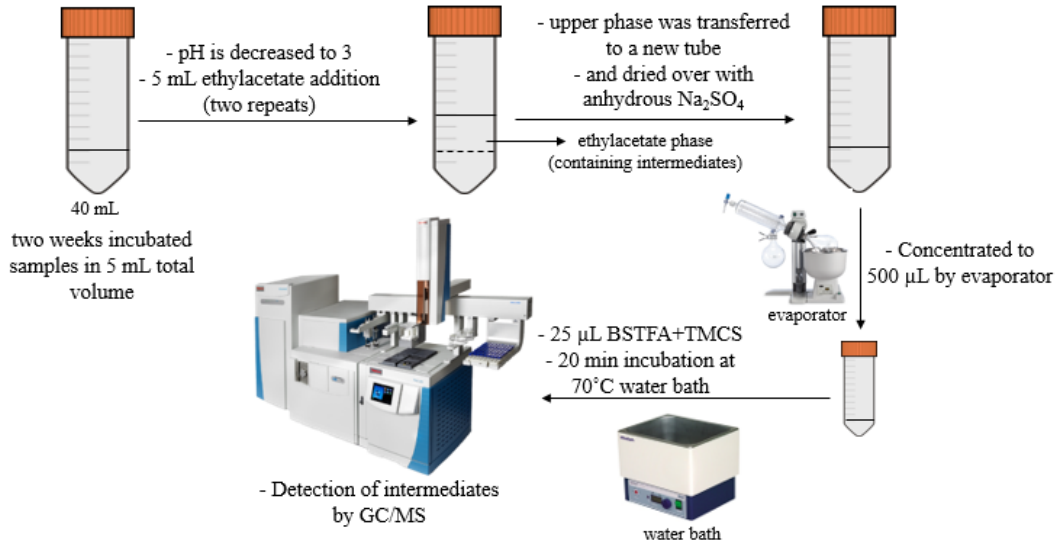


Figure 2.5 Experimental setup for the detection of intermediates in BTEX degradation

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Selection of BTEX-degrading bacteria

BTEX-degrading bacteria are widely distributed in diverse environments contaminated with petroleum hydrocarbons (Chandra *et al.*, 2013). To confirm BTEX degradation, bacterial isolates can be tested in the laboratory by growing them in BHB or MSM medium containing BTEX as sole sources of carbon (Singh & Celin, 2010). A total 22 hydrocarbon degrading bacterial strains previously isolated from Kırıkkale-Kızılırmak river by Icgen & Yilmaz (2014) were further tested for their BTEX degradation ability in this study. Except for *E. faecalis* Cr07, *E. faecalis* Pb06 and *M. luteus* Sr02, the rest of 19 bacterial strains were determined as the degrader of at least one of the BTEX compounds (Table 3.1).

Table 3.1 Selection of BTEX degraders

Bacterial strains	BTEX degradation profiles			
	benzene	toluene	ethylbenzene	xylene
Pseudomonas plecoglossicida Ag10	-	+	-	-
Raoultella planticola Ag11	+	+	+	-
Staphylococcus aureus Al11	-	+	-	-
Staphylococcus aureus Ba01	+	+	+	-
Stenotrophomons rhizophila Ba11	+	+	+	-
Delftia acidovorans Cd11	-	-	+	+
Staphylococcus warneri Co11	+	-	-	-
Enterococcus faecalis Cr07	-	-	-	-
Pseudomonas koreensis Cu12	+	-	+	+
Acinetobacter calcoaceticus Fe10	-	+	+	+
Pseudomonas koreensis Hg10	+	+	-	+
Pseudomonas koreensis Hg11	-	-	+	+
Staphylococcus aureus Li12	-	+	-	-
Serratia nematodiphila Mn11	+	+	+	-
Acinetobacter haemolyticus Mn12	+	+	+	-
Comamonas testosteroni Ni11	-	+	-	-
Enteroccus faecalis Pb06	-	-	-	-
Acinetobacter johnsonii Sb01	-	+	+	+
Pantoea agglomerans Sn11	-	+	-	-
Micrococcus luteus Sr02	-	-	-	-
Micrococcus luteus Sr11	+	+	-	-
Acinetobacter haemolyticus Zn01	-	+	-	-

+, growth; -, no growth

BTEX degradation was shown in the genus of *Pseudomonas, Raoultella, Staphylococcus, Senotrophomonas, Delftia, Acinetobacter, Serratia, Comamonas, Pantoea* and *Micrococcus* and no bacterial strain was found to degrade all BTEX compounds. Many BTEX-degrading bacteria, including different members of the genus have been isolated from diverse aerobic and anaerobic environments (Ghosal *et al*., 2016) however, so far, there has been no evidence for the degradation of the BTEX compounds by the species of *Raoultella, Pantoea* and *Micrococcus* as revealed in this study.

3.2 Determination of emulsification (E_{24}%) indices

In biodegradation, bioavailability of the hydrocarbons is considered as a key limiting factor due to their low aqueous solubility and high hydrophobicity (Olajire & Essien, 2014). To enhance the bioavailability of hydrocarbons, microorganisms produce a

dispersing agent called as biosurfactant (Ron & Rosenberg, 2002). Emulsification index (E24%) test is performed to infer the dispersing activity of biosurfactants (Peele *et al*., 2016). Therefore, BTEX degraders found in this study were further characterized for their E24% indices. The bacterial strains showed E24% activity in between 10 and 67 % for benzene, 8 and 75 % for toluene, 7 and 67 % for ethylbenzene and 21 and 83 % for xylene (Figure 3.1).

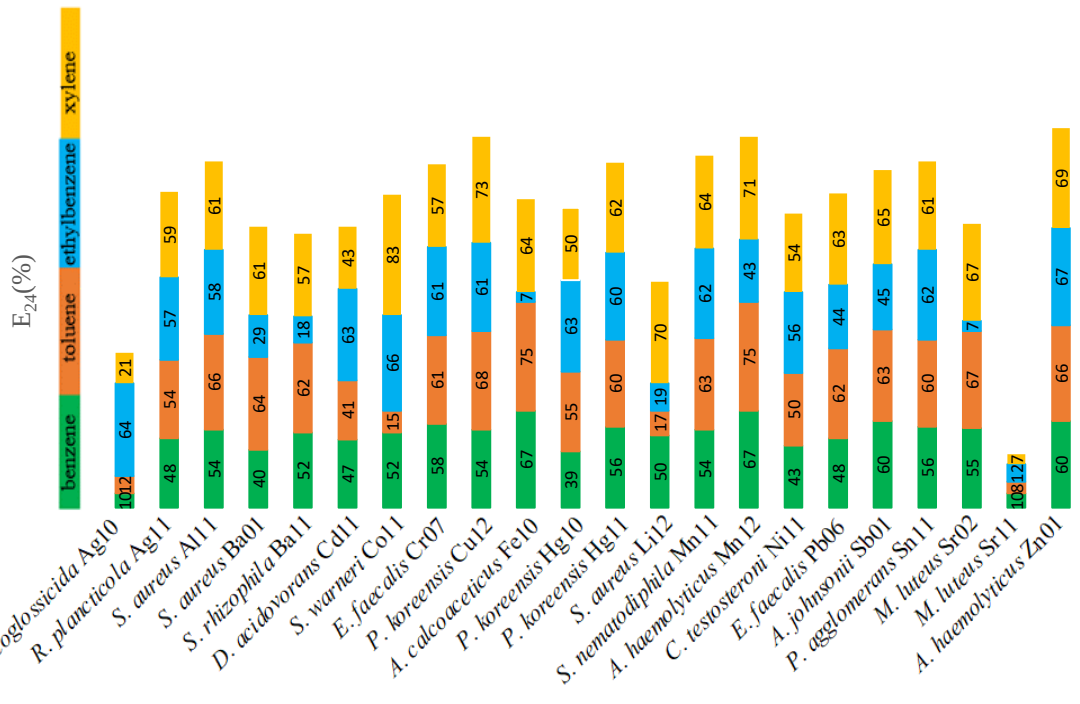


Figure 3.1 E_{24} (%) indices of the bacterial strains

13 strains for benzene and ethylbenzene, 16 strains for toluene and 20 strains for xylene showed more than 50% emulsification activity. Wide variety of bacteria like *Acinetobacter*, *Burkholderia*, *Actinomycetes*, *Bacillus*, *Aeromonas* and *Pseudomonas* have been isolated and defined as biosurfactant producer (Koshlaf & Ball, 2017). Rodríguez *et al*., (2009) indicated that bacteria showing emulsification indices higher than 50% have been designated as potential biosurfactant producers. Therefore, E24% activity of bacteria suggested to be taken into account for efficient bioremediation

process (Varjani, 2017). However, the primary selection of BTEX compounds degraders test and E24% results revealed many contradictions between degradation and emulsification activities. For example, *E. faecalis* which was determined as non-degrader for BTEX, showed higher than 50% emulsification index for each individual BTEX compound. Moreover, the highest E24% index for xylene was measured in *S. warneri* Co11 with the rate of 83% but xylene degradation was not observed in this strain. These results indicated that E24% indices tests alone were not sufficient to get information about the BTEX degradation potential of the bacteria.

3.3 Analysis of aerobic BTEX degradation abilities of bacterial strains

Monitoring of BTEX degradation can be carried out by analytical methods like GC and GC/MS. BTEX-degraders previously selected by preliminary tests were further analyzed by HS-GC/MS to determine the BTEX degradation abilities. Raw data of HS-GC/MS analyses are given in Appendix A.

Benzene degradation was measured by HS-GC/MS in 7 benzene degraders namely *R. plancticola* Ag11, *S. aureus* Ba01, *S. rhizophila* Ba11, *S. warneri* Co11, *P. koreensis* Cu12, *P. koreensis* Hg10, *S. nematodiphila* Mn11, *A. haemolyticus* Mn12 and *M. luteus* Sr11. The initial amount of benzene was 8.12 mg/mL in each test tube After 21 days of incubation, *S. nematodiphila* Mn11 removed 100% of benzene whereas *S. rhizophila* Ba11 and *M. luteus* Sr11 degraded 95% of benzene (Figure 3.2-3.3-3.10). Many bacteria have been demonstrated having the ability to degrade benzene so far (Nicholson & Fathepure, 2005; Padhi & Gokhale, 2017). However, benzene degradation by *S. nematodiphila* and *R. plancticola* was shown for the first time in this study with 100 and 75% degradation, respectively. In addition, there is no study in the literature demonstrating benzene degradation by *S. rhizophila* Ba11 and *M. luteus* Sr11 as high as 95%.

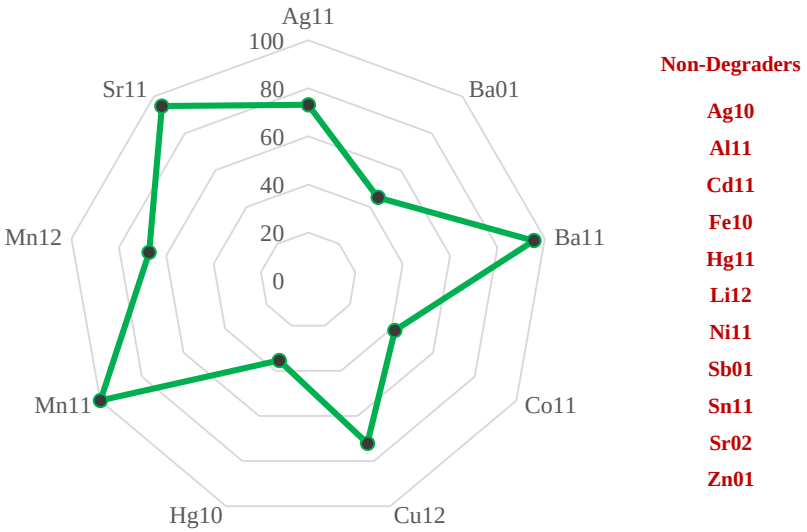
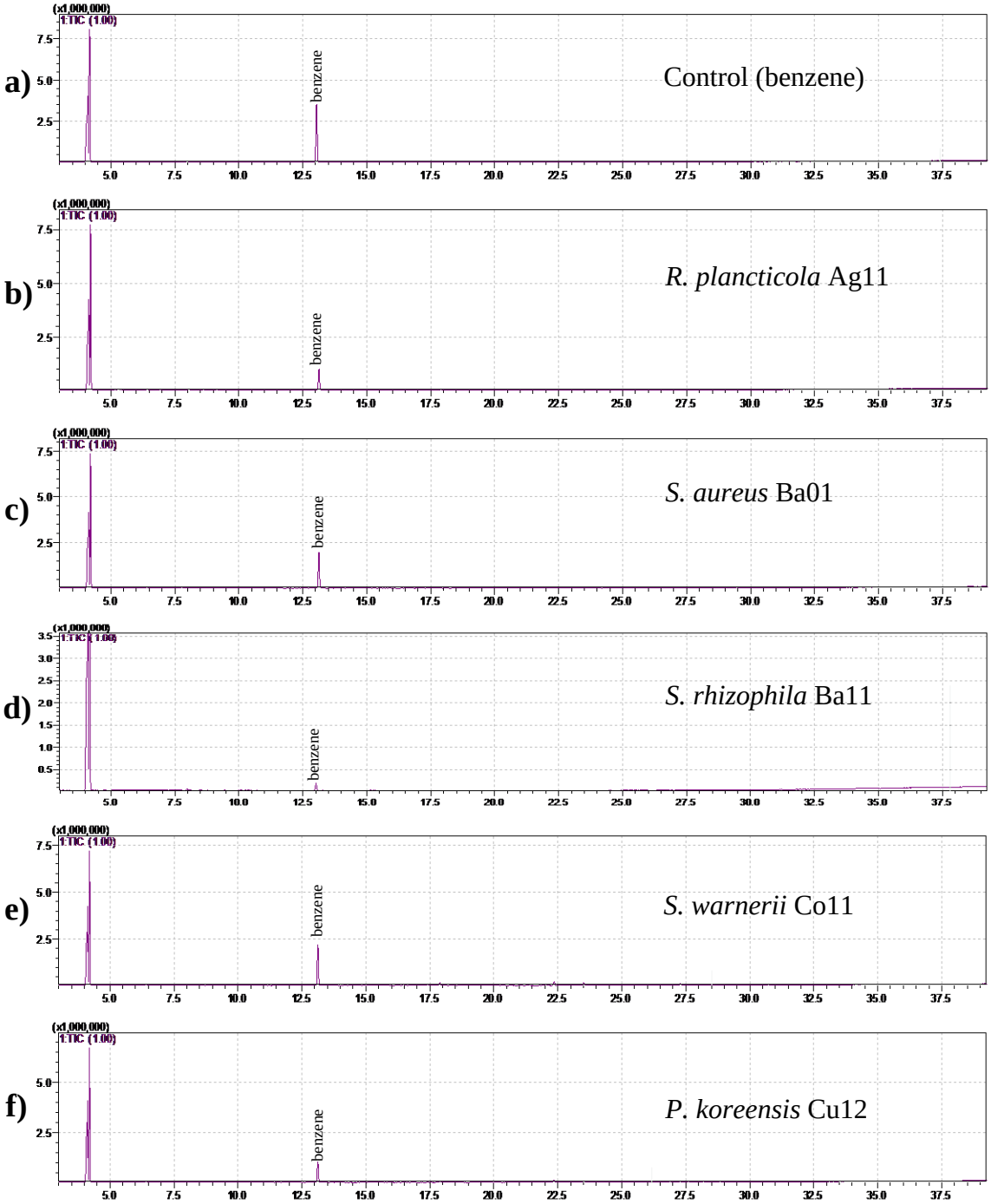


Figure 3.2 Benzene degradation abilities of the bacterial strains and non-degraders obtained from HS-GC/MS analyses


a)
Control (benzene)
b)
R. plancticola Ag11
c)
S. aureus Ba01
d)
S. rhizophila Ba11
e)
S. warnerii Co11
f)
P. koreensis Cu12
benzene

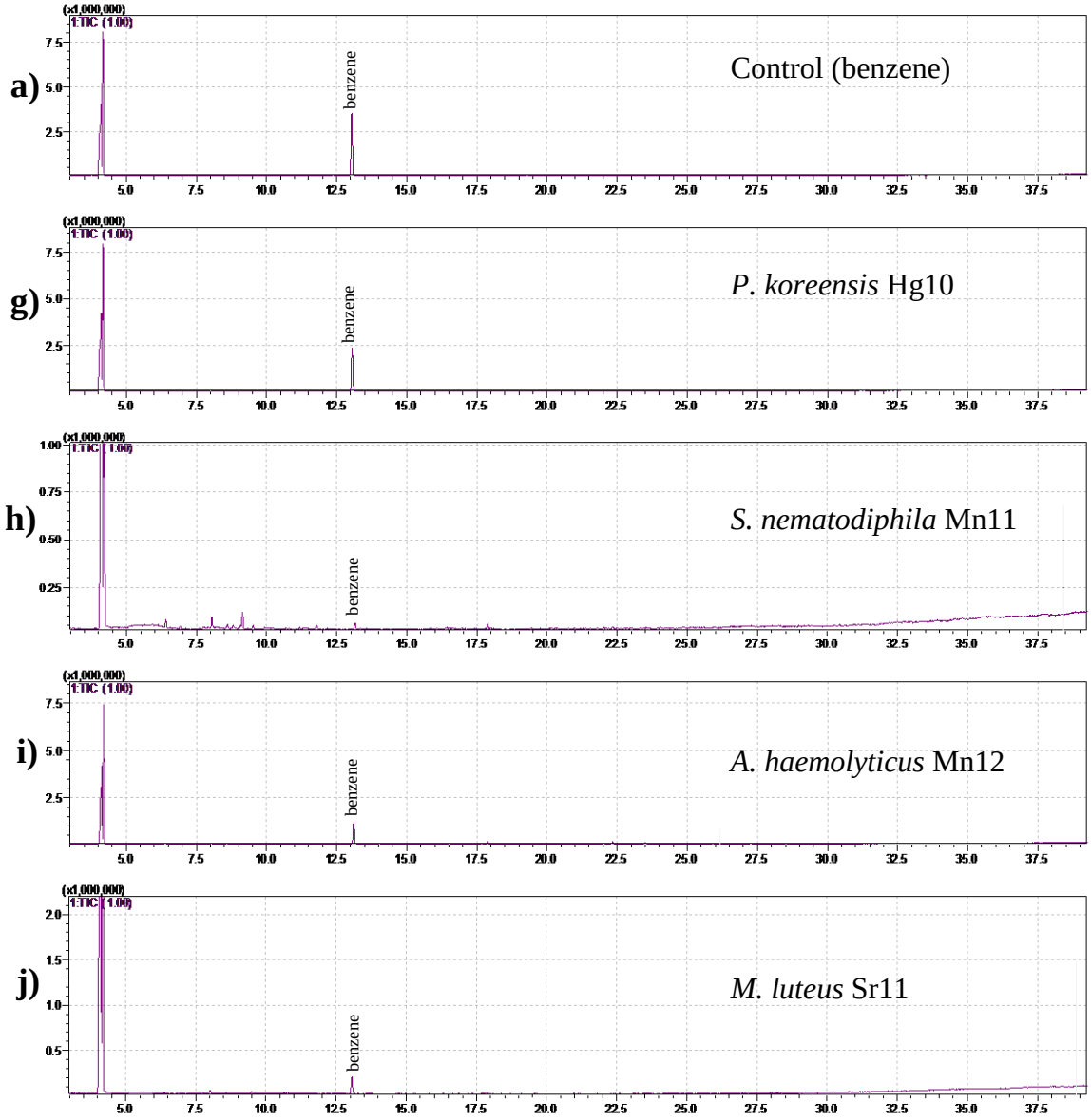


Figure 3.3 HS-GC/MS chromatograms of benzene biodegradation; control without bacterial isolates (a), incubation with *R. plancticola* Ag11 (b), *S. aureus* Ba01 (c), *S. rhizophila* Ba11 (d), *S. warnerii* Co11 (e), *P. koreensis* Cu12 (f), *P. koreensis* Hg10 (g), *S. nematodiphila* Mn11 (h), *A. haemolyticus* Mn12 (i), *M. luteus* Sr11 (j). Abundance factors of analytes are given on the upper left corner of each chromatogram.

Toluene is used as a model hydrocarbon in BTEX biodegradation studies due to its chemical properties (Armando & Díaz, 2004). Therefore, variety of bacteria have been isolated and defined as toluene degraders (Choi *et al*., 2013). By preliminary degradation tests in this study, 16 potential toluene degraders namely *P. plecoglossicida* Ag10, *R. plancticola* Ag11, *S. aureus* Al11, *S. aureus* Ba01, *S. rhizophila* Ba11, *S. warneri* Co11, *A. calcoaceticus* Fe10, *P. koreensis* Hg10, *S. aureus* Li12, *S. nematodiphila* Mn11, *A. haemolyticus* Mn12, *C. testosteroni* Ni11, *A. johnsonii* Sb01, *P. agglomerans* Sn11, *M. luteus* Sr11, *A. haemolyticus* Zn01 made toluene most prominent hydrocarbon within other BTEX compounds. Degradation of toluene was measured by HS-GC/MS and degradation rates reached up to 100 % (Figure 3.4-3.5-3.10). The initial amount of toluene was 5.26 mg/mL in each test tube. The most efficient bacterial strains were *S. aureus* Ba01, *A. calcoaceticus* Fe10 and *P. koreensis* Hg10 removed 100 % of toluene and *M. luteus* Sr11 degraded 99% of toluene. Toluene degradation by *R. plancticola* Ag11, *C. testosteroni* Ni11 and *P. agglomerans* Sb01 were also demonstrated for the first time in this study.

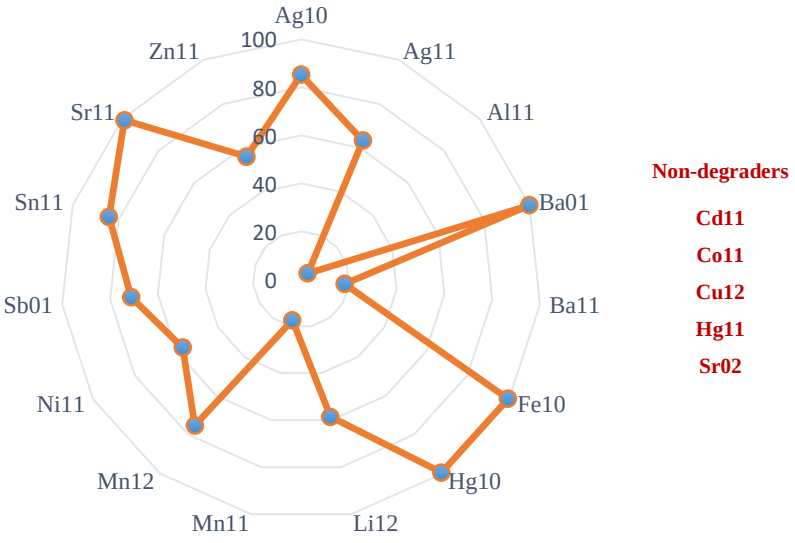
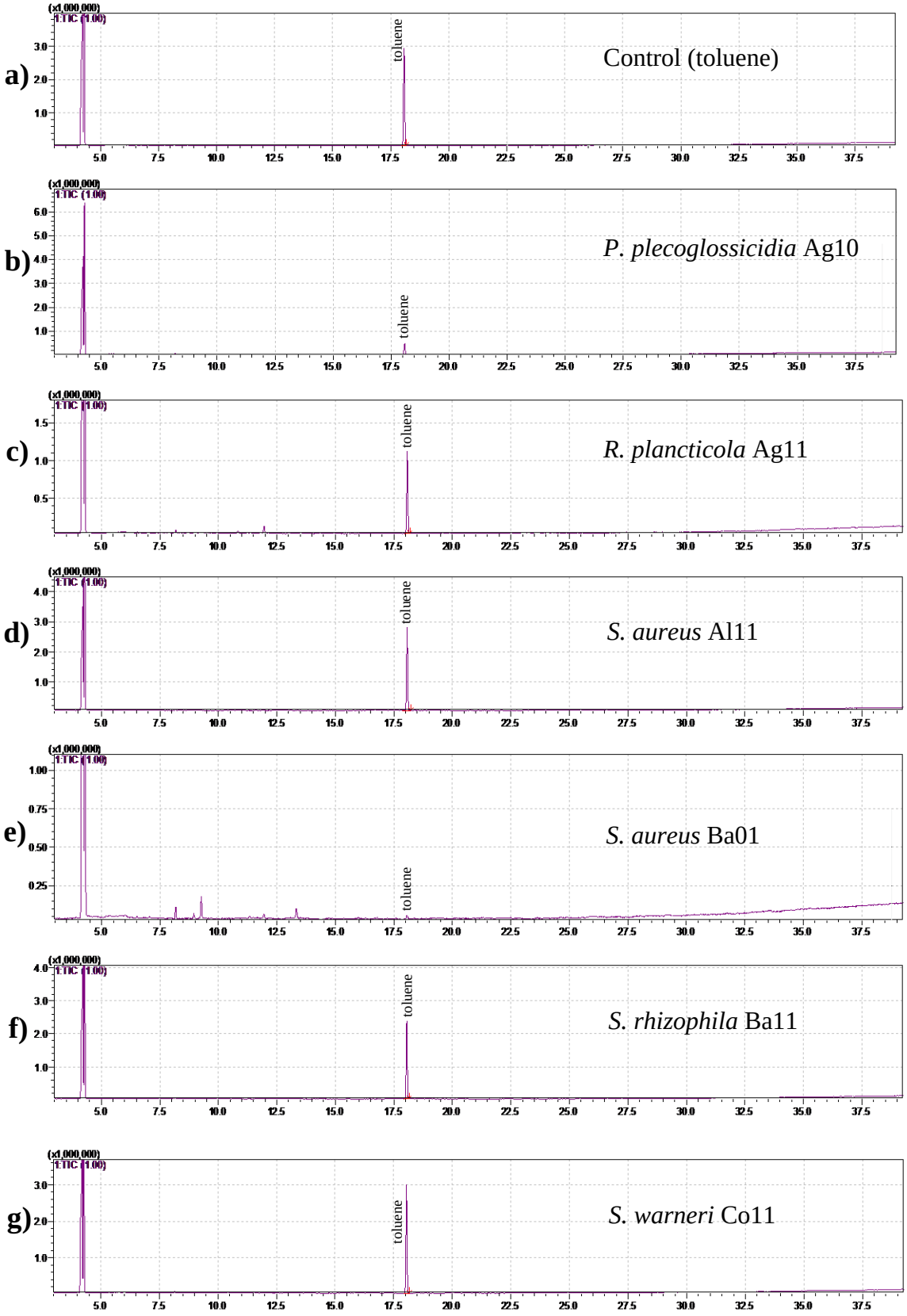
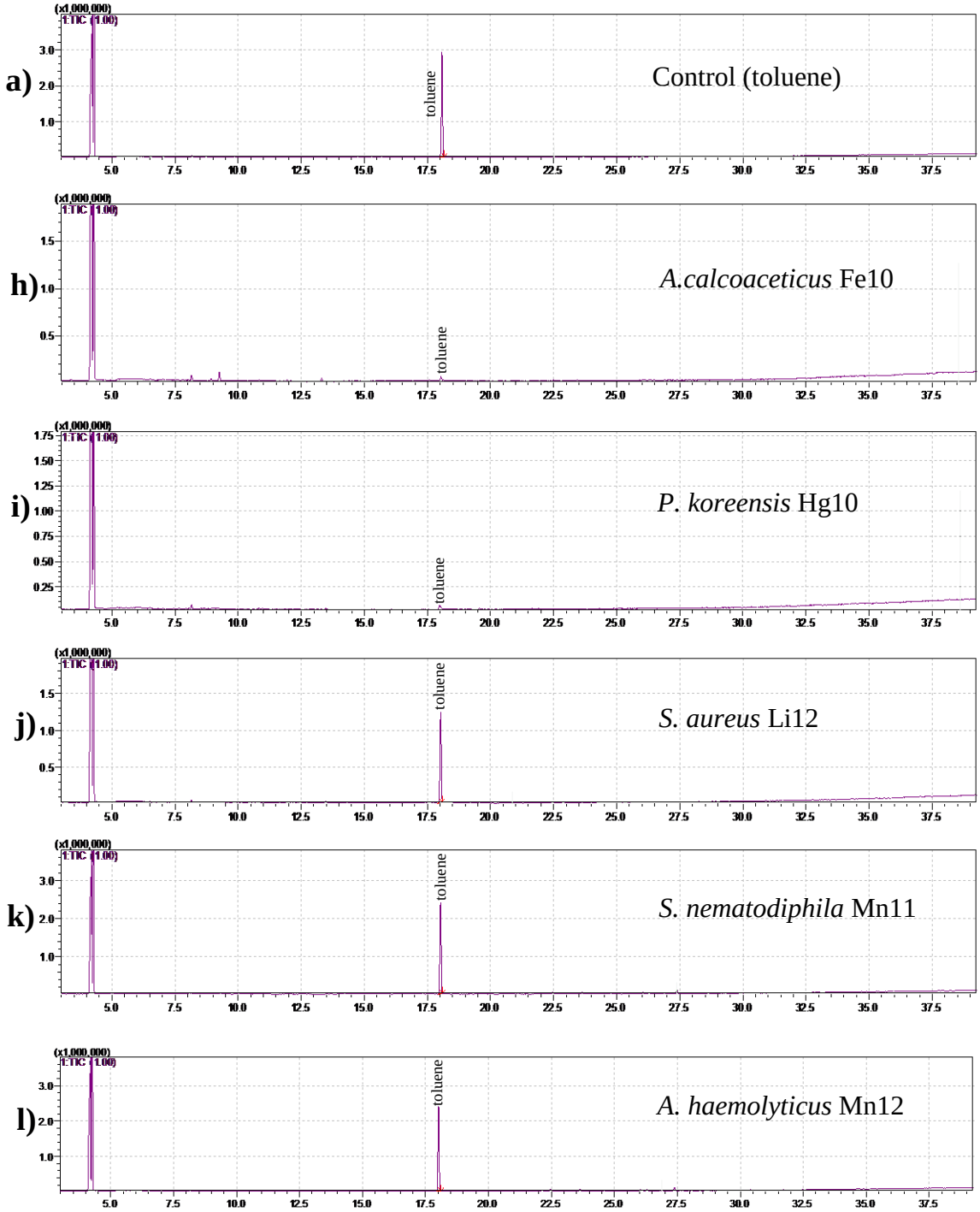


Figure 3.4 Toluene degradation abilities of the bacterial strains and non-degraders obtained from HS-GC/MS analyses


a)
Control (toluene)
b)
P. plecoglossicidia Ag10
c)
R. plancticola Ag11
d)
S. aureus Al11
e)
S. aureus Ba01
f)
S. rhizophila Ba11
g)
S. warneri Co11
toluene


a)
Control (toluene)
toluene
h)
A.calcoaceticus Fe10
toluene
i)
P. koreensis Hg10
toluene
j)
S. aureus Li12
toluene
k)
S. nematodiphila Mn11
toluene
l)
A. haemolyticus Mn12
toluene

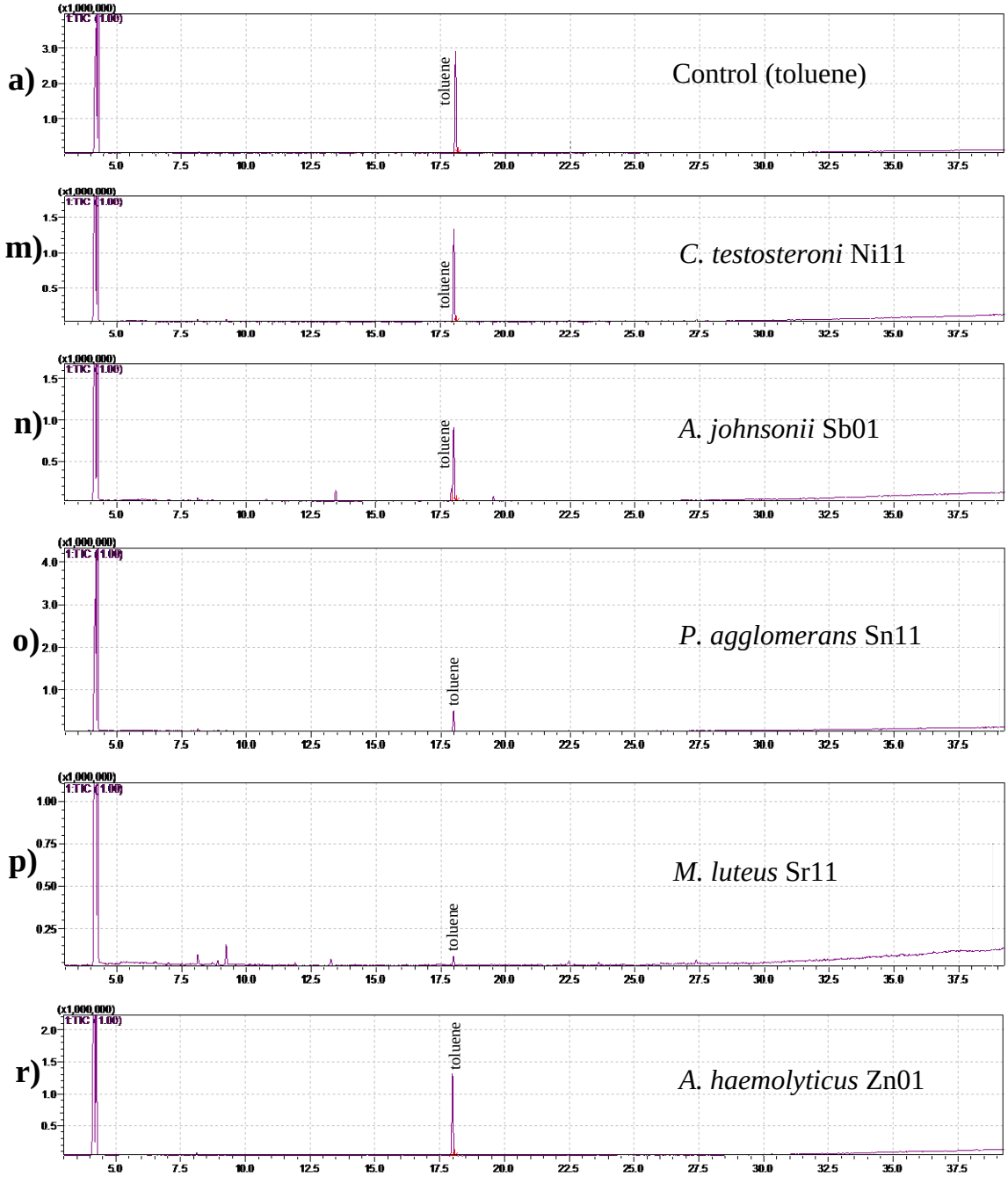


Figure 3.5 HS-GC/MS chromatograms of toluene biodegradation; control without bacterial isolates (a), *P. plecoglossicida* Ag10 (b), *R. plancticola* Ag11 (c), *S. aureus* Al11 (d), *S. aureus* Ba01 (e), *S. rhizophila* Ba11 (f), *S. warneri* Co11 (g), *A. calcoaceticus* Fe10 (h), *P. koreensis* Hg10 (i), *S. aureus* Li12 (j), *S. nematodiphila* Mn11 (k), *A. haemolyticus* Mn12 (l), *C. testosteroni* Ni11 (m), *A. johnsonii* Sb01 (n), *P. agglomerans* Sn11 (o), *M. luteus* Sr11 (p), *A. haemolyticus* Zn01 (r). Abundance factors of analytes are given on the upper left corner of each chromatogram.

Bacteria having ability to degrade benzene or toluene usually mineralize ethylbenzene as well due to similar enzymatic activity (Baldwin *et al*., 2003). HS-GC/MS was carried out to analyze the degradation rates of 10 bacteria namely *R. plancticola* Ag11, *S. aureus* Al11, *S. aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *S. warneri* Co11, *P. koreensis* Cu12, *A. calcoaceticus* Fe10, *P. koreensis* Hg11, *S. nematodiphila* Mn11, *A. haemolyticus* Mn12, *A. johnsonii* Sb01, *M. luteus* Sr11 previously found to be potential ethylbenzene degraders by preliminary degradation tests. The initial amount of ethylbenzene was 2.34 mg/mL in each test tube. Degradation rates were observed up to 100 % in bacterial strains (Figure 3.6-3.7-3.10). The most striking degradation was measured in *S. nematodiphila* Mn11 and *P. koreensis* Hg11 with 100 % and 90 % degradation rates, respectively. *R. plancticola* Ag11, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *S. nematodiphila* Mn11, *A. haemolyticus* Mn12, *A. johnsonii* Sb01 was shown as ethylbenzene degraders for the first time in this study.

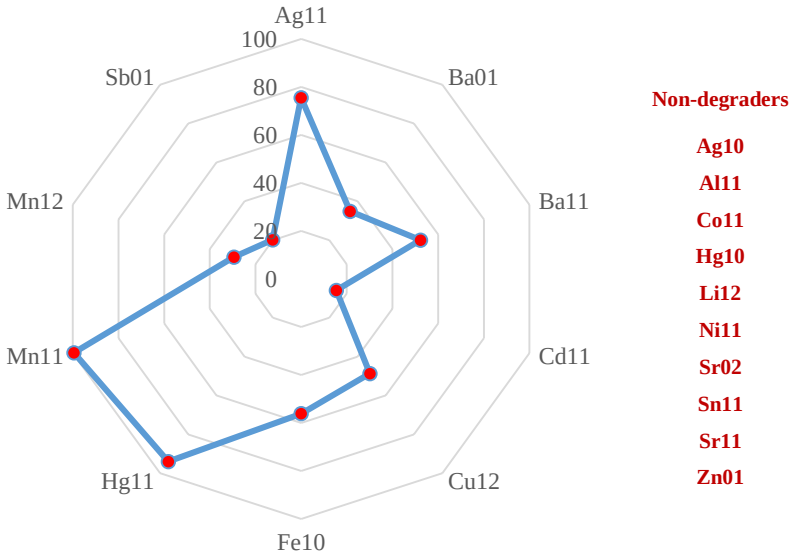
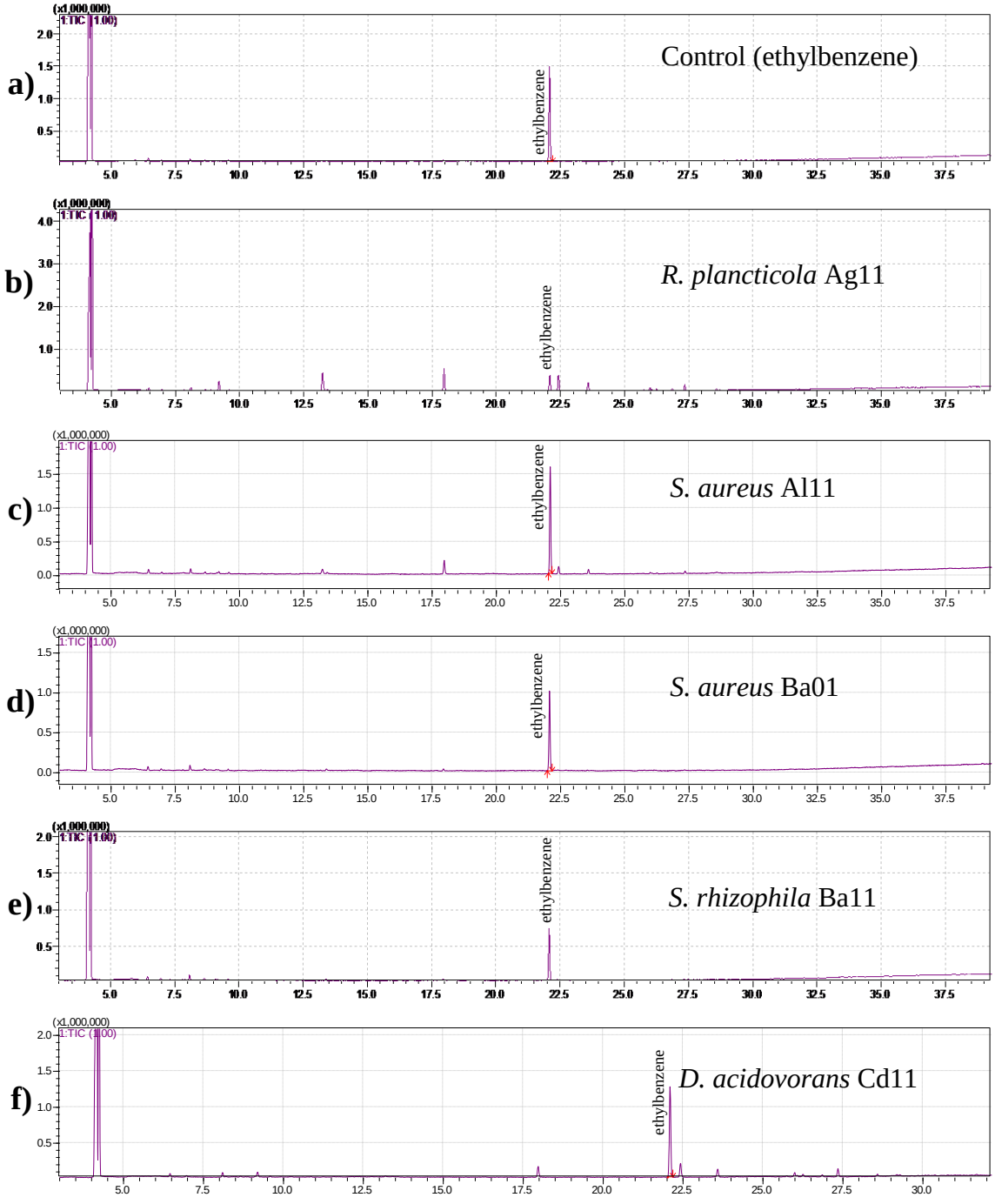
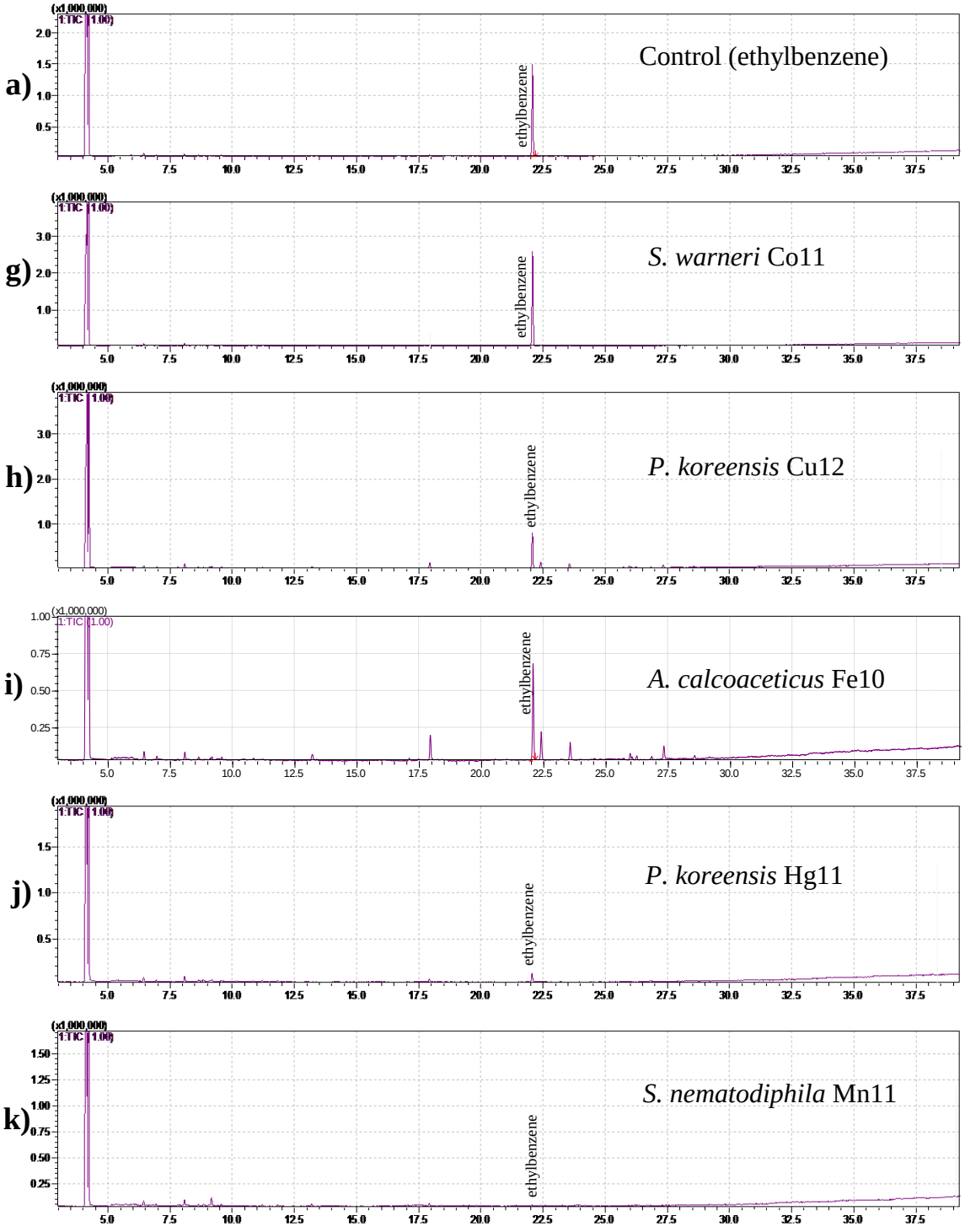


Figure 3.6 Ethylbenzene degradation abilities of the bacterial strains and non-degraders obtained from HS-GC/MS analyses


a)
Control (ethylbenzene)
ethylbenzene
b)
R. plancticola Ag11
ethylbenzene
c)
S. aureus Al11
ethylbenzene
d)
S. aureus Ba01
ethylbenzene
e)
S. rhizophila Ba11
ethylbenzene
f)
D. acidovorans Cd11
ethylbenzene


a)
Control (ethylbenzene)
g)
S. warneri Co11
h)
P. koreensis Cu12
i)
A. calcoaceticus Fe10
j)
P. koreensis Hg11
k)
S. nematodiphila Mn11
ethylbenzene

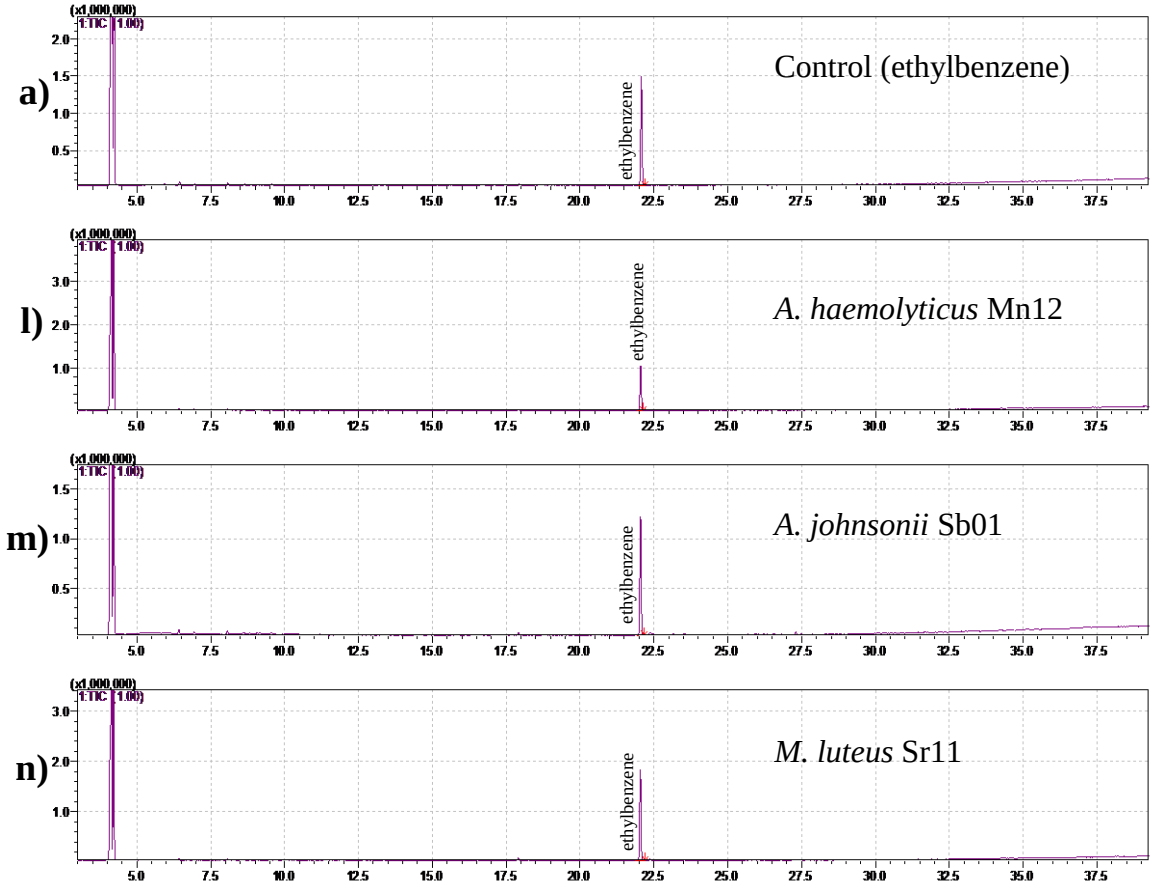


Figure 3.7 HS-GC/MS chromatograms of ethylbenzene biodegradation; control without bacterial isolates (a), *R. plancticola* Ag11 (b), *S. aureus* Al11 (c), *S. aureus* Ba01 (d), *S. rhizophila* Ba11 (e), *D. acidovorans* Cd11 (f), *S. warneri* Co11 (g), *P. koreensis* Cu12 (h), *A. calcoaceticus* Fe10 (i), *P. koreensis* Hg11 (j), *S. nematodiphila* Mn11 (k), *A. haemolyticus* Mn12 (l), *A. johnsonii* Sb01 (m), *M. luteus* Sr11 (n). Abundance factors of analytes are given on the upper left corner of each chromatogram.

Xylene has three isomers known as *-o*, *-p* and *-m* xylene and degradation characteristics of each isomer is different due to enzyme activity (Armando & Díaz, 2004). Xylene is more recalcitrant compound compared to others (Li & Goel, 2012). Results of preliminary degradation test showed that only 6 bacteria were able to degrade xylene. HS-GC/MS analyses were performed for the 6 bacterial strains (Figure 3.8-3.9-3.10). The initial amount of xylene was 8.94 mg/mL in each test tube. *A. calcoaceticus* Fe10 showed the highest degradation for xylene with 45 %. The strains *D. acidovorans* Cd11, *P. koreensis* Cu12, Hg10, Hg11 and *A. johnsonii* Sb01 were shown for the first time as xylene degraders in this study. Unlike benzene, toluene and ethylbenzene, xylene degradation is catalyzed by different enzyme systems encoded on TOL plasmid usually found in *Pseudomonas* related species *(Choi et al*., 2013). Therefore, xylene degradation is less common in bacteria (Heinaru *et al*, 2016) The usage of mix xylene as sole source of carbon in this study might be the reason of low degradation ability because *o*-xylene is very recalcitrant compound and its degradation ability has been demonstrated only for a few bacteria (Shadi *et al*., 2015).

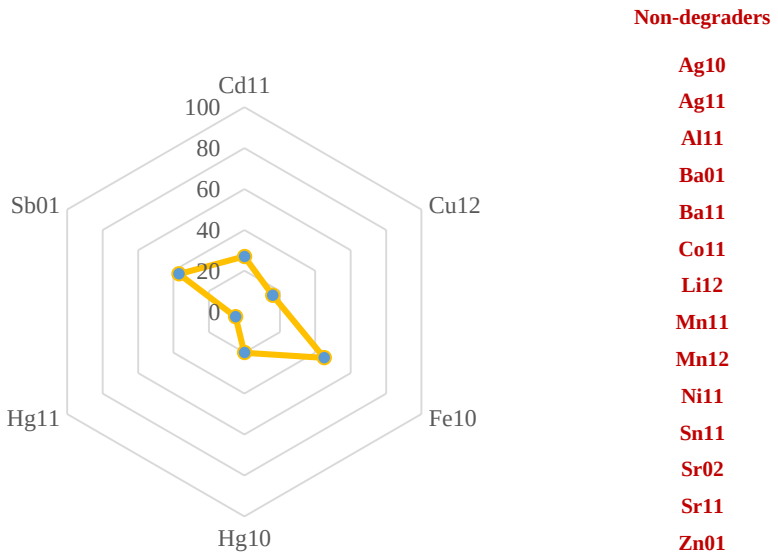


Figure 3.8 Xylene degradation rates of bacterial strains and non-degraders obtained from HS-GC/MS analyses

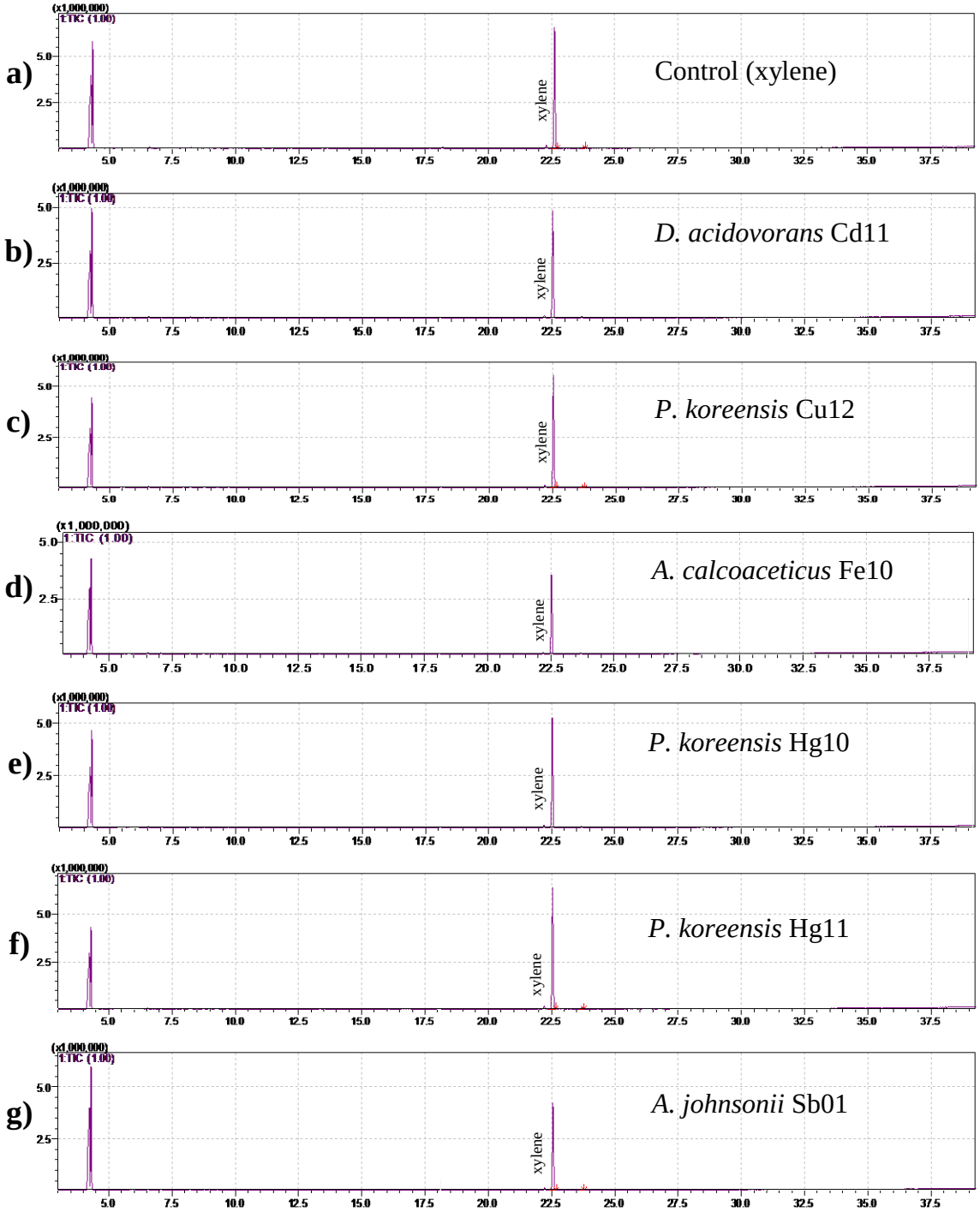


Figure 3.9 HS-GC/MS chromatograms of xylene biodegradation; control without bacterial isolates (a), *D. acidovorans* Cd11 (b), *P. koreensis* Cu12 (c), *A. calcoaceticus* Fe10 (d), *P. koreensis* Hg10 (e), *P. koreensis* Hg11 (f), *A. johnsonii* Sb01 (g). Abundance factors of analytes are given on the upper left corner of each chromatogram.

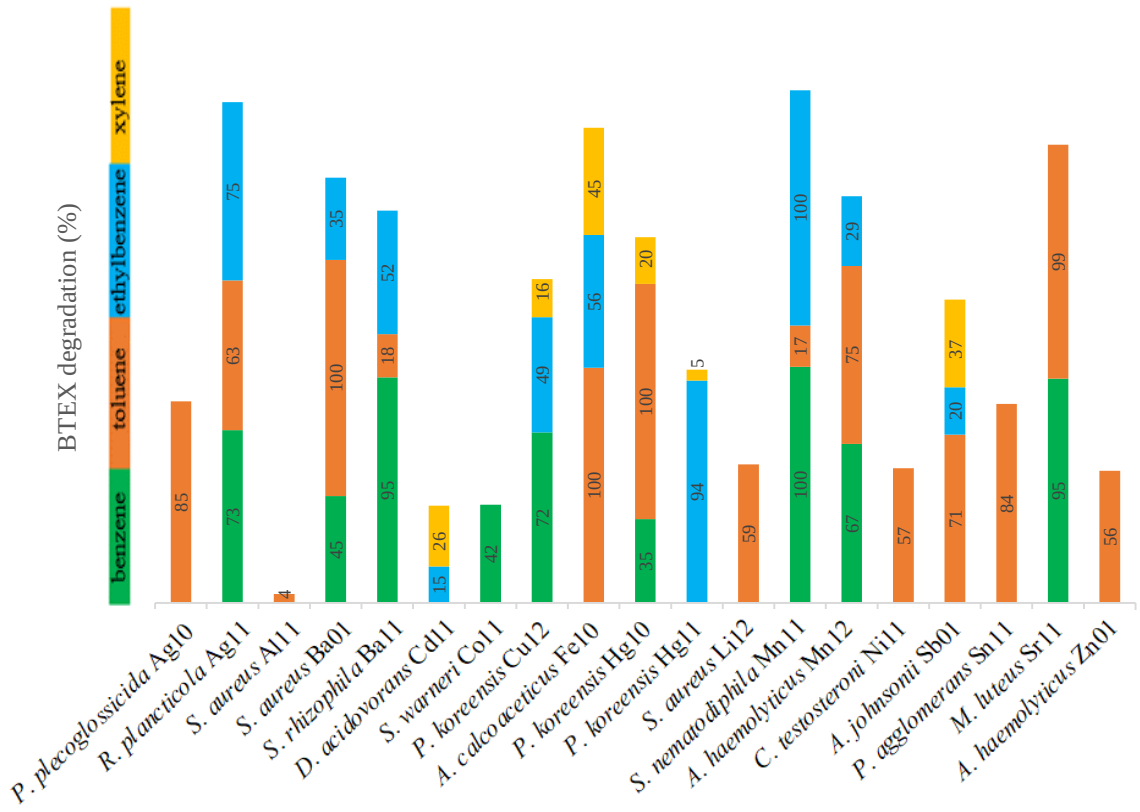


Figure 3.10 BTEX degradation percentages of all the bacterial strains used in the study

Out of 19, 9 bacterial strains were defined as efficient BTEX degraders listed in able 3.2. In addition to % BTEX degradation, degradation rates of bacterial strains were also calculated. The highest degradation rates were 0.39 mg/mL/day in *S. nematodiphila* Mn11, 0.25 mg/mL/day in *S. aureus* Ba01, *A. calcoaceticus* Fe10 and *P. koreensis* Hg10, 0.11 mg/mL/day in *S. nematodiphila* Mn11 and 0.19 mg/mL/day in *A. calcoaceticus* Fe10 for benzene, toluene, ethylbenzene and xylene, respectively. It can be seen in Figure 3.10 that lowest degradation percentage was observed in xylene. However, degradation rates revealed that its degradation was faster than ethylbenzene due to the high initial amount. The percentage of xylene degradation could be risen by decreasing the initial concentration or increasing the incubation time of degradation.

Table 3.2 Degradation characteristics of efficient BTEX compounds degrading bacterial strains

	Benzene (initial amount: 8.12 mg/mL)			**Toluene** (initial amount: 5.26 mg/mL)			**Ethylbenzene** (initial amount: 2.34 mg/mL)			**Xylene** (initial amount: 8.94)		
	Residual amount (mg/mL)	Degradation ability (%)	Degradation rate (mg/mL/day)	Residual amount (mg/mL)	Degradation ability (%)	Degradation rate (mg/mL/day)	Residual amount (mg/mL)	Degradation ability (%)	Degradation rate (mg/mL/day)	Residual amount (mg/mL)	Degradation ability (%)	Degradation rate (mg/mL/day)
R. planticola Ag11	2.18	73	0.28	1.94	63	0.16	0.58	75	0.08	8.94	0	0
S. aureus Ba01	4.44	45	0.18	0	100	0.25	1.52	34	0.03	8.94	0	0
S. rhizophila Ba11	0.37	95	0.37	4.3	18	0.04	1.12	52	0.05	8.94	0	0
A. calcoaceticus Fe10	8.12	0	0	0.02	100	0.25	1.02	56	0.06	4.90	45	0.19
P. koreensis Hg10	5.24	35	0.14	0.02	100	0.25	2.34	0	0	7.16	20	0.08
P. koreensis Hg11	8.12	0	0	5.26	0	0	0.14	94	0.10	8.52	5	0.02
S. nematodiphila Mn11	0	100	0.39	4.34	17	0.04	0	100	0.11	8.94	0	0
A. johnsonii Sb01	8.12	0	0	1.52	71	0.18	1.86	20	0.02	5.6	37	0.15
M. luteus Sr11	0.42	95	0.37	0.05	99	0.25	2.34	0	0	8.94	0	0

3.4 Analysis of the aerobic BTEX degradation pathways of the bacterial strains

There have been many studies showing that BTEX compounds are degraded through a variety of pathways (Farhadian *et al*., 2008; Choi *et al*., 2013). In the analysis of degradation pathways, one of the most powerful tool is detection of catabolic genes by PCR (Tuan *et al*., 2011). Degradation pathways predicted by genotype analysis can be additionally confirmed through the GC/MS analysis of potential metabolites produced during BTEX degradation (Choi *et al*., 2013). In this study, for deciphering the BTEX biodegradation pathways of the bacterial strains, PCR was carried out by using specific primers for the catabolic genes listed on Table 2.2 and predicted pathways were then further confirmed through detecting the intermediates by GC/MS analyses. Aerobic routes in benzene, toluene, ethylbenzene and xylene degradations are summarized in Figure 3.11.

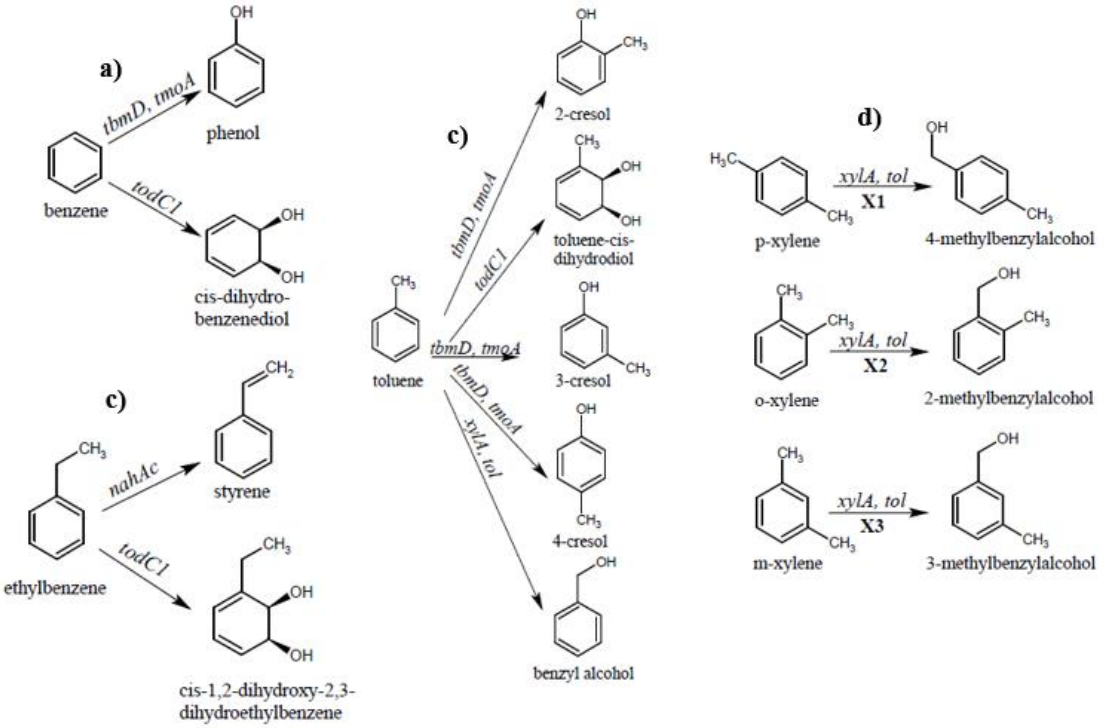


Figure 3.11 Inıtial oxidation steps of aerobic benzene (a), toluene (b), ethylbenzene(c) and xylene (d) degradation. Related genotypes are denoted on the reaction arrows. (modified from KEGG)

3.4.1 PCR analyses of catabolic genes

Aerobic BTEX degradation genes are encoded either on chromosomal or plasmid DNA (Hendrickx *et al*., 2006). Total DNA, therefore, was isolated to obtain both chromosomal and plasmid DNA of the bacterial strains for PCR analysis.

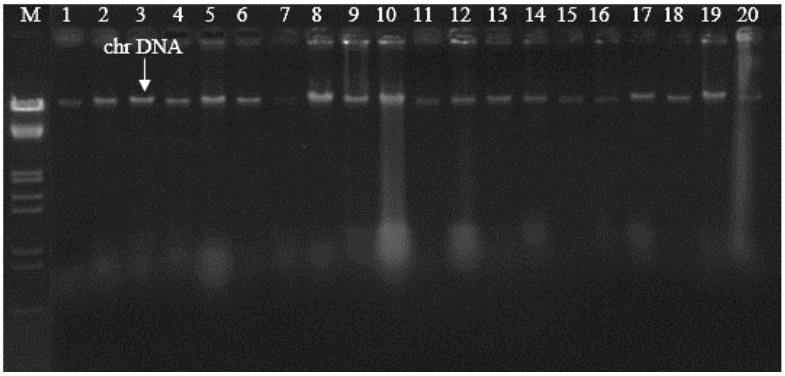


Figure 3.12 Agarose gel electrophoresis of chromosomal DNA extracted from the bacterial strains used in the study; *P. plecoglossicida* Ag10 (lane 1), *R. planticola* Ag11 (lane 2), *S. aureus* Al11 (lane 3), *S. aureus* Ba01 (lane 4), *S. rhizophila* Ba11 (lane 5), *D. acidovorans* (lane 6), *S. warneri* Co11 (lane 7), *P. koreensis* Cu12 (lane 8), *A. calcoaceticus* Fe10 (lane 9), *P. koreensis* Hg10 (lane10), *P. koreensis* Hg11 (lane 11), *S. aureus* Li12 (lane 12), *S. nematodiphila* Mn11 (lane 13), *A. haemolyticus* Mn12 (lane 14), *C. testosteroni* Ni11 (lane 15), *A. johnsonii* Sb01 (lane 16), *P. agglomerans* Sn11 (lane 17), *M. luteus* Sr02 (lane 18), *M. luteus* Sr11 (lane 19), *A. haemolyticus* Zn01 (lane 20). M, Thermo Fisher Lambda DNA/HindIII DNA ladder; from bottom to top: 125, 564, 2027, 2322, 4361, 6557, 9416 and 23130 bp, respectively. chr, chromosomal DNA.

3.4.1.1 PCR analysis of initial attack genes

Aerobic BTEX degradation is initiated by different enzyme systems including ring hydroxylating monooxygenases, side chain monooxygenases and ring hydroxylating dioxygenases (Jiménez *et al*., 2002). These enzymes are encoded by catabolic genes located on chromosomal and/or plasmid DNA (Armando & Díaz, 2004). To date, numerous catabolic genes related to BTEX degradation have been characterized by PCR (Baldwin *et al*., 2003). Therefore, to predict the degradation pathways of the bacterial strains in this study, initial oxidation genes *tbmD*, *tmoA*, *phhN*, *xylA*, *tol*, *todC1* and *nahAc* were analyzed by PCR amplification.

3.4.1.1.1 PCR analysis of *tbmD* gene

Ring hydroxylating mononxygenases catalyze the first step of aerobic BTEX degradation by incorporating oxygen atom into different positions on aromatic ring (Johnson & Olsen, 1995). For the detection of *tbmD* gene (Figure 3.13) in the bacterial strains, PCR was performed. *A. calcoaceticus* was used to optimize PCR conditions by changing the annealing temperature ranging from 63˚C to 66˚C and $MgCl_2$ concentration ranging from 1.75 to 3 mM (Figure 3.14). Best results were obtained at 66˚C temperature and 1.75 mM $MgCl_2$ concentration and preferred as optimum conditions for PCR-detection of *tbmD* gene. The *tbmD* gene was detected in only *A. calcoaceticus* Fe10 and *M. luteus* Sr11 by the amplification of PCR products with the expected sizes of 640 bp (Figure 3.15).

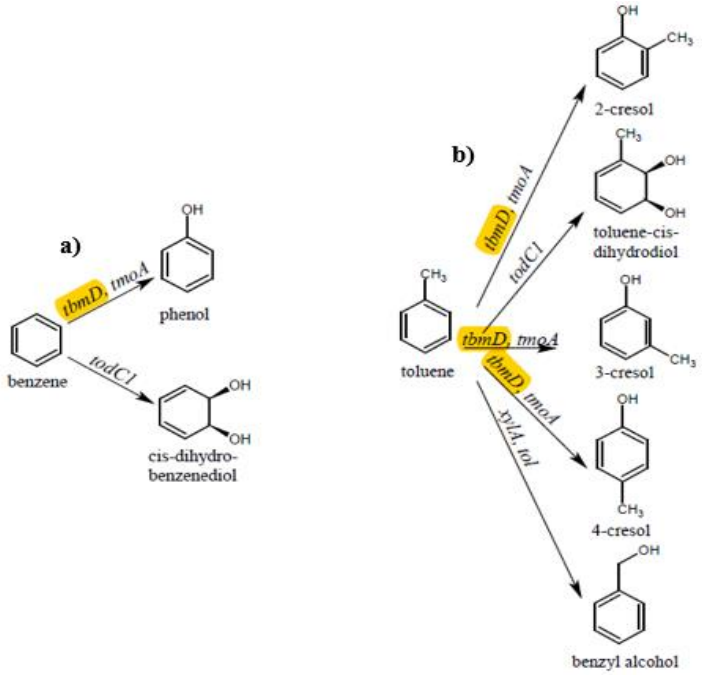


Figure 3.13 The *tbmD* gene in the first oxidation step of benzene (a) and toluene (b) degradation

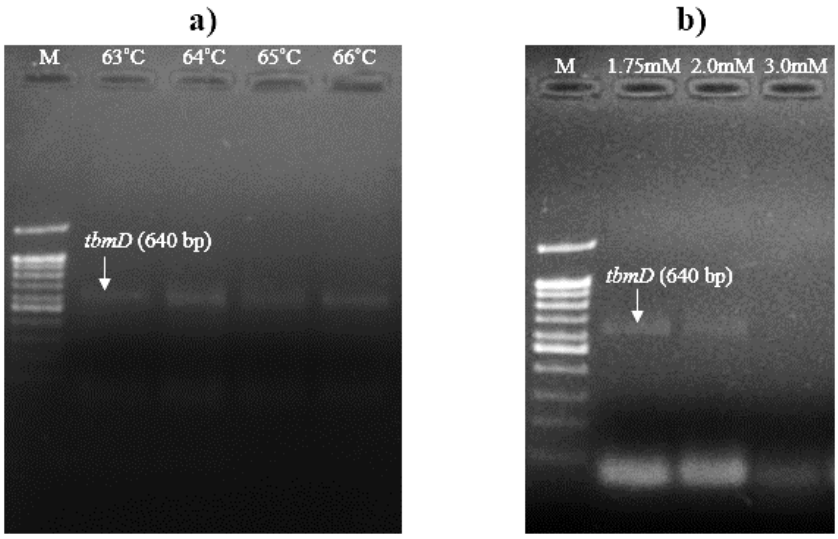


Figure 3.14 Optimization of PCR conditions at various annealing temperatures (˚C) (a) and $MgCl_2$ concentrations (mM) (b) for *A. calcoaceticus* Fe10. M, Quick-Load 100 bp DNA ladder from bottom to top 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 bp, respectively.

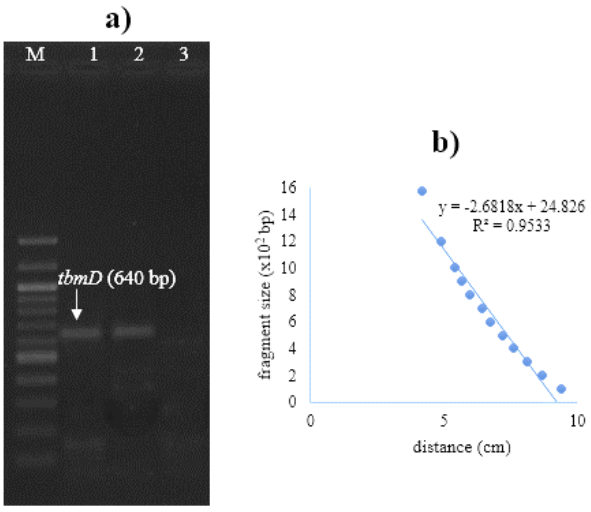


Figure 3.15 The *tbmD* gene harboring bacterial strains (a) *A. calcoaceticus* Fe10 (lane 1), *M. luteus* (lane 2), negative control (lane 3). M, NEB 3231 100 bp DNA ladder from bottom to top 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200 and 1517 bp, respectively (a). Standard curve of agarose gel for molecular weight estimation (b).

Due to strong DNA and protein homology among ring hydroxylating monooxygenases it is not possible to design a primer set targeting specific gene (Baldwin *et al.*, 2003). There have been many studies focusing on the catabolic genes of the bacterial degraders (Choi *et al*., 2013). Degenerated primers are mainly used in PCR targeting the conserved regions of catabolic genes (Hendrickx *et al*., 2006). The *tbmD* primer set was used to detect all possible genes encoding α-subunit of subfamily 1 of the hydroxylase component of aromatic monooxygenases (Figure 1.9) (Baldwin *et al*., 2003). By using *tbmD* primer set Hendrickx *et al*., (2006) amplified *tbmD* gene in different bacterial species like *Pseudomonas putida*, *Stenotrophomonas* sp., *Pseudomonas veronii*, *Ralstonia eutropha*, *Variovorax* sp., *Pseudomonas marginalis*. In this study, the presence of *tbmD* gene in and *M. luteus* Sr11 was demonstrated for the first time. Alternative to *tbmD*, *tmoA* and *phhN* primers were used to detect different subfamilies of ring hydroxylating monooxygenase genes but no PCR products were obtained. By using tbmD primer, all possible ring hydroxylating monooxygenase sequences were targeted including toluene monooxygenases and phenol hydroxylases. Further phylogeny analysis PCR amplicons is needed to understand the sequence homology between all possible ring hydroxylating monooxygenases.

3.4.1.1.2 Sequence analysis of *tbmD* amplicons

Two *tbmD* sequences were compared to 10 different *tbmD*-like sequences obtained from NCBI database and phylogenetic tree was constructed accordingly (Figure 3.16). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to assume phylogenetic tree. Multiple sequence alignment and pairwise distance matrix are given in Appendix B. As can be seen on phylogenetic tree, *tbmD* sequences showed sequence similarities with phenol hydroxylase sequences in between 72 and 99%. These results revealed that *tbmD* sequences in this study belonged to R.1 type phenol hydroxylases, also demonstrated by Baldwin *et al*. (2003).

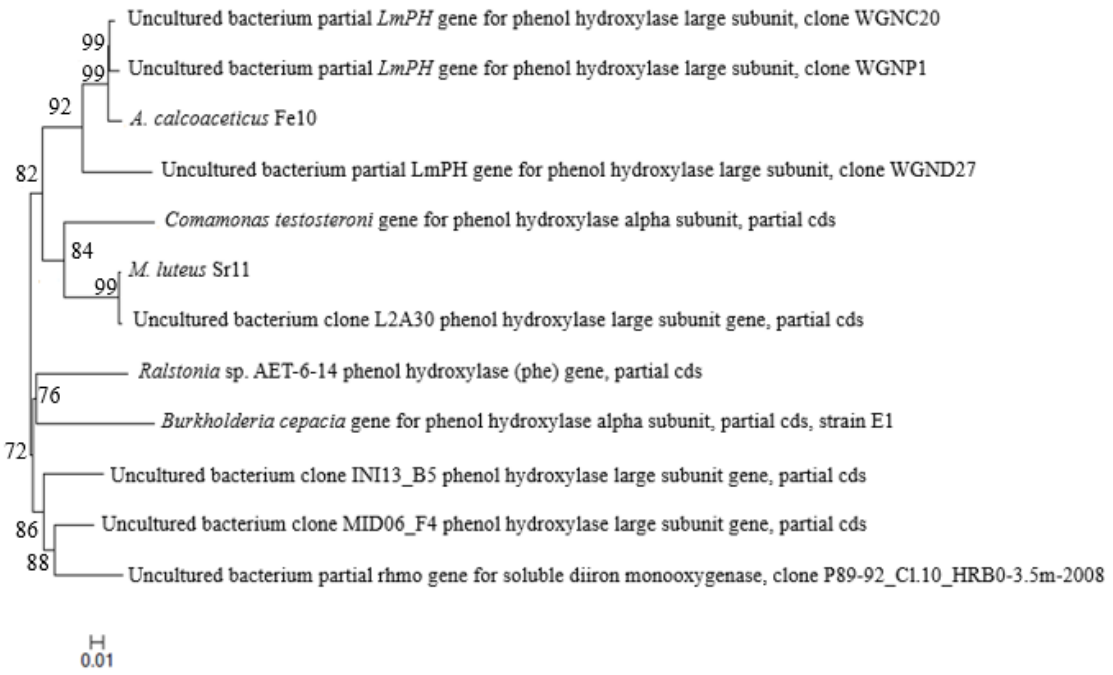


Figure 3.16 Phylogenetic analysis of *tbmD* (ring hydroxylating monooxygenase) genes among bacterial strains. Scale bar at the bottom of the tree stands for nucleotide changes per site.

3.4.1.1.3 PCR analysis of *todC1* gene

Aromatic hydrocarbon degradation is also catalyzed by ring hydroxylating dioxygenases including benzene dioxygenase, toluene dioxygenase, ethylbenzene dioxygenase, naphthalene dioxygenase etc. (Witzig *et al*., 2006). These enzymes are generally encoded on chromosomal DNA (Armando & Díaz, 2004). For the detection of *todC1* gene (Figure 3.17) in the bacterial strains used in the study, PCR was performed. *P. koreensis* Hg10 was used to optimize PCR conditions by changing the annealing temperature ranging from 64˚C to 67˚C and $MgCl_2$ concentration ranging from 2.0 to 3.0 mM (Figure 3.18). Best results were obtained at 65˚C temperature and 2.0 mM $MgCl_2$ concentration and chose as optimum conditions for PCR-detection of *todC1* gene. The *todC1* gene was detected in 14 bacterial strains namely *R. planticola* Ag11, *S. rhizophila* Ba11, *S. warneri* Co11, *P. koreensis* Cu12, *A. calcoaceticus* Fe10, *P. koreensis* Hg10, *P. koreensis* Hg11, *S. aureus* Li12, *S. nematodiphila* Mn11, *A. haemolyticus* Mn12, *C.*

testosteroni Ni11, *A. johnsonii* Sb01, *P. agglomerans* Sn11, *M. luteus* Sr11 by PCR amplification with the expected sizes of 510 bp (Figure 3.19).

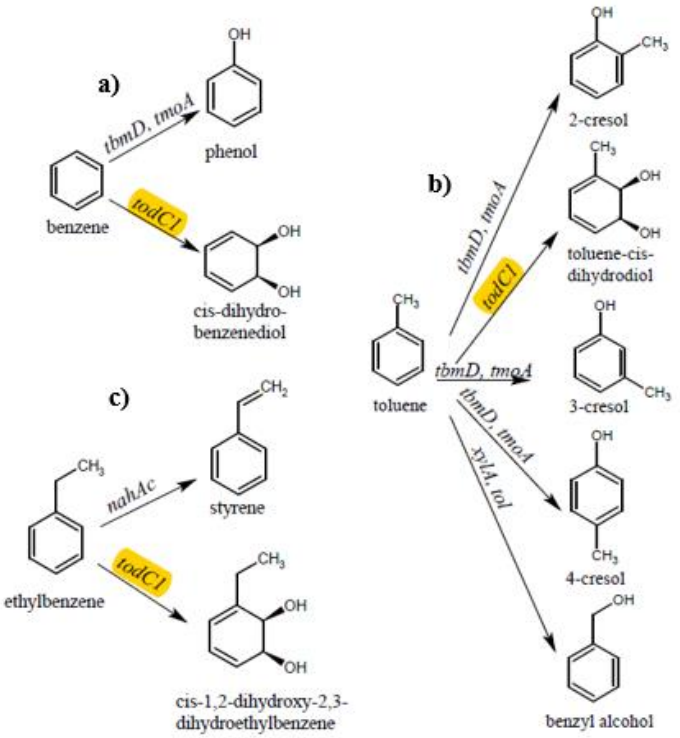


Figure 3.17 The *todC1* gene in the first oxidation step of benzene (a), toluene (b) and ethylbenzene (c) degradation

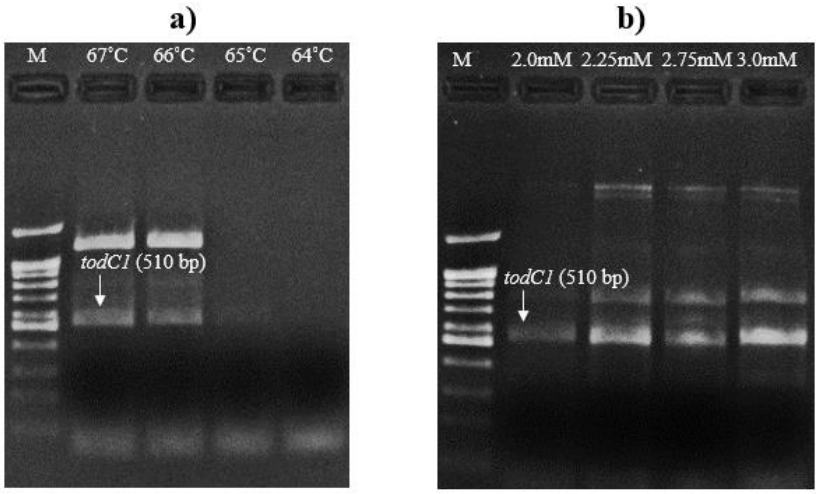


Figure 3.18 Optimization of PCR conditions at various annealing temperatures (˚C) (a) and $MgCl_2$ concentrations (mM (b) for *P. koreensis* Hg10. M, Quick-Load 100 bp DNA ladder from bottom to top 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 bp, respectively.

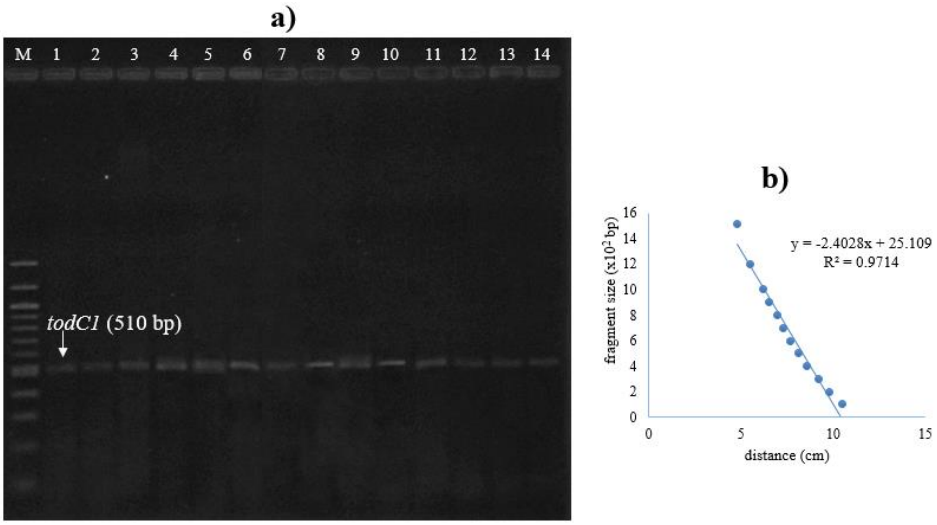


Figure 3.19 The *todC1* gene harboring bacterial strains (a) *R. planticola* Ag11 (lane 1), *S. rhizophila* Ba11 (lane 2), *S. warneri* Co11 (lane 3), *P. koreensis* Cu12 (lane 4), *A. calcoaceticus* Fe10 (lane 5), *P. koreensis* Hg10 (lane 6), *P. koreensis* Hg11 (lane 7), *S. aureus* Li12 (lane 8), *S. nematodiphila* Mn11 (lane 9), *A. haemolyticus* Mn12 (lane 10), *C. testosteroni* Ni11 (lane 11), *A. johnsonii* Sb01 (lane 12), *P. agglomerans* Sn11 (lane 13), *M. luteus* Sr11 (lane 14). M, NEB 3231 100 bp DNA Ladder from bottom to top 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200 and 1517 bp, respectively. Standard curve of agarose gel for molecular weight estimation (b).

todC1 primer set was used in this study previously designed by Hendrickx *et al*., (2006). This primer set targets the all five subfamilies (Figure 1.15) of the iron–sulfur oxygenase a-subunits of the ring hydroxylating dioxygenases involved in BTE degradation (Hendrickx *et al*., 2006). *todC1* and *todC1*-like genes were previously shown in *Pseudomonas putida, Pseudomonas jesenii, Pseudomonas marginalis* (Dejonghe, *et al*., 2006), *Thauera* sp. (Shinoda *et al*., 2004), *Klebsiella pneumonia, Raoultella ornithinolytica* (Rodrigues *et al*.,, 2009), *Serratia marcescens* (González *et al*., 2013), *Pseudoxanthomonas spadix* (Kim *et al*., 2008), *Acinetobacter* sp. (Kim & Lee, 2011). The *todC1* gene in *P. agglomerans* was detected for the first time in this study. Alternative to *todC1*, *nahAc* primer was also used to amplify naphthalene dioxygenase genes but no PCR detection was obtained. By using *todC1* primer, all possible ring hydroxylating dioxygenases like toluene, benzene, ethylbenzene, naphthalene and biphenyl dioxygenases (Baldwin *et al*., 2003). Therefore, PCR products amplified by *todC1* primer need further phylogeny analysis to make a comparison among sequences.

3.4.1.1.4 Sequence analysis of *todC1* amplicons

todC1 gene was found to be harbored in 14 bacterial strains namely *R. planticola* Ag11, *S. rhizophila* Ba11, *S. warneri* Co11, *P. koreensis* Cu12, *A. calcoaceticus* Fe10, *P. koreensis* Hg10, *P. koreensis* Hg11, *S. aureus* Li12, *S. nematodiphila* Mn11, *A. haemolyticus* Mn12, *C. testosteroni* Ni11, *A. johnsonii* Sb01, *P. agglomerans* Sn11, *M. luteus* Sr11. Each *todC1* amplicon was sequenced and phylogenetic tree was constructed among bacterial strains to analyze the evolutionary relationship (Figure 3.20). Sequence similarities for *todC1* amplicons in bacterial strains ranged in between 62 and 99%. The closest *todC1* sequence homology was found in between *A. haemolyticus* Mn12 and S. *warneri* Co11 with 99% similarity. Multiple sequence alignment and pairwise distance matrix are given in Appendix B. This result might be an indication for the occurrence of horizontal transfer of BTEX catabolic genes among the aquifer community. The presence of catabolic genes in phylogenetically distant bacteria reveals the occurrence of horizontal transfer of these genes in the aquifer (Hendrickx *et al*., 2006). The same observation was also done by Cavalca *et al*. (2004) who proposed interspecies transfer

of the *tmoA* genes in a BTEX contaminated aquifer based on the occurrence of *tmoA* genes in different BTEX degrading bacteria like *Mycobacterium*, *Pseudomonas,* and *Bradyrhizobium*.

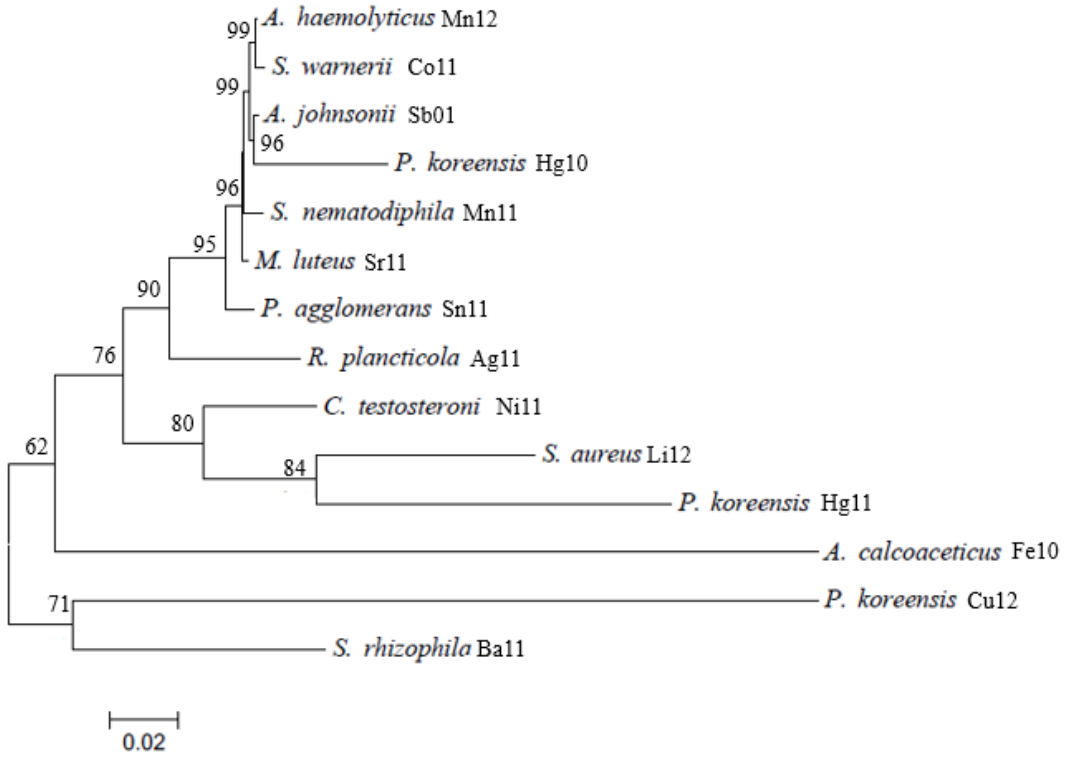


Figure 3.20 Phylogenetic analysis of *todC1* genes (ring hydroxylating dioxygenase) among bacterial strains. Scale bar at the bottom of the tree stands for nucleotide changes per site

3.4.1.1.5 PCR analysis of *xyl* genes

Toluene and xylene degradation can be catalyzed by different enzyme system known as side chain monooxygenases, encoded on TOL plasmid (Heinaru *et al*., 2016). This plasmid is commonly harbored in *Pseudomonas* sp. and other related bacteria (Hendrickx *et al*., 2006). Two different primer sets namely *xylA* (Hendrickx *et al*., 2006) and *tol* (Baldwin *et al*., 2003) were used in this study to detect all possible *xyl*-like genes in the bacterial strains. However, no PCR-detection was observed. By using these

primers Hendrickx *et al*., (2006) were amplified *xylA* genes in many *Pseudomonas* species.

3.4.1.2 PCR analyses of ring cleavage genes

As can be shown in Figure 3.21, a common step in aromatic hydrocarbon degradation is intradiol or extradiol cleavage of catechol-like intermediates by catechol dioxygenase enzymes (Tuan *et al*., 2011). Catechol dioxygenase genes used as marker for monitoring of aromatic hydrocarbon degradation (Lillis *et al*., 2010). Many comprehensive studies have been performed to detect catechol dioxygenase genes by PCR-based methods (Silva *et al*., 2012; Wojcieszyńska *et al*., 2012; Guzik *et al*., 2013). Therefore, detection of catechol 1,2 dioxygenase (*catA*) and catechol 2,3 dioxygenase (*C23O*) genes were also performed in this study.

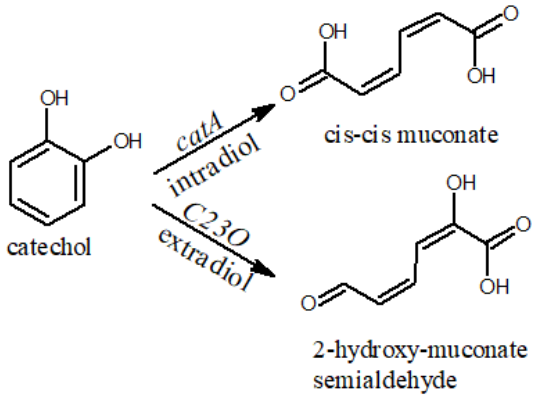


Figure 3.21 The routes of aerobic aromatic ring cleavage

3.4.1.2.1 PCR analysis of *catA* gene

Intradiol cleavage of catechol and its derivatives can be catalyzed by catechol 1,2 dioxygenase (Guzik *et al*., 2011). The genes encoding the catechol 1,2 dioxygenase enzyme are located on *cat* operon (Nelson *et al*., 2002). *catA* primer was used to amplify the partial region of catechol 1,2 dioxygenase gene involved in intradiol cleavage (Figure 3.22). Optimization of PCR conditions was carried out in using *R. plancticola*

Ag11 by changing annealing temperature of primer ranging from 58˚C to 60˚C and $MgCl_2$ concentration ranging from 2.0 to 3.0 Mm (Figure 3.23). Best results were obtained at 60˚C temperature and 2.0 mM $MgCl_2$ concentration and preferred as optimum conditions for PCR amplification of *catA* gene.

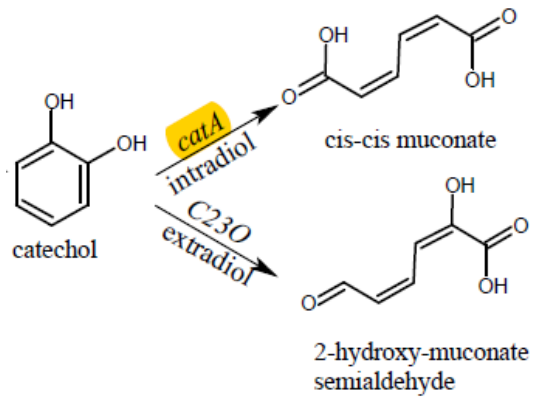


Figure 3.22 The *catA* gene in the intradiol cleavage of aromatic ring

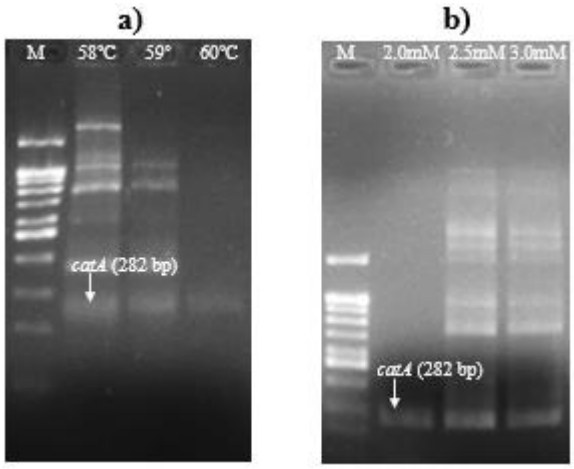


Figure 3.23 Optimization of PCR conditions at various annealing temperatures (˚C) (a) and $MgCl_2$ concentrations (mM) (b) for *R. plancticola* Ag11. M, Quick-Load 100 bp DNA ladder from bottom to top 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 bp, respectively.

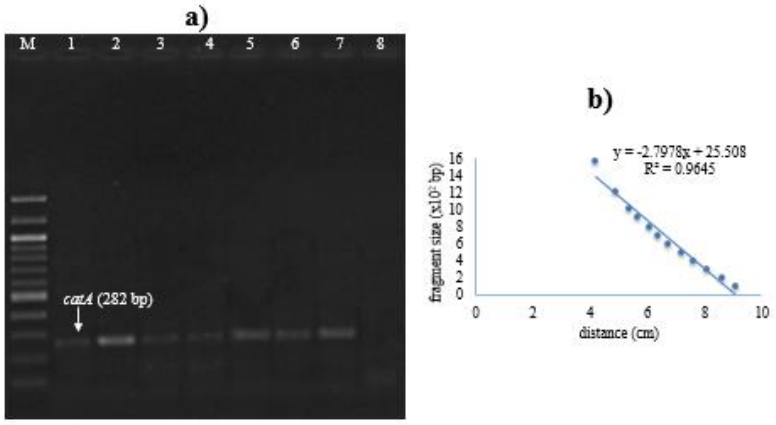


Figure 3.24 The *catA* gene harboring bacterial strains (a) *R. plancticola* Ag11 (lane 1), *S. aureus* Ba01 (lane 2), *D. acidovorans* Cd11 (lane 3), *A. calcoaceicus* Fe10 (lane 4), *P. koreensis* Hg11 (lane 5), *S. nematodiphila* Mn11 (lane 6), *M. luteus* Sr11 (lane 7), negative control (lane 8). M, NEB 3231 100 bp DNA ladder from bottom to top 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200 and 1517 bp, respectively (a). Standard curve of agarose gel for molecular weight estimation (b).

PCR products with the expected sizes of 282 bp were confirmed in *R. plancticola* Ag11, *S. aureus* Ba01, *D. acidovorans* Cd11, *A. calcoaceticus* Fe10, *P. koreensis* Hg11, *S. nematodiphila* Mn11 and *M. luteus* Sr11 (Figure 3.24). Catechol 1,2 dioxygenase enzymes are very common in aromatic hydrocarbon degradation, and it has also been demonstrated in variety of bacteria so far (Saxena & Thakur, 2005; Giedraityte & Kalėdienė, 2009). However, the *catA* gene was detected for the first time by this study in *R. plancticola* Ag11.

3.4.1.2.2 PCR analysis of *C23O* gene

Catechol 2,3 dioxygenase, one of the key enzymes in aerobic BTEX degradation, catalyze the extradiol cleavage of the aromatic ring (Borowski & Siegbahn, 2006) as shown in . Genes encoding the catechol 2,3 dioxygenase enzymes located on chromosomal (Hendrickx *et al*., 2006) and plasmid DNA (Táncsics *et al*., 2012). *C23O*-I primer set (Mesarch *et al*., 2000) was used in the study for targeting the subfamily I.2.C

related catechol dioxygenase genes suggested to be widely distributed in BTEX degrading bacteria (Mesarch *et al*., 2000).

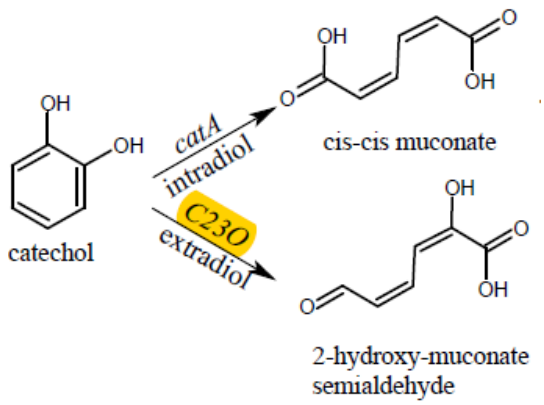


Figure 3.25 The *C23O* gene in the extradiol cleavage of aromatic ring

For the detection of *C230* gene in bacterial strains, PCR was performed. *R. plancticola* was used for the optimization of PCR conditions by changing the annealing temperature ranging from 54˚C to 56˚C and $MgCl_2$ concentration ranging from 2.0 to 3.0 mM (Figure 3.26). Best results were obtained at 55˚C temperature and 2.0 mM $MgCl_2$ concentration and preferred as optimum conditions for PCR amplification of the *C23O* gene. The *C23O* gene was detected in 5 bacterial strains namely *P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. rhizophila* Ba11, *A. calcoaceticus* Fe10, *P. koreensis* Hg11 by PCR detection with the expected sizes of 216 bp (Figure 3.27). *C23O*-II primer (Hendrickx *et al*., 2006) was also used for targeting the subfamily I.2.A related catechol dioxygenases. However, no PCR detection was obtained within the bacterial strains. Since the *C23O* genes have strong diversity with their different subfamilies, alternative primer sets have been developed to detect them through PCR. Bacterial strains used in this study found to mainly belong to the subfamily I.2.C. This subfamily includes *C23O* sequences involved in BTEX degradation derived from a wide variety of other bacterial genera (*Pseudomonas, Comamonas, Burkholderia and Ralstonia*) (Hendrickx *et al*., 2006). For the detection of all possible *C23O* sequences belong to different subfamilies

in bacterial strains, therefore, alternative primer sets should be used or new primer sets should be designed.

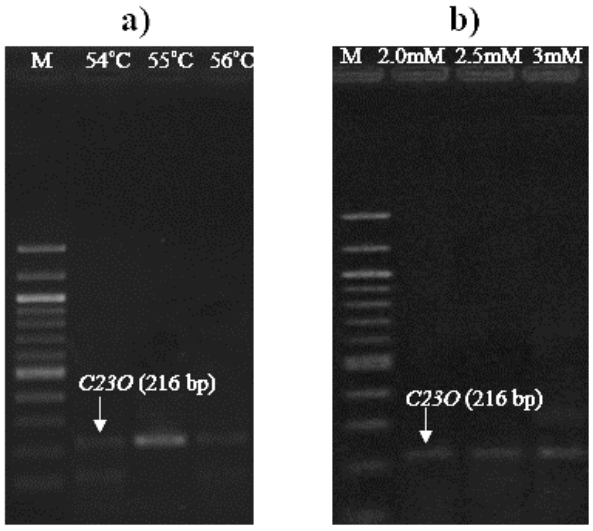


Figure 3.26 Optimization of PCR conditions at various annealing temperatures (˚C) (a) and $MgCl_2$ concentrations (mM) (b) for *R. plancticola* Ag11. M, NEB 3231 100 bp DNA ladder from bottom to top 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200 and 1517 bp, respectively.

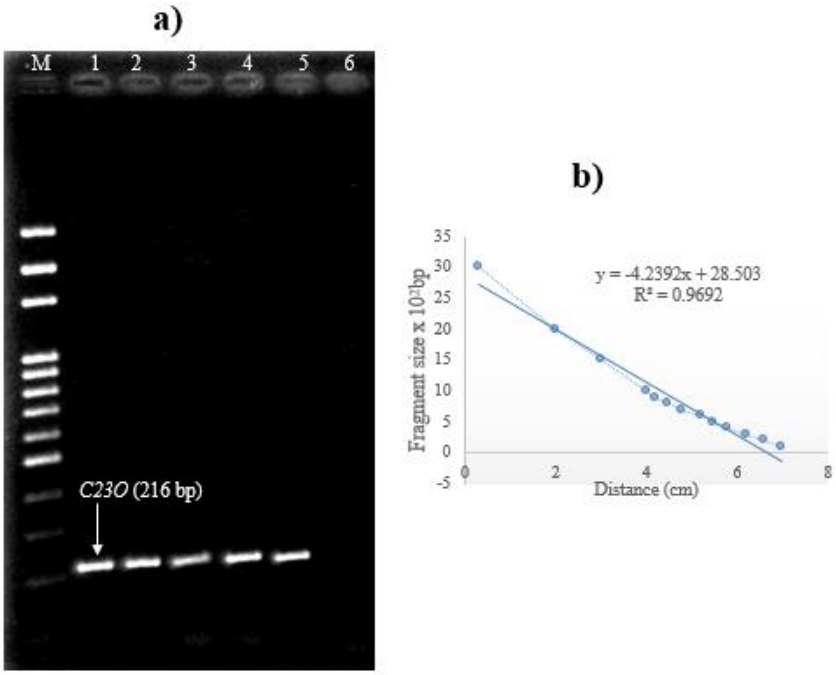


Figure 3.27 The *c23o* gene harboring bacterial strains (a) *P. plecoglossicida* Ag10 (lane 1), *R. planticola* Ag11 (lane 2), *S. rhizophila* Ba11 (lane 3), *A. calcoaceticus* Fe10 (lane 4), *P. koreensis* Hg11 (lane 5), negative control (lane 6). M, Solis Biodyne 100 bp DNA ladder from bottom to top 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000 and 3000 bp, respectively. Standard curve of agarose gel for molecular weight estimation (b).

The BTEX compounds degradation abilities and corresponding genotypes of the bacterial strains were summarized in Table 3.3. Out of 20, 9 bacterial strains, (highlighted in yellow) namely *Raoultella planticola* Ag11, *Staphylococcus aureus* Ba01, *Stenotrophomonas rhizophila* Ba11, *Acinetobacter calcoaceticus* Fe10, *Pseudomonas koreensis* Hg10, *Pseudomonas koreensis* Hg11, *Serratia nematodiphila* Mn11, *Acinetobacter johnsonii* Sb01, *Micrococcus luteus* Sr11 were chosen as efficient BTEX degraders and further analyzed for their pathway used.

Table 3.3 Description of BTEX degradation profiles of all the bacterial strains used in the study

Bacterial strains	% BTEX degradation				Initial attack genes							Cleavage genes		
	benzene	toluene	ethylbenzene	xylene	*tbmD*	*tmoA*	*todC1*	*xylA*	*tol*	*nahAc*	*phhN*	*catA*	*C23O*-II	*C23O*-I
Pseudomonas plecoglossicida Ag10	-	85	-	-	-	-	-	-	-	-	-	-	-	+
Raoultella planticola Ag11	73	63	75	-	-	-	+	-	-	-	-	+	-	+
Staphylococcus aureus Al11	-	4	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus Ba01	45	100	34	-	-	-	-	-	-	-	-	+	-	-
Stenotrophomonas rhizophila Ba11	95	18	52	-	-	-	+	-	-	-	-	-	-	+
Delftia acidovorans Cd11	-	-	15	26	-	-	-	-	-	-	-	+	-	-
Staphylococcus warneri Co11	41	-	-	-	-	-	+	-	-	-	-	-	-	-
Pseudomonas koreensis Cu12	72	-	49	16	-	-	+	-	-	-	-	-	-	-
Acinetobacter calcoaceticus Fe10	-	99	56	45	+	-	+	-	-	-	-	+	-	+
Pseudomonas koreensis Hg10	35	99	-	19	-	-	+	-	-	-	-	-	-	-
Pseudomonas koreensis Hg11	-	-	94	5	-	-	+	-	-	-	-	+	-	+
Staphylococcus aureus Li12	-	59	-	-	-	-	+	-	-	-	-	-	-	-
Serratia nematodiphila Mn11	100	17	99	-	-	-	+	-	-	-	-	+	-	-
Acinetobacter haemolyticus Mn12	67	75	29	-	-	-	+	-	-	-	-	-	-	-
Comamonas testosteroni Ni11	-	57	-	-	-	-	+	-	-	-	-	-	-	-
Acinetobacter johnsonii Sb01	-	71	20	37	-	-	+	-	-	-	-	-	-	-
Pantoea agglomerans Sn11	-	84	-	-	-	-	+	-	-	-	-	-	-	-
Micrococcus luteus Sr11	95	99	-	-	+	-	+	-	-	-	-	+	-	-
Acinetobacter haemolyticus Zn01	-	56	-	-	-	-	-	-	-	-	-	-	-	-

todC1, ring hydroxylating dioxygenase genes; *tbmD*, *tmoA* and *phhN* ring hydroxylating monooxygenase genes; *xylA* and *tol*, side chain monooxygenase genes; *nahAc*, naphthalene dioxygenase gene; *catA*, catechol 1,2 dioxygenase gene; *c23o*, catechol 2,3 dioxygenase genes; -, not detected; +, detect

3.4.2 Detection of intermediates during BTEX degradation

Aerobic BTEX degradation pathways of the bacterial strains were first predicted by PCR analyses of the catabolic genes. Due to diversity of genes and corresponding enzymes in BTEX degradation, further analyses were needed as suggested by Jiménez *et al*. (2002). For deciphering the actively used degradation pathways of efficient BTEX degraders (Table 3.3), intermediates during the degradation of BTEX compounds were detected through GC/MS analyses. Raw data of GC/MS chromatograms and MS ionization are given in Appendix C.

3.4.2.1 Detection of intermediates during benzene degradation

Benzene is degraded to phenol or cis-benzenedihydrodiol and catechol is produced at the end of each route as an identical product (Armando & Díaz, 2004). Four bacterial strains namely *R. plancticola* Ag11, *S. rhizophila* Ba11, *S. nematodiphila* Mn11 and *M. luteus* Sr11 were previously determined as efficient benzene degraders. The bacterial strains were further used to analyze the intermediates produced during benzene degradation. As an intermediary product phenol was detected in the degradation by *R. plancticola* Ag11 and *M. luteus* Sr11. No identical intermediate was detected in the degradation by *S. nematodiphila* Ba11. Catechol was detected in the degradation by S. nematodiphila (Figure 3.28). Similarly, phenol and catechol were detected as intermediary products in benzene degrading *Pseudoxanthomonas spadix* BD-a59 in the study done by Choi *et al*. (2013).

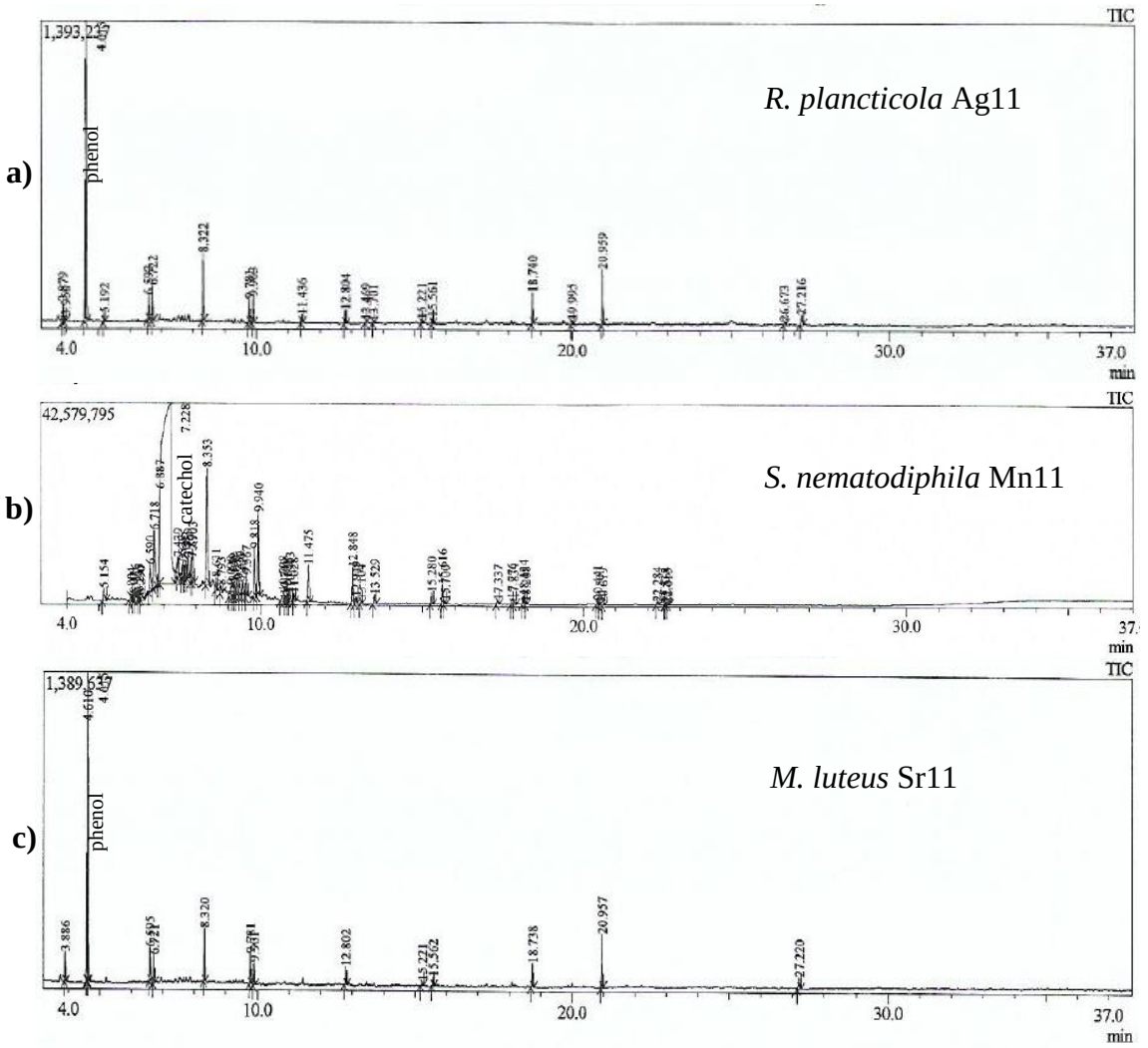


Figure 3.28 GC/MS chromatograms showing the intermediate products during benzene degradation while incubation with *R. plancticola* Ag11 (a), *S. nematodiphila* Mn11 (b) and *M. luteus* Sr11 (c). Main intermediates detected are indicated on each chromatogram.

3.4.2.2 Detection of intermediates during toluene degradation

Toluene has five different biodegradation pathways catalyzed by variety of enzyme systems (Leusch & Bartkow, 2010). Cresols are produced by the activity of monooxygenation (González *et al*., 2013). Side chain monooxygenation leads to production of benzyl alcohol (Heinaru *et al*., 2016) and dioxygenation is ended up with cis-toluenedihydrodiol (Rodrigues *et al*., 2009). At the end of each pathway catechol derivatives are formed. Five bacterial strains in this study were defined as efficient toluene degraders namely *R. plancticola* Ag11, *S. aureus* Ba01, *A. calcoaceticus* Fe10, *P. koreensis* Hg10 and *M. luteus* Sr11. Their intermediates were analyzed by GC/MS during toluene degradation. Benzyl alcohol and 4-cresol were detected in the degradation of toluene by *A. calcoaceticus* Fe10 and *M. luteus*, respectively (Figure 3.29). Choi *et al*. (2013) were reported the detection of 4-hydroxytoluene, 4-hydroxybenzaldehyde, and 4-hydroxybenzoate were detected as intermediates in in toluene degrading *Pseudomonas* sp. In the degradation of toluene by other toluene degraders, no related intermediate could be detected.

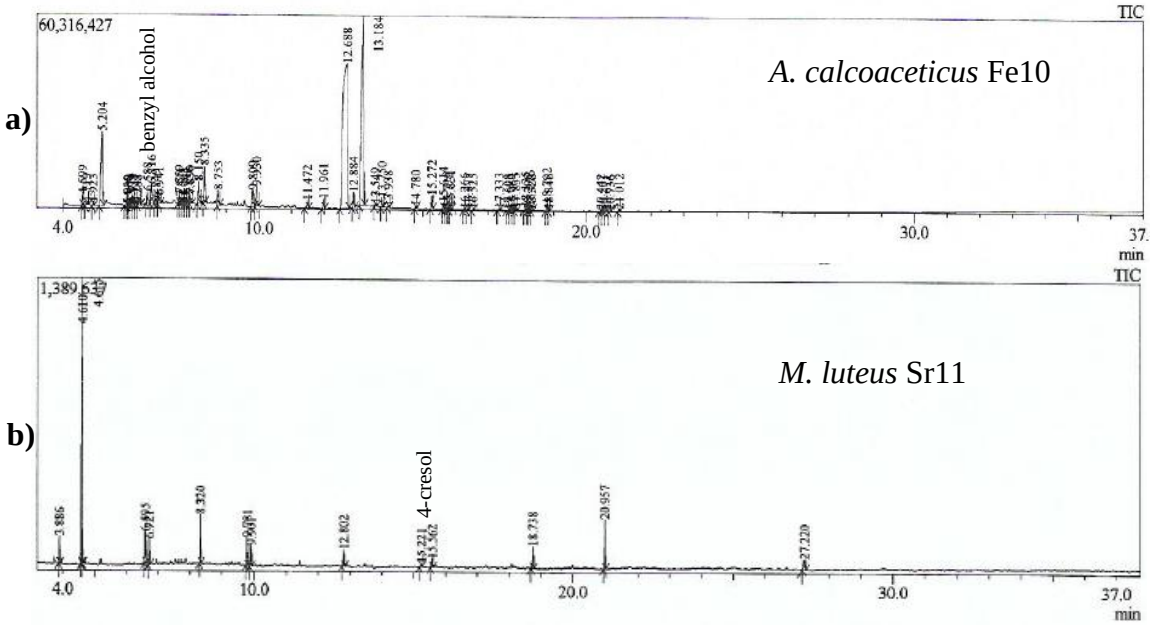


Figure 3.29 GC/MS chromatograms showing the intermediate products during toluene degradation while incubation with *A. calcoaceticus* Fe10 (a) and *M. luteus* Sr11 (b). Main intermediates detected are indicated on each chromatogram.

3.4.2.3 Detection of intermediates during ethylbenzene degradation

Ethylbenzene can be catalyzed by dioxygenases including toluene dioxygenase and naphthalene dioxygenase producing cis-ethylbenzenedihydrodiol and styrene, respectively (Ma *et al*., 2007). *R. plancticola* Ag11, *P. koreensis* Hg11, *S. nematodiphila* Mn11 were previously selected as efficient ethylbenzene degraders due to GC/MS analyses. In the degradation of ethylbenzene by *R. plancticola* Ag11, homogentisic acid was detected. By GC/MS analyses, lactic acid and acetoacetic acid were also detected in the ethylbenzene degradation by *P. koreensis* Hg11. As a metabolite, phenylacetic acid was found in the degradation of ethylbenzene by *S. nematodiphila* Mn11 (Figure 3.30). Metabolites of styrene pathway were also previously demonstrated in *Pseudoxanthomonas spadix* BD-a59 by Choi *et al*., (2013).

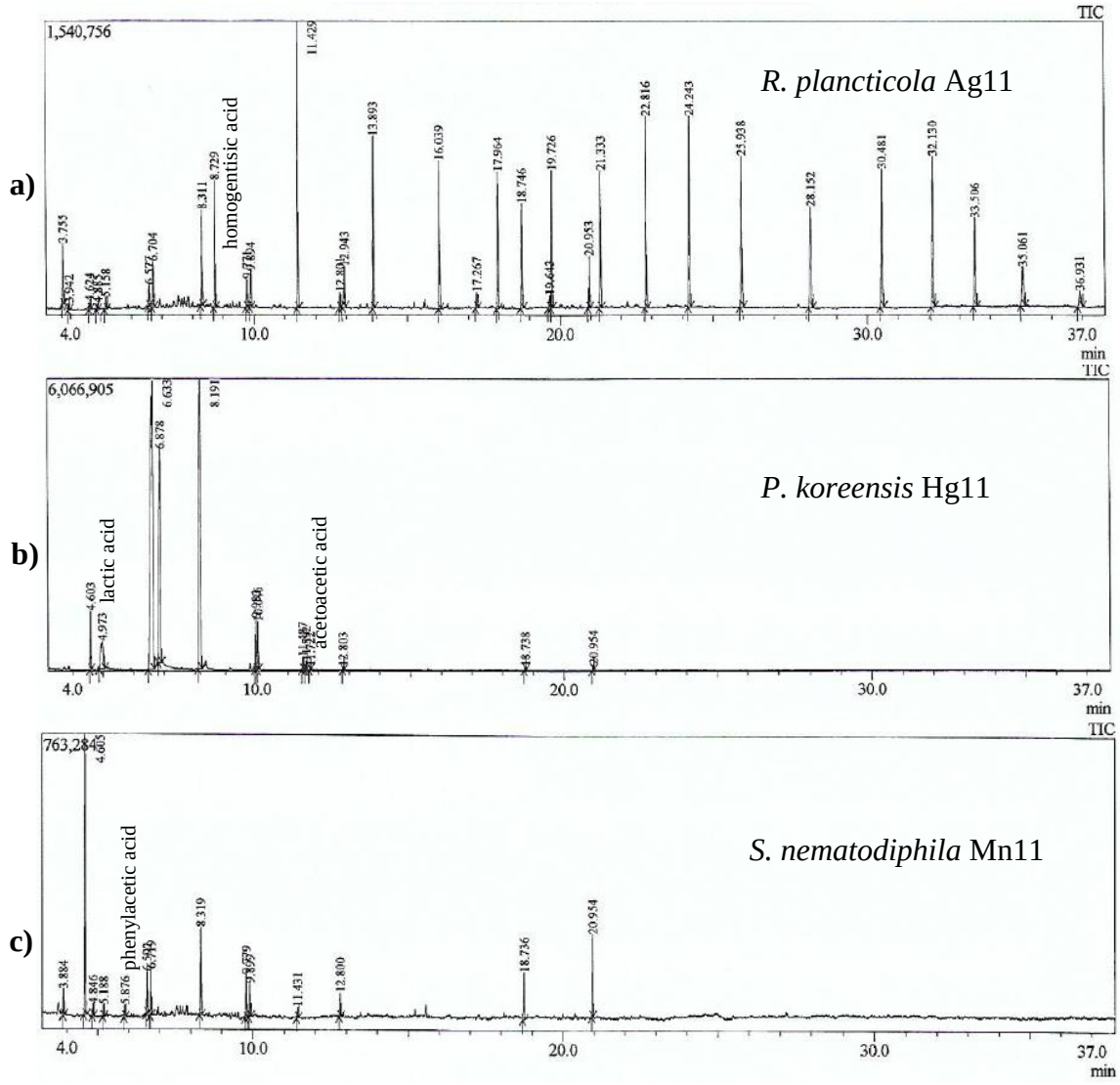


Figure 3.30 GC/MS chromatograms showing the intermediate products during ethylbenzene degradation while incubation with *R. plancticola* Ag11 (a), *P. koreensis* Hg11 (b), *S. nematodiphila* Mn11 (c). Main intermediates detected are indicated on each chromatogram.

3.4.2.4 Detection of intermediates during xylene degradation

Xylenes are metabolized to methylbenzyl alcohols and methyl benzoates subsequently (Choi *et al*., 2013). Mixed form of xylene was used in this study comprising *-o, -m* ad *-p* isomers. The previously defined bacterial strains namely *A. calcoaceticus* Fe10 and *A. johnsonii* were selected from xylene degraders and intermediates produced during xylene degradation by these two strains were determined by GC/MS. Three form of methyl benzoates (-2, -3, -4) were detected in the degradation of xylenes by *A. calcoaceticus* Fe10 (Figure 3.31) and none of the metabolite was detected in the degradation of xylene by *A. johnsonii* Sb01. The degradation of *o*-xylene in *B. cepacia* MB2 catalyzes through the oxidation of a methyl substituent of *o*-xylene to 2-methylbenzyl alcohol by xylene monooxygenase, which is followed by the subsequent conversion of 2-methylbenzyl alcohol to 2-methylbenzaldehyde and 2-methylbenzoate as reported by Jørgensen (1995).

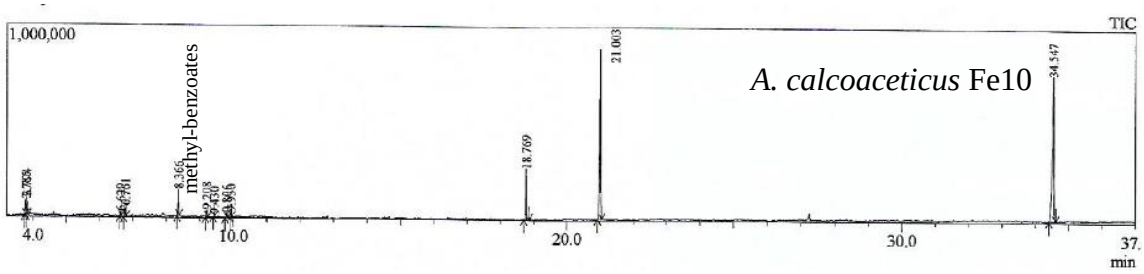


Figure 3.31 GC/MS chromatogram showing the intermediate products during xylene degradation while incubation with *A. calcoaceticus* Fe10. Main intermediates detected are indicated on each chromatogram.

Aerobic BTEX degradation pathways of efficient BTEX degraders were first predicted by PCR detection of the initial attack genes and then confirmed by intermediate analyses produced during degradation. The degradation characteristics of BTEX compounds by efficient degraders were summarized in Table 3.4 including degradation percentages, intermediates detected by GC/MS analysis during degradation, corresponding genotypes, and identified biodegradation pathways.

Table 3.4 Degradation characteristics of BTEX compounds by efficient degraders and their degradation pathways

Bacterial strains	Substrates	Degradation (%)	Intermediates	Initial attack genes							Cleavage genes			Pathway used
				todC1	*tbmD*	*tmoA*	*xylA*	*tol*	*phhN*	*nahAc*	*catA*	*C23O*-I	*C23O*-II	
R. planticola Ag11	B	73	phenol	+	-	-	-	-	-	-	+	-	+	B1
	T	63	-											T2
	E	75	homogentisic acid											E1.1
	X	-	na											nd
A. calcoaceticus Fe10	B	-	na	+	+	-	-	-	-	-	+	-	+	B2
	T	99	benzyl alcohol											T5
	E	56	na											E2
	X	45	methyl-benzoates											X1/X2/X3
P. koreensis Hg11	B	-	na	+	-	-	-	-	-	-	-	-	+	B2
	T	-	na											T2
	E	94	acetoacetate, lactic acid											E1.1/E1.2
	X	5	na											nd
S. nematodiphila Mn11	B	100	catechol	+	-	-	-	-	-	-	+	-	-	B2
	T	17	na											T2
	E	99	phenylacetic acid											E1.1
	X	-	na											nd
M. luteus Sr11	B	95	phenol	+	+	-	-	-	-	-	+	-	-	B1
	T	99	4-cresol											T4
	E	-	na											E2
	X	-	na											nd
S. aureus Ba01	B	45	na	-	-	-	-	-	-	-	+	-	-	nd
	T	100	-											nd
	E	34	na											nd
	X	-	na											nd
S. rhizophila Ba11	B	95	-	+	-	-	-	-	-	-	-	-	+	B2
	T	18	na											T2
	E	52	na											E2
	X		na											nd
P. koreensis Hg10	B	35	na	+	-	-	-	-	-	-	-	-	-	B2
	T	99	-											T2
	E	19	na											E2
	X	-	na											nd
A. johnsonii Sb01	B	-	na	+	-	-	-	-	-	-	-	-	-	B2
	T	71	na											T2
	E	20	na											E2
	X	37	-											nd

b, benzene; t, toluene; e, ethylbenzene; x, xylene; *todC1*, ring hydroxylating dioxygenase genes; *tbmD, tmoA* and *phhN*, ring hydroxylating monooxygenase genes; *xylA* and *tol*, side chain monooxygenase genes; *nahAc*, naphthalene dioxygenase gene; *catA*, catechol 1,2 dioxygenase genes; *C23O*, catechol 2,3 dioxygenase genes; -, not detected; +, detected; na, not analyzed; nd, not determined; for pathway abbreviations see Figure 3.28-29.

With these results, *R. plancticola* Ag11 degraded benzene by monooxygenation pathway, annotated as B1 (Figure 3.32). However, none of the genes related to monooxygenation pathways were detected by PCR. These results indicated that primers *tbmD, tmoA* and *phhN* were not suitable for the detection of all possible monooxygenase genes in the bacterial strains used in the study. The *todC1* gene involved in BTE degradation was detected in *R. plancticola* Ag11 but no intermediate was found related to dioxygenation pathway during the degradation of BTE. This may be due to the unstable characteristic of *cis*-toluene dihydrodiol as suggested by Jouanneau & Meyer (2006), the first produced metabolite of dioxygenation pathway. Detection of homogentisic acid justified that *R. plancticola* Ag11 metabolizes ethylbenzene by utilizing styrene pathway (E1.1) (Figure 3.35).

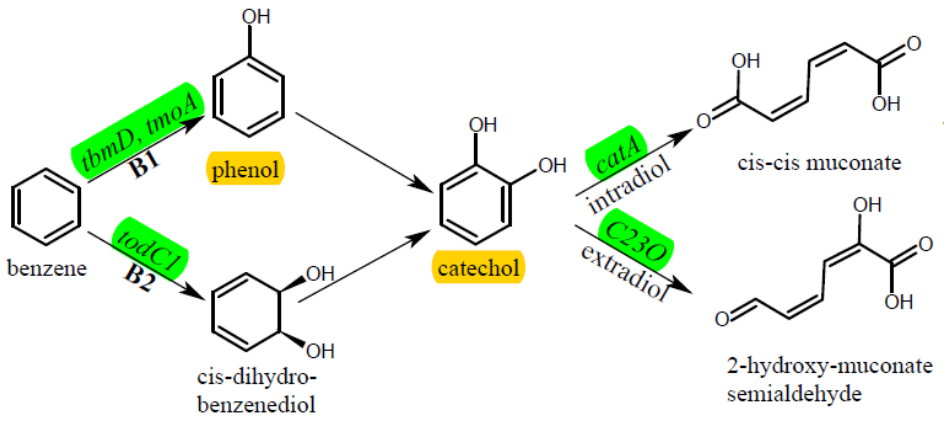


Figure 3.32 Schematic representation of aerobic benzene degradation (modified from KEGG)

An efficient benzene and toluene degrading strain *M. luteus* Sr11 was also evaluated by means of intermediates produced during degradation. In benzene and toluene degradation, detection of phenol and 4-cresol, respectively as key intermediates showed that degradations were initiated through monooxygenation pathways B1 and T4 (Figure 3.32-33). The presence of *tbmD* gene in *M. luteus* Sr11 was further supported the confirmation of monooxygenation pathway identified.

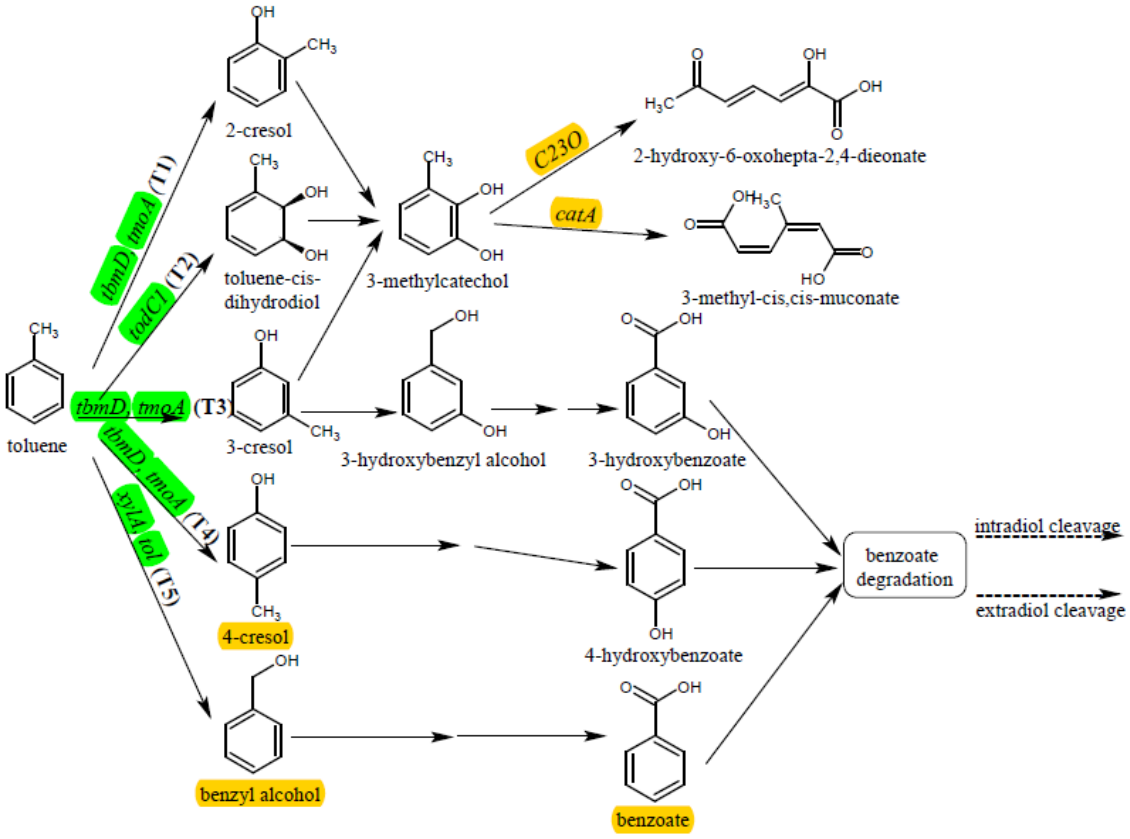


Figure 3.33 Schematic representation of aerobic toluene degradation (modified from KEGG)

In *A. calcoaceticus* Fe10, it was confirmed that, toluene and xylene degradation were catalyzed by TOL pathway annotated as T5 and X1/2/3, respectively (Figure 3.33-34) due to detection of benzylalcohol during toluene degradation and methylbenzoates during xylene degradation. The *todC1* and *tbmD* genes were detected but none of the *xyl* gene was observed related to T5 pathway. These results led to idea that primers *xylA* and *tol* were not sufficient for the detection of all possible *xyl* genes for the bacterial strains used in the study.

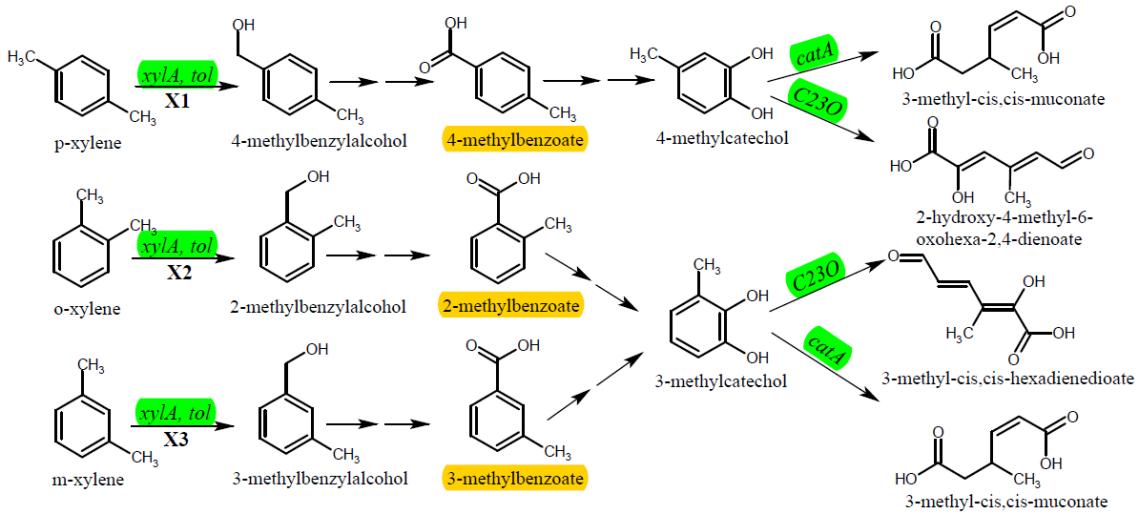


Figure 3.34 Schematic representation of aerobic toluene degradation (modified from KEGG)

In the degradation of ethylbenzene by *P. koreensis* Hg11, acetoacetic acid and lactic acid were found as intermediates. From these results, it was confirmed that ethylbenzene was degraded through styrene pathway and then divided into two branches namely E1.1 and E1.2 as shown in Figure 3.35.

Intermediates produced during the degradation of benzene and ethylbenzene by *S. nematodiphila* Mn11 were also analyzed. Detection of catechol in benzene degradation could not give any clue about the exact initial degradation pathway because catechol was produced in both monooxygenation and dioxygenation pathways (Figure 3.32). Presence of *todC1* in *S. nematodiphila* rendered the usage of dioxygenation pathway more favorable. Phenylacetic acid was also detected in ethylbenzene degradation by *S. nematodiphila* Mn11. Thus, it can be concluded that *S. nematodiphila* Mn11 degraded ethylbenzene through styrene pathway E1 (Figure 3.35).

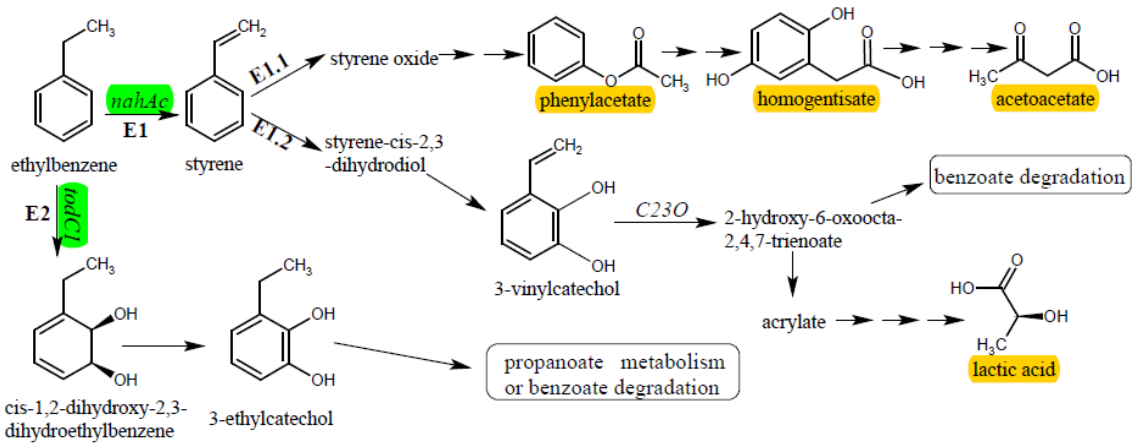


Figure 3.35 Schematic representation of aerobic ethylbenzene degradation (modified from KEGG)

PCR-detection results showed that *todC1* was found to be most common gene among the bacterial strains used in the study. Therefore, it was predicted that bacteria harboring the *todC1* gene used ring dioxygenation pathway. However, the intermediate cis-dihyrdodiols could not be detected through GC/MS analyses. This was attributed to the unstable behavior of *cis*-dihydrodiols made its detection difficult as confirmed in the study done by Kim *et al*. (2002). Therefore, the experimental conditions of GC/MS should be improved to detect unstable metabolites during degradation.

3.5 Conclusion

1. Out of 22 hydrocarbon degrading bacterial strains, 19 were selected as potential BTEX degrader belonging to the genera *Pseudomonas*, *Raoultella*, *Staphylococcus*, *Stenotrophomonas*, *Delftia*, *Acinetobacter*, *Serratia*, *Comamonas*, *Pantoea* and *Micrococcus*.
2. HS-GC/MS analyses were carried out to precisely determine the BTEX degradation abilities of bacterial strains. Almost 100 % of benzene by *S. nematodiphila* Mn11; 100 % of toluene by *A. calcoaceticus* Fe10, *M. luteus* Sr11, *S. aureus* Ba01 and *P. koreensis* Hg10; 100 % of ethylbenzene by *S. nematodiphila* Mn11; and 45 % of xylene by *A. calcoaceticus* Fe10 was removed. *S. nematodiphila* Mn11 showed the highest degradation rates in

benzene with 0.39 mg/mL/day and ethylbenzene degradation with 0.11 mg/mL/day, whereas the highest toluene and xylene degradation rates were observed in *A. calcoaceticus* Fe10 with 0.25 and 0.19 mg/mL/day respectively.

3. Phenol was detected in the degradation of benzene by *R. plancticola* Ag11 and *M. luteus* Sr11. Based on these results, *M. luteus* Sr11 and *R. plancticola* Ag11 degraded benzene over the monooxygenation pathway although *R. plancticola* Ag11 had *todC1* gene. Other efficient benzene degraders most likely used dioxygenation pathway due to the presence of *todC1* gene.
4. Detection of benzyl alcohol and 4-cresol in *A. calcoaceticus* Fe10 and *M. luteus* Sr11, revealed that *A. calcoaceticus* Fe10 metabolized toluene by using side chain monooxygenation pathway and *M. luteus* Sr11 convert toluene to 4-cresol through the monooxidation of the ring at 4^{th} position. Except for *S. aureus* Ba01, all other efficient toluene degraders contained *todC1* gene, therefore, it was predicted that dioxygenation pathway was used in toluene degradation.
5. In ethylbenzene degradation by *R. plancticola* Ag11, *P. koreensis* Hg11 and *S. nematodiphila* Mn11 homogentisic acid, acetoacetic acid/lactic acid and phenylacetic acid were detected, respectively. Based on these results, it was concluded that styrene pathway was used for the degradation of ethylbenzene by these strains.
6. In the degradation of xylene by *A. calcoaceticus* Fe10 detection of methylbenzoates led to the conclusion that xylene was metabolized through side-chain monooxygenation pathway by *A. calcoaceticus* Fe10.
7. Different *todC1* amplicons in 14 bacterial strains were compared to database and strong homology was found with toluene/benzene dioxygenase genes among all these strains. The sequence homology ranged in between 62 and 99 %. These results pointed out the horizontal transfer of dioxygenase genes among bacterial strains studied.

3.6 Future prospects and recommendations

- The degradation patterns of BTEX degraders should be evaluated in different mediums combined with BTEX mixtures for efficient bioremediation of all the BTEX compounds together.
- Bioavailability of BTEX is limited for microorganism due to hydrophobic nature. Before the field scale applications, biosurfactant producing abilities of bacteria and type of biosurfactants should be determined for efficient bioremediation.
- To prove the efficiency of bioremediation process, besides genetic and pathway analysis, the effects of environmental factors such as pH, temperature, presence of other substrates and their concentration, oxygen demand and dynamics of microbial consortia should be evaluated.
- The activity of efficient BTEX degrading bacteria should be determined in the presence of other bacteria.
- Genetically modified organisms can also be used to improve the hydrocarbon degradation efficiency, but other limitations may complicate the procedure, such as problems with international legislations
- Crude enzymes may also be used to transform the contaminant into less-toxic or nontoxic compounds. Enzymatic remediation can be simpler than microbial one. The use of crude enzymes does not generate toxic byproducts and whole cell competitiveness is not necessary.

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APPENDIX A

RAW DATA OF BTEX DEGRADATION

Table A.1 Amount of benzene measured by HS-GC/MS after degradation by bacterial strains

Samples	Benzene (diluted) (µg)	Dilution factor	Benzene (µg)
R. plancticola Ag11	4.36656	2500	10916.4
S. aureus Ba01	8.9034	2500	22258.5
S. rhizophila Ba11	0.74843	2500	1871.08
S. warneri Co11	9.48227	2500	23705.7
P. koreensis Cu12	4.51952	2500	11298.8
P. koreensis Hg10	10.4861	2500	26215.3
S. nematodiphila Mn11	0.01735	2500	43.3750
A. haemolyticus Mn12	5.30898	2500	13272.5
M. luteus Sr11	0.84071	2500	2101.78
Control (benzene)	16.23633	2500	40590.8

Table A.2 Amount of toluene measured by HS-GC/MS after degradation by bacterial strains

Samples	Toluene (diluted) (µg)	Dilution factor	Toluene (µg)
P. plecoglossicida Ag10	1.55136	2500	3878.40
R. plancticola Ag11	3.86647	2500	9666.18
S. aureus Al11	10.13648	2500	25341.2
S. aureus Ba01	0.00282	2500	7.05000
S. rhizophila Ba11	8.60595	2500	21514.9
S. warneri Co11	10.67474	2500	26686.9
A. calcoaceticus Fe10	0.05186	2500	129.650
P. koreensis Hg10	0.05183	2500	129.575
S. aureus Li12	4.36011	2500	10900.3
S. nematodiphila Mn11	8.69222	2500	21730.6
A. haemolyticus Mn12	2.60603	2500	6515.08
C. testosteroni Ni11	4.53679	2500	11342.0
A. johnsonii Sb01	3.0364	2500	7591.00
P. agglomerans Sn11	1.666	2500	4165.00
M. luteus Sr11	0.10595	2500	264.875
A. haemolyticus Zn01	4.64667	2500	11616.7
Control (toluene)	10.52967	2500	26324.2

Table A.3 Amount of ethylbenzene measured by HS-GC/MS after degradation by bacterial strains

Samples	Ethylbenzene (dilution) (µg)	Dilution factor	Ethylbenzene (µg)
R. plancticola Ag11	1.1517	2500	2879.25
S. aureus Ba01	3.06264	2500	7656.60
S. rhizophila Ba11	2.24013	2500	5600.33
D. acidovorans Cd11	3.96848	2500	9921.20
P. koreensis Cu12	2.40491	2500	6012.28
A. calcoaceticus Fe10	2.0568	2500	5142.00
P. koreensis Hg11	0.27942	2500	698.550
S. nematodiphila Mn11	0.02263	2500	56.5750
A. haemolyticus Mn12	3.3126	2500	8281.50
A. johnsonii Sb01	3.74994	2500	9374.85
Control (Ethylbenzene)	4.69412	2500	11735.3

Table A.4 Amount of xylene measured by HS-GC/MS after degradation by bacterial strains

Samples	Xylene (diluted) (µg)	Dilution factor	Xylene (µg)
D. acidovorans Cd11	13.29611	2500	33240.3
P. koreensis Cu12	15.02216	2500	37555.4
A. calcoaceticus Fe10	9.78636	2500	24465.9
P. koreensis Hg10	14.34366	2500	35859.2
P. koreensis Hg11	17.05074	2500	42626.9
A. johnsonii Sb01	11.26997	2500	28174.9
Control (Xylene)	17.88847	2500	44721.2

APPENDIX B

MULTIPLE SEQUENCE ALIGNMENTS OF PCR AMPLICONS

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AB017631.1   ------------------------------------------CCGCCTGACCATGGAC
GQ870381.1   ------------------------------------------------------------
Sr11-tbmD_C9 ------------------------------------------------------------
LT604176.1   ------------------------------------------------------------
Fe10-tbmD_C9 ------------------------------------------------------------
LT604161.1   ------------------------------------------------------------
LT604143.1   ------------------------------------------------------------
AB016861.1   ------------------------------------------CCGCCTGACGATGGAT
KT021791.1   ----------------------------------------------TGACCATGGAT
HF561957.1   GAGGGGATCAGGGTCCACGATTGGGACAAGTGGGGAGACCCTTTCCGTCTGACCATGGAC
JF806589.1   ------------------------------------------------------------
JQ069989.1   ------------------------------------------------------------

AB017631.1   GCCTACTGGAAGTACCAGGGCGAAAAAGAGAAAAAGCTCTACGCCGTGATCGAGGCCTTT
GQ870381.1   --------------CCAGGGCGAGAAGGAGAGGAAGTTCTACGCCGTGATCGAGGCCTTC
Sr11-tbmD_C9 ---AGTCTTCGTGTTAAGTCTCATGCAGGCGAGGAACTTTACGCCGTGATCGACGCGTTC
LT604176.1   --------------CCAGGGTGAGAAGGAGAGGAAGCTCTACGCCGTGATCGACGCGTTT
Fe10-tbmD_C9 ------------CAGTTCGCTCATACAGGCGAGGAGCTTTACGCCGTGATCGACGCGTTC
LT604161.1   --------------CCAGGCGGAGAAGGAGAGGAAGCTCTACGCCGTGATCGACGCGTTC
LT604143.1   --------------CCAGGGGGAAAAGGAGAGGAAACTCTACGCCGTGATCGACGCGTTC
AB016861.1   GCGTACTGGAAATACCAGGGCGAGAAGGAGAAGAAGCTGTACGCGGTGCTCGATGCGTTC
KT021791.1   GCGTACTGGAAGTATCAGGGCGAAAAGGAGAAGAAGCTCTACGCCGTGATTGAGGCTTTT
HF561957.1   GCCTACTGGAAGTACCAGGGCGAGAAGGAGAAGAAGCTCTACGCGGTGATCGAGGCCTTC
JF806589.1   ----------------------------------------TACGCGGTGATCGAGGCCTTC
JQ069989.1   ----------------------------------------TACGCCGTGATCGAGGCCTTC
                                                     ***** *** * ** ** **

AB017631.1   GCCCAGAACAACGGCCAGTTGGGCGTGACCGATGCGCGCTATCTCAACGCGCTCAAGCTG
GQ870381.1   GCCCAGAACAACGGCCAGCTGGGCGTGACCGACGCGCGCTACGTCAACGCGCTCAAGCTG
Sr11-tbmD_C9 GCGCAGAACAACGGCCAGCTGGAGGTGAGCGACGCGCGCCACATCAACGCGCTCAAGCTG
LT604176.1   GCGCAGAATAACGGCCAGTTGGGTGTGAGTGACGCCCGTTACATCAACGCCCTGAAGCTG
Fe10-tbmD_C9 GCGCAGAACAACGGCCAGCTGGGGGTGAGCGACGCGCGCTACATCAACGCGCTCAAGCTG
LT604161.1   GCGCAGAACAACGGCCAGCTGGGCGTGAGCGACGCGCGCTACATCAACGCGCTCAAGCTG
LT604143.1   GCGCAGAACAACGGCCAGCTGGGCGTGAGCGACGCGCGCTACATCAACGCGCTCAAGCTG
AB016861.1   ACGCAGAACAACGCGTTCCTCGGCGTGACCGACGCGCGCTACATCAACGCGCTGAAGCTG
KT021791.1   GCGCAGAACAACGGTCAGCTCGGCATCAGCGATGCGCGCTACGTCAACGCGCTCAAGCTG
HF561957.1   GCGCAGAACAACGGCCAGCTCGGCGTCAGCGACGCGCGCTACATCAACGCGCTGAAGCTC
JF806589.1   GCGCAGAACAACGGCCAGCTCGGCGTGGCCGACGCGCGCTACATCAATGCGCTGAAGCTC
JQ069989.1   GCGCAGAACAACGGCCAGCTCGGCGTGGCCGACGCGCGCTACGTCAACGCGCTCAAGCTG
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AB017631.1   TTCATTCAGGGCGTGGTGCCGCTGGAGTATTACGCCCACCGCGGTTTTGCCCATGCGGGC
GQ870381.1   TTCCTCCAGGGCGTGACGCCGCTGGAGTACGGCGCGCACCGGGGCTTCGCCCACGTGGGC
Sr11-tbmD_C9 TTCATCCAGGGCGTGTCGCCGCTCGAGTATTACGCGCACCACGGCTTCGCCCACGTCGGC
LT604176.1   TTCATCCAGGGTGTATCACCGCTCGAGTACTACGCGCACCGTGGTTTCGCCCATGTGGGC
Fe10-tbmD_C9 TTCATCCAGGGCGTGTCGCCGCTCGAGTATTACGCGCACCGCGGCTTCGCCCACGTCGGC
LT604161.1   TTCATCCAGGGCGTGTCGCCGCTCGAGTACTACGCGCACCGCGGCTTCGCCCACGTGGGC
LT604143.1   TTCATCCAGGGCGTGTCGCCGCTCGAGTACTACGCGCACCGCGGCTTCGCCCACGTCGGC
AB016861.1   TTCATCCAGGGCGTGACGCCGCTTGAATATCTCGCGCATCGCGGTTTCGCGCACGTCGGC
KT021791.1   TTCATCCAGGGCGTCACGCCACTGGAATACAACGCGCACCGTGGGTTTGCCCACGTCGGC
HF561957.1   TTCATCCAGGGCGTGACGCCGCTCGAGTACTACGCGCACCGCGGTTATGCGCATGTCGGC
JF806589.1   TTCATCCAGGGCGTGACGCCGCTGGAGTACTACGCGCACCGCGGCTTCGCCCACGTGGGC
JQ069989.1   TTCATCCAGGGCGTGACGCCGCTGGAGTACTACGCGCACCGCGGCTTCGCCCACGTGGGC
             *** * ***** **    ** ** ** **   *** ** *  ** *  ** ** *  ***

AB017631.1   CGGCATTTCACCGGTGCGGGGGCACGCGTGGCGGCGCAGATGCAGTCCATCGACGAGCTG
GQ870381.1   CGGCATTTCACGGGGGCCGGCGCGCGCGTGGCCGCGCAGATGCGGTCCATCGACGAGCTG
Sr11-tbmD_C9 CGGCAATTTACCGGCGCTGGCCCGCGCGTAGCAGCGCAGATGCAGTCGGTCGACGAACTG
LT604176.1   CGGCATTTCACCGGCGCCGGAGCGCGGGTGGCGGCGCAGATGCAGTCGATCGACGAACTG
Fe10-tbmD_C9 CGGCAATTTACCGGCGCTGGCGCGCGCGTAGCAGCGCAGATGCAGTCGATCGACGAACTG
LT604161.1   CGGCAATTTACCGGCGCCGGCGCGCGCGTGGCAGCGCAGATGCAGTCGATCGACGAACTG
LT604143.1   CGGCAATTTACCGGCGCCGGCGCGCGCGTGGCGGCGCAGATGCAGTCGATCGACGAACTG
AB016861.1   CGGCACTTCACCGGCGAGGGCGCGCGCATCGCGTGCCAGATGCAGTCGATCGACGAGTTG
KT021791.1   CGTCAGTTCACGGGCGAAGGTGCGCGCGTGGCTGCGCAGATGCAGTCCATCGACGAACTG
HF561957.1   CGGCAGTTCACCGGCGAAGGCGCGCGCGTGGCAGCGCAGATGCAGAGCATCGACGAGTTG
JF806589.1   CGGCAGTTCACCGGCGAAGGCGCCCGTGTGGCGGCGCAGATGCAGTCGATCGACGAGCTG
JQ069989.1   CGCCAGTTCACCGGTGAAGGTGCCCGCGTCGCCGCGCAGATGCAGTCCATCGACGAGCTG
             ** ** ** ** ** * **  * **  * **    ******* *    *******  **

AB017631.1   CGCCACTTCCAGACCGAGACCCATGCGCTGTCCAACTACAACAAGTACTTCAACGGCATG
GQ870381.1   CGCCATTTCCAGACAGAGACGCATGCGCTGAGCCACTACAACAAGTACTTCAACGGCATG
Sr11-tbmD_C9 CGTCACTACCAGACGCAGGCGCACGCCGTCAGCCACTACAACAAGCACTCTAACGGCATG
LT604176.1   CGCCATTACCAGACGCAGGCGCACGCGGTCAGCCACTACAACAAGCACTTCAACGGGATG
Fe10-tbmD_C9 CGTCATTACCAGACGCAGGCGCACGCCGTCAGCCACTACAACAAGCACTTTAACGGCATG
LT604161.1   CGTCACTACCAGACGCAGGCGCACGCCGTCAGCCACTACAACAAGCACTTCAACGGCATG
LT604143.1   CGTCACTACCAGACGCAGGCGCACGCCGTCAGCCACTACAACAAGCACTTTAACGGCATG
AB016861.1   CGGCACTACCAGACCGAAACGCACGCGATGTCGACGTACAACAAGTTCTTCAACGGCTTC
KT021791.1   CGCCATTACCAGACCGAGACGCATGCGATCTCGCACTACAACAAGTACTTCAACGGCATG
HF561957.1   CGGCACTACCAGACCGAAACGCACGCGCTCTCGCACTACAACAAGTACTTCAACGGCCTG
JF806589.1   CGCCACTACCAGACCGAGACCCACGCGATCTCGCACTACAACAAGTACTTCAACGGCATG
JQ069989.1   CGCCACTACCAGACCGAGACGCACGCGATCAGCCACTACAACAAGTACTTCAACGGCATG
             ** ** * ******  *  * ** **  *       *********  **  *****  *

AB017631.1   CACAGCTCCAG-CCACTGGTTCGACCGGGTCTGGTTTCTGTCGGT--GCCCAAGTCCTTT
GQ870381.1   CACAACGCATC-GCAGTGGTACGACCGCGTCTGGTACCTGTCCGT--GCCCAAGTCCTTC
Sr11-tbmD_C9 CACAGCGCCGAACCACTGGTTCGACCGCATGTGGTACCTGTCGATGTGCCGAAGTCGTTC
LT604176.1   CACAGCGCCAA-CCATTGGTTCGACCGCGTGTGGTACCTGTCGGT--TCCGAAGTCGTTC
Fe10-tbmD_C9 CACAGCCCGAA-CCACTGGTTCGACCGCGTGTGGTACCTGTCGGT--GCCGAAGTCGTTC
LT604161.1   CACAGCCCGAA-CCACTGGTTCGACCGCGTGTGGTACCTGTCGGT--GCCGAAGTCGTTC
LT604143.1   CACAGCCCGAA-CCACTGGTTCGACCGCGTGTGGTATCTGTCGGT--GCCGAAGTCGTTC
AB016861.1   CATCATTCGAA-CCACTGGTTCGACCGCGTCTGGTACCTGTCGGT--GCCGAAGTCGTTC
KT021791.1   CACAGCCCGAA-CCACTGGTTCGACCGTGTCTGGTACCTGTCGGT--GCCGAAGTCGTTC
HF561957.1   CACCAGTCGAA-CCACTGGTTCGACCGCGTCTGGTACCTCTCGGT--GCCGAAGTCCTTC
JF806589.1   CACCACTCGAA-CCACTGGTTCGACCGCGTGTGGTACCTGTCGGT--GCCGAAGTCCTTC
JQ069989.1   CACCACTCGAA-CCACTGGTTCGACCGTGTGTGGTACCTGTCGGT--GCCAAAGTCCTTC
             **     *     ** **** ******  * ****  ** **  *   ** ***** **
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AB017631.1   TTTGAAGATG--CGCTGAGCGCAGGCCCGTTCGAGTTCCTCACGGCAGTGAGCTTCTCCT
GQ870381.1   TTCGAGGACG--CGATGACGGCCGGCCCCTTCGAGTTCCTCACGGCCGTGAGCTTCTCGT
Sr11-tbmD_C9 TTCGAGGACGTCAACACGGCGCCGGGCCGTTCGAGTTCCTCACCGCGGTGAGCTTTTCGT
LT604176.1   TTTGAGGATG--CGAACACCGCCGGGCCGTTCGAGTTCCTCACCGCGGTGAGCTTTTCGT
Fe10-tbmD_C9 TTCGAGGACG--CCAACAGCGCCGGGCCGTTCGAGTTCCTCACCGCGGTGAGCTTTTCGT
LT604161.1   TTCGAGGATG--CCAACAGCGCCGGGCCGTTCGAGTTCCTCACCGCGGTGAGCTTTTCGT
LT604143.1   TTCGAGGATG--CCAACAGCGCCGGGCCGTTCGAGTTCCTCGTCGCGGTGAGCTTCTCGT
AB016861.1   TTCGAGGACG--CGTATTCGTCGGGGCCGTTCGAGTTCCTGACGGCGGTCAGCTTCTCGT
KT021791.1   TTCGAAGACG--CATGCACGGCCGGTCCGTTCGAGTTTCTGACAGCGGTCAGCTTCTCGT
HF561957.1   TTCGAGGACG--CGATCACCGGTGGGCCGTTCGAGTTCCTGTGTGCCGTCAGCTTCTCCT
JF806589.1   TTCGAGGATG--CCAACAGCGCCGGGCCGTTCGAGTTCCTCACCGCAGTGAGCTTCTCGT
JQ069989.1   TTCGAAGACG--CCTGCACCGGCGGCCCGTTCGAGTTCCTGACCGCGGTCAGTTTCTCGT
             ** ** ** *             ** ** ******** **    ** ** ** ** ** *

AB017631.1   TCGAATATGTGCTGACCAATCTGCTGTTCGTGCCCTTCATGTCGGGCGCGGCGCACAACG
GQ870381.1   TCGAATATGTGCTGACCAACCTGCTGTTCGTGCCCTTCATGTCGGGCGCGGCGCACAACG
Sr11-tbmD_C9 TCGAATACGTGCTCACCAACCTGCTGTTCGTGCCCTTCATGTCCGGCGCAGCGCACAACA
LT604176.1   TCGAATACGTTTTGACCAACCTGCTGTTCGTGCCCTTCATGTCCGGTGCGGCGCACAACG
Fe10-tbmD_C9 TCGAATACGTGCTCACCAACCTGCTGTTCGTGCCCTTCATGTCGGGCGCAGCGCACAACG
LT604161.1   TCGAATACGTGCTCACCAACCTGCTGTTCGTGCCCTTCATGTCGGGCGCAGCGCACAACG
LT604143.1   TCGAATACGTGCTCACCAACCTGCTGTTCGTGCCCTTCATGTCGGGCGCAGCGCACAACG
AB016861.1   TCGAATACGTGCTGACGAACCTGCTGTTCGTGCCGTTCATGTCGGGCGCCGCGTACAACG
KT021791.1   TCGAATACGTACTGACCAACCTGTTGTTCGTGCCGTTCATGTCCGGCGCCGCGCACAACG
HF561957.1   TCGAGTACGTGCTGACGAACCTGCTCTTCGTGCCCTTCATGAGCGGCGCGGCGCACAACG
JF806589.1   TCGAATACGTGCTGACCAACCTGCTGTTTGTGCCCTTCATGAGCGGCGCGGCGCACAACG
JQ069989.1   TCGAGTACGTGCTGACCAACCTACTGTTCGTGCCTTTCATGAGCGGCGCGGCGCACAACG
             **** ** **  * ** ** **  * ** ***** ******   ** ** *** ****

AB017631.1   GCGATCTGTCCACCGTGACCTTTGGCTTCTCGGCGCAAAGCGATGAGTCTCGCCATATGA
GQ870381.1   GCGACCTGTCCACGGTGACCTTCGGCTTCTCGGCGCAGAGCGACGAGTCGCGCCACATGA
Sr11-tbmD_C9 GCGACCTGGCCACGATGACCTCCGGCTTCAGCGCACAGAGCGACGAGTCGCGCCACATGA
LT604176.1   GCGACTTGGCCACGGTGACCTTCGGCTTCAGCGCTCAGAGCGACGAGTCGCGCCACATGA
Fe10-tbmD_C9 GCGACCTGGCCACGGTGACCTTCGGCTTCAGCGCACAGAGCGACGAGTCGCGCCACATGA
LT604161.1   GCGACCTGGCCACGGTGACCTTCGGCTTCAGCGCACAGAGCGACGAGTCGCGCCACATGA
LT604143.1   GCGACCTGGCCACGGTGACCTTCGGCTTCAGCGCACAGAGCGACGAGTCGCGCCACATGA
AB016861.1   GCGACATGTCCACCGTCACGTTCGGCTTTTCCGCGCAGTCGGACGAATCGCGTCACATGA
KT021791.1   GCGACATGAGTACCGTCACCTTCGGCTTCTCTGCACAGTCCGATGAGTCGCGCCACATGA
HF561957.1   GCGACATGTCGACCGTGACCTTCGGCTTCAGCGCGCAGAGCGACGAGAGCCGCCACATGA
JF806589.1   GCGACATGTCCACCGTGACCTTCGGGTTCAGCGCGCAGAGCGACGAGAGCCGCCACATGA
JQ069989.1   GCGACATGTCCACCGTCACCTTCGGCTTCTCGGCCCAGTCCGACGAAAGCCGCCACATGA
             ****  **   **  * ** *  ** **    ** **    ** **    ** ** ****

AB017631.1   CGCTGGGCATCGAATGCATCAA----GTTCATGCTGGAGCAGGACCCGGGCAATGTGCCC
GQ870381.1   CGCTGGGCATCGAGTGCATCAA----GTTCATGCTGGAGCAGGACCCGGCCAACGTGCCC
Sr11-tbmD_C9 CGCTGGACATCGAGTGCATCATTTGAGTTCCTGCTCGAGCAGGACCCGGCCAACGTGCCC
LT604176.1   CCCTGGGCATCGAGTGCATCAA----GTTCCTGTTGGAACAGGACCCGGCCAACGTGCCG
Fe10-tbmD_C9 CGCTGGGCATCGAGTGCATCAA----GTTCCTGCTCGAGCAGGACCCGGCCAACGTGCCC
LT604161.1   CGCTGGGCATCGAGTGCATCAA----GTTCCTGCTCGAGCAGGACCCGGCCAACGTGCCC
LT604143.1   CGCTGGGCATCGAGTGCATCAA----GTTCCTGCTCGAGCAGGACCCGGCCAACGTGCCC
AB016861.1   CGCTCGGCATCGAGTGCATCAA----GTTCCTGCTCGAGCAGGACCCGGACAACGTGCCG
KT021791.1   CGCTGGGCATCGAATGCATCAA----GTTCATGCTGGAGCAGGATCCCGACAACGTGCCC
HF561957.1   CACTCGGCATCGAGTGCATCAA----GTTCATGCTCGAGCAGGACCCGGCCAACGTGCCG
JF806589.1   CGCTGGGCATCGAGTGCATCAA----GTTCATGCTCGAGCAGGACCCGGCCAACGTGCCC
JQ069989.1   CGCTGGGGATCGAGTGCATCAA----GTTCCTGCTGGAGCAGGACCCGGACAACGTGCCG
             * ** *  ***** *******     **** ** * ** ***** ** * *** *****
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AB017631.1   ATCGTGCAGCGCTGGATCGACAAATGGTTCTGGCGCGGCTACCGCGTGCTGACCCTGGTG
GQ870381.1   ATCGTGCAGCGCTGGATCGACAAGTGGTTCTGGCGCGGCTACCG----------------
Sr11-tbmD_C9 ATCGTGCAGGGCTGGATCGACAAGTGGTTTCTGGGAAGCCCGGAAGCG------------
LT604176.1   ATCGTGCAGGGTTGGATCGACAAGTGGTTCTGGCGCGGCTTCCG----------------
Fe10-tbmD_C9 ATCGTGCAGGGCTGGATCGACAAGTGGTTTCTGGGGGAAGCCCGGGGGCG----------
LT604161.1   ATCGTGCAGGGCTGGATCGACAAGTGGTTCTGGCGCGGCTTCCG----------------
LT604143.1   ATCGTGCAGGGCTGGATCGACAAGTGGTTCTGGCGCGGCTACCG----------------
AB016861.1   ATCGTGCAGCGCTGGATCGACAAGTGGTTCTGGCGCGGCTACCGGCTGCTCACGCTGGTC
KT021791.1   ATCGTTCAGCGCTGGATCGACAAGTGGTTCTGGCGCGGCTACCGGCTGCTGACCATCGTC
HF561957.1   ATCGTGCAGAAGTGGATCGACAAGTGGTTCTGGCGCGGCTACCGCCTGCTCACGCTCGTC
JF806589.1   ATCGTGCAGCAGTGGATCGACAAG------------------------------------
JQ069989.1   ATCGTGCAGAAGTGGATCGACAAG------------------------------------
             ***** ***   ***********

AB017631.1   GCCATGATGCAGGATTACATGCTGCCCAAGCGCGTGATGAGCTGGAAGGAAGCCTGGGAG
GQ870381.1   ------------------------------------------------------------
Sr11-tbmD_C9 ------------------------------------------------------------
LT604176.1   ------------------------------------------------------------
Fe10-tbmD_C9 ------------------------------------------------------------
LT604161.1   ------------------------------------------------------------
LT604143.1   ------------------------------------------------------------
AB016861.1   GCGATGATGATGGACTACATGCAGCCGAAGCGCGTGATGAGCTGGCGCGAGTCGTGGGAG
KT021791.1   GCGATGATGCAGG------------------------------------------------
HF561957.1   GCGATGATGGACTACATGCTCCCG-------------------------------------
JF806589.1   ------------------------------------------------------------
JQ069989.1   ------------------------------------------------------------

AB017631.1   ATGTATGCCGAGGAAAACGGCGGCGCTCTGTTCCGCGACCTGGCGCGCTACGGCATTCGC
GQ870381.1   ------------------------------------------------------------
Sr11-tbmD_C9 ------------------------------------------------------------
LT604176.1   ------------------------------------------------------------
Fe10-tbmD_C9 ------------------------------------------------------------
LT604161.1   ------------------------------------------------------------
LT604143.1   ------------------------------------------------------------
AB016861.1   ATGTACGCGGAGCAGAACGGCGGCGCGCTGTTCAAGGATCTCGCGCGCTACGGCATTCGC
KT021791.1   ------------------------------------------------------------
HF561957.1   ------------------------------------------------------------
JF806589.1   ------------------------------------------------------------
JQ069989.1   ------------------------------------------------------------

AB017631.1   GAGCCCGCAGGCTGGAAGCTGGCCTGCGAAGGTAAGGACCACATCAGCCACCAGGCCTGG
GQ870381.1   ------------------------------------------------------------
Sr11-tbmD_C9 ------------------------------------------------------------
LT604176.1   ------------------------------------------------------------
Fe10-tbmD_C9 ------------------------------------------------------------
LT604161.1   ------------------------------------------------------------
LT604143.1   ------------------------------------------------------------
AB016861.1   GAGCCGAAGGGCTGGCAGGACGCCTGCGAAGGCAAGGACCACATCAGCCACCAGGCATGG
KT021791.1   ------------------------------------------------------------
HF561957.1   ------------------------------------------------------------
JF806589.1   ------------------------------------------------------------
JQ069989.1   ------------------------------------------------------------

AB017631.1   AACATCTTCTACAACTATACGGCGGCCGCACCTTTTCACACCTGGGTGCCCGGCGAGCAG
GQ870381.1   ------------------------------------------------------------
Sr11-tbmD_C9 ------------------------------------------------------------
LT604176.1   ------------------------------------------------------------
Fe10-tbmD_C9 ------------------------------------------------------------
LT604161.1   ------------------------------------------------------------
LT604143.1   ------------------------------------------------------------
AB016861.1   TCGACATTCTACGGCTTCAACGCGGCGTCGGCGTTCCACACGTGGGTGCCGACCGAGGAC
KT021791.1   ------------------------------------------------------------
```

```
HF561957.1   ------------------------------------------------------------
JF806589.1   ------------------------------------------------------------
JQ069989.1   ------------------------------------------------------------

AB017631.1   GAGATGCAGTGGCTGTCCGAAAAATACCCGGACAGCTTTGACAAGCACTACCGTCCCCGC
GQ870381.1   ------------------------------------------------------------
Sr11-tbmD_C9 ------------------------------------------------------------
LT604176.1   ------------------------------------------------------------
Fe10-tbmD_C9 ------------------------------------------------------------
LT604161.1   ------------------------------------------------------------
LT604143.1   ------------------------------------------------------------
AB016861.1   GAGATGGCGTGGCTGTCCGCGAAGTATCCCGACTCGTTCGACCGCTATTACCGCCCGCGC
KT021791.1   ------------------------------------------------------------
HF561957.1   ------------------------------------------------------------
JF806589.1   ------------------------------------------------------------
JQ069989.1   ------------------------------------------------------------

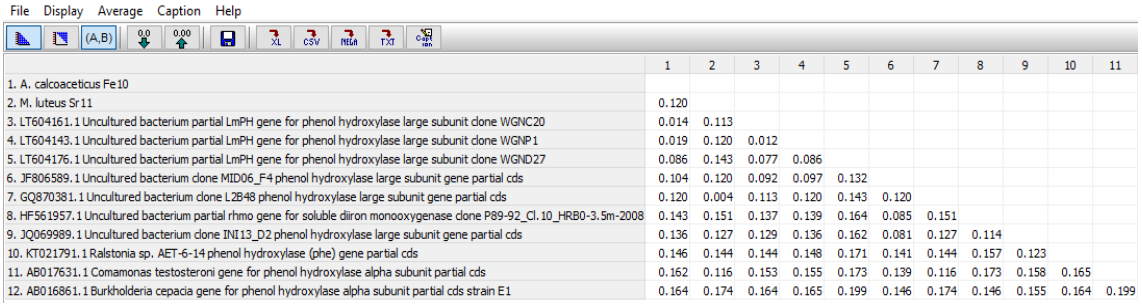
AB017631.1   CTGGAGCACTACCTTGCCGAGCAGCAGGCCGGCAAGCGCTTTTACAGCAAGACCCTGCCC
GQ870381.1   ------------------------------------------------------------
Sr11-tbmD_C9 ------------------------------------------------------------
LT604176.1   ------------------------------------------------------------
Fe10-tbmD_C9 ------------------------------------------------------------
LT604161.1   ------------------------------------------------------------
LT604143.1   ------------------------------------------------------------
AB016861.1   TTCGACTACTGGGGAGAGCAGGCGAAGGCCGGCAACCGCTTCTACATGAAGACGCTGCCG
KT021791.1   ------------------------------------------------------------
HF561957.1   ------------------------------------------------------------
JF806589.1   ------------------------------------------------------------
JQ069989.1   ------------------------------------------------------------

AB017631.1   ATGCTGTGCACCACCTGCCAGATCCCTATGGGCTTCACCGAGCCCGGTGACGCCACCAAG
GQ870381.1   ------------------------------------------------------------
Sr11-tbmD_C9 ------------------------------------------------------------
LT604176.1   ------------------------------------------------------------
Fe10-tbmD_C9 ------------------------------------------------------------
LT604161.1   ------------------------------------------------------------
LT604143.1   ------------------------------------------------------------
AB016861.1   ATGCTGTGCCAGACCTGCCAGATCCCGATGCTGTTCACCGAGCCCGGCAACCCGACGAAG
KT021791.1   ------------------------------------------------------------
HF561957.1   ------------------------------------------------------------
JF806589.1   ------------------------------------------------------------
JQ069989.1   ------------------------------------------------------------

AB017631.1   ATCTGCTATCGCGAGTCCGACTACGAGGGCAGCAAATACCACTTCTGCAGCGACGGCTGC
GQ870381.1   ------------------------------------------------------------
Sr11-tbmD_C9 ------------------------------------------------------------
LT604176.1   ------------------------------------------------------------
Fe10-tbmD_C9 ------------------------------------------------------------
LT604161.1   ------------------------------------------------------------
LT604143.1   ------------------------------------------------------------
AB016861.1   ATCGGCGCGCGCGAGTCGAACTACCTCGGC----------------------------
KT021791.1   ------------------------------------------------------------
HF561957.1   ------------------------------------------------------------
JF806589.1   ------------------------------------------------------------
JQ069989.1   ------------------------------------------------------------
```

```
AB017631.1    AAGCATG
GQ870381.1    -------
Sr11-tbmD_C9  -------
LT604176.1    -------
Fe10-tbmD_C9  -------
LT604161.1    -------
LT604143.1    -------
AB016861.1    -------
KT021791.1    -------
HF561957.1    -------
JF806589.1    -------
JQ069989.1    -------
```

Figure B.1 Multiple sequence alignment of partial *tbmD* sequences by Clustal Omega. Asterisks denote identical nucleotides.



	1	2	3	4	5	6	7	8	9	10	11
1. A. calcoaceticus Fe10											
2. M. luteus Sr11	0.120										
3. LT604161.1 Uncultured bacterium partial LmPH gene for phenol hydroxylase large subunit clone WGNC20	0.014	0.113									
4. LT604143.1 Uncultured bacterium partial LmPH gene for phenol hydroxylase large subunit clone WGNP1	0.019	0.120	0.012								
5. LT604176.1 Uncultured bacterium partial LmPH gene for phenol hydroxylase large subunit clone WGND27	0.086	0.143	0.077	0.086							
6. JF806589.1 Uncultured bacterium clone MID06_F4 phenol hydroxylase large subunit gene partial cds	0.104	0.120	0.092	0.097	0.132						
7. GQ870381.1 Uncultured bacterium clone L2B48 phenol hydroxylase large subunit gene partial cds	0.120	0.004	0.113	0.120	0.143	0.120					
8. HF561957.1 Uncultured bacterium partial rhmo gene for soluble diiron monooxygenase clone P89-92_Cl.10_HRB0-3.5m-2008	0.143	0.151	0.137	0.139	0.164	0.085	0.151				
9. JQ069989.1 Uncultured bacterium clone INI13_D2 phenol hydroxylase large subunit gene partial cds	0.136	0.127	0.129	0.136	0.162	0.081	0.127	0.114			
10. KT021791.1 Ralstonia sp. AET-6-14 phenol hydroxylase (phe) gene partial cds	0.146	0.144	0.144	0.148	0.171	0.141	0.144	0.157	0.123		
11. AB017631.1 Comamonas testosteroni gene for phenol hydroxylase alpha subunit partial cds	0.162	0.116	0.153	0.155	0.173	0.139	0.116	0.173	0.158	0.165	
12. AB016861.1 Burkholderia cepacia gene for phenol hydroxylase alpha subunit partial cds strain E1	0.164	0.174	0.164	0.165	0.199	0.146	0.174	0.146	0.155	0.164	0.199

Figure B.2 Pairwise distance matrix of partial *tbmD* sequences among bacterial strains and reference bacteria

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Cu12-todC1-f_F7 -CGA---TAGATTGAACGATGGAGG-CCAG---GCAAGCGCGCCCCAGCGGAGGGGAAGG
Fe10-todC1-f_G7 CCGG------------GGCTGGTACTCCGA-------------AGATCGGCCCCAGAAGG
Ba11-todC1-f_D7 ------------GGCGGTAATGGAACAGGTAGCTGCAGCATGGTGCTAGGGCTGGACTAG
Hg11-todC1-f_A8 -------TAGGGTCCCGGCGGGCCGGACAT-GCAG-CTTCCTTGACTGACCAGTCTACTT
Li12-todC1-f_B8 -------TGGAACCCAGT-CGGGGGGACGT-GGAAGTTTGCGTGTATG-GGTACACGAGT
Mn11-todC1-f_C8 --------------GGGGGTGAAGGGGCGA-GAACGAAAGCTTTCAAAGAGGCAGGCCGG
Mn12-todC1-f_D8 ------TTGCACGGCAGTGAGGCGGGACGT-GGATACATCGTGGTATGGGGTGGATTGGA
Ag11-todC1-f_B7 ------TTGGCCACAGGAGAGGCAGAAAGT---GCAGCTATCGTGGTAGGGGAGTGCATG
Hg10-todC1-f_H7 -------------CGGGGCATGCAAGTAC----TG-CACGCGTTGCTAGCGCTACC-ACT
Sr11-todC1-f_H8 ----------ACGGGGGTAATGAAAGGAGT---GCAGCTGTGTTCATACTGGCTGGCCAG
Sn11-todC1-f_G8 -----AGATGTGGAGTGGAAAGTAGCTGCA-GCCGTGCCTCCTATGGGGTGTGCGTAGGG
Ni11-todC1-f_E8 CAGGCCCGCTAGAAGGGGCGGGAAGCGGAC---AGCGTGGTCCTAGTGGCTGTACCAGGT
Co11-todC1-f_E7 --GGG--GTGAGGTCATGCATGGCG-CTGC-ACTGCGTTCCTTGCAGC-TACCACCAGGT
Sb01-todC1-f_F8 CAAGG--GTGAGTAGTGGACAGGAGCTGCA-GCTGTGGTCGTAGTGGCTACCGCCAGTTG

Cu12-todC1-f_F7 GGAGGCAGGGAAATGG--AACCTTTTTGTCCTGCGGGTGTCGG----------------G
Fe10-todC1-f_G7 G--GAG---TAGCTCC----CATACGTTGTCGCCGTCATTCGAGCCCGCAGGCTCCGGTG
Ba11-todC1-f_D7 GTCGTCTCAGTACTCGCCGGCATTCTTGCCATGTGCCTTACGAGGCCG-------------
Hg11-todC1-f_A8 G--------GACACT-----GGACCGATACTGGTGCCCTACCGCAGCTCCTTCTCT---G
Li12-todC1-f_B8 GGTGGAAGTGGAAACCACAGATGCTCGAGCCGGAACCGCGTCCCACCG-------------
Mn11-todC1-f_C8 GGGCGGAATGACAAGGGGAACATCTTGTCAATGTGCCTTACGAGGCCG-------------
Mn12-todC1-f_D8 GGTGGCAAGTGGAAGGCACAGGTGCTTGAATTGAACCGAACAAAGCCG-------------
Ag11-todC1-f_B7 GTGGTGTAATACACCCCCCAGGTGCTTGTCTTGTACCTTACAAGGCCG-------------
Hg10-todC1-f_H7 GGTGGGTTACGACCCGCAAGTGATCTTGTCATGTGCCTTACGAGGCCG-------------
Sr11-todC1-f_H8 GGGTCTTACGACAACGCACACATCTTGTCAATGTGCCTTACGAGGCCG-------------
Sn11-todC1-f_G8 GGTGGCAGGAGAAAGCACATGATCTTGTCAATGTACCTTACAAGGCCG-------------
Ni11-todC1-f_E8 GAGCTCACGACACCGCAGGCAATCTTGTCAATGTGCCTTACGAGGCCG-------------
Co11-todC1-f_E7 GAGCTTACGACACCGCCGGCGATCTTGTCAATGTGCCTTACGAGGCCG-------------
Sb01-todC1-f_F8 CAGCTCACGACACCGCCAGCAATCTTGTCAATGTGCCTTACGAGGCCG-------------

Cu12-todC1-f_F7 GGCCGGCCTTCGCGTG--CCTCCACACTAAGGAATGGGAGTTCCCGCTAACGCCCGGGTA
Fe10-todC1-f_G7 CAACGGCAGTCCCACCCATCGGGACTGCGAGCG--AG-ACCTTCGCCGTATGGCGAAGCT
Ba11-todC1-f_D7 AATCC---TTCACGTG--CCTGAACTGAAGGAAT-G--ACCTT-CGCTGATGGCCCGGCT
Hg11-todC1-f_A8 AATGG---TAAAGGCAATCCGGAGGAAAAGGATGTGG-ATAC--CTCCCAAGTCCTGTAT
Li12-todC1-f_B8 AATCC---TTCTCGTA--TTTGAACTCCATCCGCCAG-ATCCA-CGTGAATTT--GCTAA
Mn11-todC1-f_C8 AATCC---TTCGCGTG--CATGAACAAGAAGGAATGG-AGCCC-GCTGAAGGCCCGGGTA
Mn12-todC1-f_D8 AATCC---TTCTCGTG--CCTGAACTACATCGACCGG-ATCCC-GGTGAATGCCCTAATA
Ag11-todC1-f_B7 AATCC---TTCTCGTG--CCTGACCTCGATTGAATGG-ATGCC-GCTGAAAGCCCCAGTA
Hg10-todC1-f_H7 ATTCC---TTCGCGTG--CCTGAACAACAAGAAATGG-AGCCCGCCTGAAGGCCCGGGTA
Sr11-todC1-f_H8 AATCC---TTCGCGTG--CCTGAACAAGAAGGAATGG-AGCCC-GCTGAAGGCCCGGGTA
Sn11-todC1-f_G8 AATCC---TTCACGTG--CCTGAACAAGAAGGAATGG-AGCCC-GCTGAAGGCCCGGGTA
Ni11-todC1-f_E8 AATCC---TTCGCGTG--CCTGAACAAGAAGGAATGG-AGCCC-GCTGAAGGCCCGGGTA
Co11-todC1-f_E7 AATCC---TTCGCGTG--CCTGAACAAGAAGGAATGG-ATCCC-GCTGAAGGCCCGGGTA
Sb01-todC1-f_F8 AATCC---TTCGCGTG--CCTGAACAAGAAGGAATGG-ATCCC-GCTGAAGGCCCGGGTA
                                                      *          *
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Cu12-todC1-f_F7 CAAACCCC-TCTCGGCCTTCTTTTCTCCTCCTTCCCTCATCAACACTCTATACCACAAGA
Fe10-todC1-f_G7 CTGTCCCCGGATCCCCCCGATCCT--CCGTT-GCGGAT--CCTCGCCGTCCGGCTTGAGG
Ba11-todC1-f_D7 CTGTCCTA-CAATGGCCTGATTTTCCCCAAC-TGGGATGAGAACGCCCGGCTCCTCGCAT
Hg11-todC1-f_A8 GAATGGGA-ACAGGGATTTTCTCACTCCTTC-ACTCATGAACAAGTAGCAAACCTCGACA
Li12-todC1-f_B8 TTTTTGCT-TGAGGGTGGACTTTTCGGGACG-GGCTAGGAAAGGGAAGTATACCTCGACA
Mn11-todC1-f_C8 GAAACCTA-CAAGGGCCTGATTTTCGCCAAC-TGGGATGAGAACGCTGTAGACCTCGACA
Mn12-todC1-f_D8 TAAACCTA-CTAGGGCCTGATTTTCGGGAAC-TGGGATGAGAACGAAGT-AACCTCGACA
Ag11-todC1-f_B7 GAAACCTA-CAATGGCCTGATTTTCGCCAAC-TGGGATGAGAACCATGTAGACCTCGACA
Hg10-todC1-f_H7 GAAACCTA-CAAGGGCCTGATTTTCGCCAAC-TGGGATGAGAACGCTGTAGACCTCGACA
Sr11-todC1-f_H8 GAAACCTA-CAAGGGCCTGATTTTCGCCAAC-TGGGATGAGAACGCTGTAGACCTCGACA
Sn11-todC1-f_G8 GAAACCTA-CAAGGGCCTGATTTTCGCCAAC-TGGGATGAGAACGCTGTAGACCTCGACA
Ni11-todC1-f_E8 GAAACCTA-CAAGGGCCTGATTTTCGCCAAC-TGGGATGAGAACGCTGTAGACCTCGACA
Co11-todC1-f_E7 TAAACCTA-CAAGGGCCTGATTTTCGCCAAC-TGGGATGAGAACGCTGTAGACCTCGACA
Sb01-todC1-f_F8 GAAAC-CA-CAAGGGCCTGATTTTCGCCAAC-TGGGATGAGAACGCTGTAGACCTCGACA
                                                                *

Cu12-todC1-f_F7 CCTATCTTTGGCGAGGCGCAAATTCTACATGTCACCAACCTGCTTCTATCGCCCCCGCGG
Fe10-todC1-f_G7 CATTCTATTTTTCCAACGGACAAACCCAATGCAGACGATCGGTAA----TGTGTGGTTGT
Ba11-todC1-f_D7 TCTATTCTTTCCCAAGGACAAATTCTATGTGGACCACATGCTCTG----TGTGGTTGTGG
Hg11-todC1-f_A8 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
Li12-todC1-f_B8 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
Mn11-todC1-f_C8 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
Mn12-todC1-f_D8 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
Ag11-todC1-f_B7 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
Hg10-todC1-f_H7 CGTA--TCTGGGCGAGGCTAAGTTCTACATGGACCACCTGCTCCA----CCGCACCGAG-
Sr11-todC1-f_H8 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
Sn11-todC1-f_G8 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
Ni11-todC1-f_E8 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
Co11-todC1-f_E7 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
Sb01-todC1-f_F8 CGTA--TCTGGGCGAGGCGAAGTTCTACATGGACCACATGCTCGA----CCGCACCGAG-
                  *     *          *    *    **                          *

Cu12-todC1-f_F7 CCGCGCCCCCAAGCCATCCCGGGCGTGCTGAATTGG--TCCTTCCCTGTACTGTTAATTC
Fe10-todC1-f_G7 GGGGCGGACATGGAAATGGCAGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Ba11-todC1-f_D7 CCGGCACCATGAGCAATCCCAGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Hg11-todC1-f_A8 GCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Li12-todC1-f_B8 GCCGGCACCGAACCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Mn11-todC1-f_C8 GCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Mn12-todC1-f_D8 GCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Ag11-todC1-f_B7 GCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Hg10-todC1-f_H7 GCCGGCACCGAAGCGATCCCGGGCGTGCACAAATGAGTCATTCCCTGTAACTGGAAATTC
Sr11-todC1-f_H8 GCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Sn11-todC1-f_G8 GCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGAAAATTC
Ni11-todC1-f_E8 GCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Co11-todC1-f_E7 GCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
Sb01-todC1-f_F8 GCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTCATTCCCTGTAACTGGAAATTC
                              **  * *******  **  **     * **    ****  *****
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Cu12-todC1-f_F7 CCCGCAGATCACTTTTGCACCTACATGTTCCATGCCGGGATAACTTCTCTCTTTT-TGGT
Fe10-todC1-f_G7 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Ba11-todC1-f_D7 GCCGCAAAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Hg11-todC1-f_A8 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Li12-todC1-f_B8 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Mn11-todC1-f_C8 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Mn12-todC1-f_D8 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Ag11-todC1-f_B7 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Hg10-todC1-f_H7 GCCGCACAGCAGTTTTGCAGCGACCTGTACCATGCCGGGACGACCTCTCATCTGTCTGGC
Sr11-todC1-f_H8 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Sn11-todC1-f_G8 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Ni11-todC1-f_E8 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Co11-todC1-f_E7 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
Sb01-todC1-f_F8 GCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGGACGACCTCGCATCTGTCTGGC
                 ***** * ** ******* * ** *** ***********  ** ** *   * * ***

Cu12-todC1-f_F7 ATCTGGGCAGACCTGCTAAAAAACTTTTTATGGCCGTTTCTGTC--CTCCGCCGTGTAGC
Fe10-todC1-f_G7 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Ba11-todC1-f_D7 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Hg11-todC1-f_A8 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Li12-todC1-f_B8 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Mn11-todC1-f_C8 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Mn12-todC1-f_D8 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Ag11-todC1-f_B7 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Hg10-todC1-f_H7 ATCCTGGCAGGCCTGCCATAATACCTTGAAATGGCCGACCTTGCTCCGCCAACATTTGGC
Sr11-todC1-f_H8 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Sn11-todC1-f_G8 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Ni11-todC1-f_E8 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Co11-todC1-f_E7 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
Sb01-todC1-f_F8 ATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGACCTTGCTCCGCCGACAGTTGGC
                 ***  ***** ***** * ** ** **  *  * *    **  *  * **  *   * **

Cu12-todC1-f_F7 TAGTCCTACGCTCTTGATTGAAGGGGTCAGTGAATGTAGAAAAAATTTACCGCCCCCCTT
Fe10-todC1-f_G7 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCAAAGGTTAGCGATTGAATAG
Ba11-todC1-f_D7 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCAAAGCCTCTCGCGTCCTGTT
Hg11-todC1-f_A8 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCAAAGGGTGGTGGGGGG----
Li12-todC1-f_B8 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCAACGCAGACCGGTTTTGAGG
Mn11-todC1-f_C8 AAGCAGTACCGTGCGTCATGGGGCGGACTGGCAGT-GGCATCTGGTGCTTGCATGTGCGC
Mn12-todC1-f_D8 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCAACACTGACCGGTTTTGAGG
Ag11-todC1-f_B7 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCCAACAGCTGCTTTTAAGGTG
Hg10-todC1-f_H7 AAGCAGTACCGTGCGTCATGGGGCGGACTGGAAAG-TGGCAATGGTCGGTCACTCCTGGG
Sr11-todC1-f_H8 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCAAACAGGTGTCTTTTGCCTC
Sn11-todC1-f_G8 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCCAACCATTATTGTGGCCCCC
Ni11-todC1-f_E8 AAGCAGTACCGTGCGTCATGGGGCGGACTGGAAAG-TGGCAAAGGTCCGTCACTCGTGGG
Co11-todC1-f_E7 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCAATGGTCGGTCACTCGTGGG
Sb01-todC1-f_F8 AAGCAGTACCGTGCGTCATGGGGCGGACATGGAAG-TGGCAAAGGTCGGTCACTCGTGGG
                 **   ***  *      **  * ** *    *
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Cu12-todC1-f_F7 TACTACAGTGTGCTCGGTTACTTTTTTTACTGCTCAA----------------------
Fe10-todC1-f_G7 GCTTTAGAAGTA------------------------------------------------
Ba11-todC1-f_D7 GGTGGGGGGGAGATGGAACTAACAGAGAGAAGAGCTCGCTATTTTCCTTTCGTTTTTT--
Hg11-todC1-f_A8 ------------------------------------------------------------
Li12-todC1-f_B8 TTAAACCGGCAATCCTGATGATTCTGGCCGGCGTCTACCCTGAAGAGGCGCAGTTGCGCT
Mn11-todC1-f_C8 CAGGGGGAAGATGGGAATGTGAGAGTGGAGACTGCGTCTACTCCGGCTGCCGGTTGCTGG
Mn12-todC1-f_D8 TTAAACCGGCAATCCTGATGATTCTGGCCGGCGTCGACGCTGAAAAGGCGCAGTTGCGCT
Ag11-todC1-f_B7 AAATGGGGAATACTGATGATTCAGACCTCTCGACGCT----GAAGAGG-CGCATTGCGCT
Hg10-todC1-f_H7 GCGGACATGGAAGTGGCACCACTTGACATAGCGCCCG----GTGTAGGCCGGCATCCGCG
Sr11-todC1-f_H8 CTATGTGACGTTTCCAAATTTTAGATCTAAGACCCCT----------------------
Sn11-todC1-f_G8 CAATGGGGGTTGACCTTTTAGCCAAAGTGTTGATACT----TTCTTTAAAACAATATCGA
Ni11-todC1-f_E8 GCGGACATGGAAG-----TGGC--AAGAGGTCAGAGG----TTGTCCAATTCC-------
Co11-todC1-f_E7 GCGGACATGGAAG-----TGGC--AACTCCGGGGACT----AGTAG---------------
Sb01-todC1-f_F8 GCGGACATGGAAG-----TGGC--AATTG--------------------------------

Cu12-todC1-f_F7 ----CCTACTACGATATAATTTGCTGCCA---TCGATTT---------------------
Fe10-todC1-f_G7 ------------------------------------------------------------
Ba11-todC1-f_D7 ----------TTTTGTTTTTTTTTTT-----------------------------------
Hg11-todC1-f_A8 ------------------------------------------------------------
Li12-todC1-f_B8 TAAAGCAGCACGACGGCTATTTACGCGCGGCGT----TGACGCGCTAACGACACCTGGGG
Mn11-todC1-f_C8 GAAAACCACACCGGCAAATTCTCGGATGGCCAGCTACCCGTCTTTCAAAGAGAAA-----
Mn12-todC1-f_D8 TAAAGCAGCACGACGGCTATTTACGCGCG---G-CGTTGACGCGCTAACGACACCTGGGG
Ag11-todC1-f_B7 TAAAGCAGCCGACGGCTATTTCGCGCGGGGTGACGCCTAA--------CGACACCTGGGG
Hg10-todC1-f_H7 GCGGGCCGCCGATCGTACCTTGGGTATCGGCACGGCTGAAACGCACCCCTTGAGCTGCGA
Sr11-todC1-f_H8 ----------ATTTATTTGCTTTTCTACCGCAAATTTTAGCCGGTTCCCGCTCAT----C
Sn11-todC1-f_G8 G---------ATTCATTGAAATATGATCGCCAAGTCTTATTTGGACCGTTATCAA----C
Ni11-todC1-f_E8 ------------------------------------------------------------
Co11-todC1-f_E7 ------------------------------------------------------------
Sb01-todC1-f_F8 ------------------------------------------------------------

Cu12-todC1-f_F7 ------------------------------------------------------------
Fe10-todC1-f_G7 ------------------------------------------------------------
Ba11-todC1-f_D7 ------------------------------------------------------------
Hg11-todC1-f_A8 ------------------------------------------------------------
Li12-todC1-f_B8 CGGACATGGAAGTGGCAATTGGTTTTATGGTGTCATTGTGTATG----TGTGTGGTTGTT
Mn11-todC1-f_C8 -AACTATGGCAACCCCCTGCAAAAGATATCCCGCAACGTGTGCAGCAGCGACAGCTCCAA
Mn12-todC1-f_D8 CGGACATGGAATTGGCAGTGGTG--------------------------------------
Ag11-todC1-f_B7 CGGACATGGAAATGGCAC------------------------------------------
Hg10-todC1-f_H7 GGCGATCGATATTCGGTGTATGCAGATGCGGGTGTCCGTAGCAGGACAGGTAATCGCGG-
Sr11-todC1-f_H8 CGGCAGTAA---------------------------------------------------
Sn11-todC1-f_G8 -GGAAGTGATGTTTGCTTTAAAAAGTGGTTGG-------GGGGGGACATGGGAAATTGG-
Ni11-todC1-f_E8 ------------------------------------------------------------
Co11-todC1-f_E7 ------------------------------------------------------------
Sb01-todC1-f_F8 ------------------------------------------------------------
```

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Cu12-todC1-f_F7 ------------------------------------------------------------
Fe10-todC1-f_G7 ------------------------------------------------------------
Ba11-todC1-f_D7 ------------------------------------------------------------
Hg11-todC1-f_A8 ------------------------------------------------------------
Li12-todC1-f_B8 GTCGCTTCATGTGCCTTTAACCCGTTCTCGTTCTCTTTAGCGGTTAGGTGG---------
Mn11-todC1-f_C8 CCAGGTTCAACGGT------TCAAACCCGGTCGTGGTGATGGTGGGGCTGGAACATAACT
Mn12-todC1-f_D8 -------GGTCGTTTTTTTTTTTCGGTTTTTTGTGTTGT-G-------------------
Ag11-todC1-f_B7 ------------------------------------------------------------
Hg10-todC1-f_H7 CGCAGTTGATCGCACATGATGTACAGCACGTTGCGCACG-GGGTTGGATGGGGCGTACAT
Sr11-todC1-f_H8 ------------------------------------------------------------
Sn11-todC1-f_G8 CC----------------------------------------------------------
Ni11-todC1-f_E8 ------------------------------------------------------------
Co11-todC1-f_E7 ------------------------------------------------------------
Sb01-todC1-f_F8 ------------------------------------------------------------

Cu12-todC1-f_F7 ------------------------------------------------------------
Fe10-todC1-f_G7 ------------------------------------------------------------
Ba11-todC1-f_D7 ------------------------------------------------------------
Hg11-todC1-f_A8 ------------------------------------------------------------
Li12-todC1-f_B8 ------------------------------------------------------------
Mn11-todC1-f_C8 GGATTCGGCCTGATACCGTGTTGCA---------------CACCCGCCGTATCGGTGAA-
Mn12-todC1-f_D8 -------GCTGTTTTTGGGTTTTGTGCTTGGTGTTTTTTGTTTTT---------------
Ag11-todC1-f_B7 ------------------------------------------------------------
Hg10-todC1-f_H7 GGAACTGGCAGCTTGTTTGGTTTCTGGCTCGCTTGTTGACCTCTTAAGGAATGGGTTAAT
Sr11-todC1-f_H8 ------------------------------------------------------------
Sn11-todC1-f_G8 ------------------------------------------------------------
Ni11-todC1-f_E8 ------------------------------------------------------------
Co11-todC1-f_E7 ------------------------------------------------------------
Sb01-todC1-f_F8 ------------------------------------------------------------

Cu12-todC1-f_F7 ------------------------------------------------------------
Fe10-todC1-f_G7 ------------------------------------------------------------
Ba11-todC1-f_D7 ------------------------------------------------------------
Hg11-todC1-f_A8 ------------------------------------------------------------
Li12-todC1-f_B8 ------------------------------------------------------------
Mn11-todC1-f_C8 CGGTCACCTGATCAAGGACTCCGCCTTGGCCAACTGACCATCACCGGTGTGTGCAAATTC
Mn12-todC1-f_D8 ------------------------------------------------------------
Ag11-todC1-f_B7 ------------------------------------------------------------
Hg10-todC1-f_H7 TGGATCCCTGGTCGAGTTTTGTAACATCGAGAGGCAGGTACCGCTTTTGTTTTCCCGCTC
Sr11-todC1-f_H8 ------------------------------------------------------------
Sn11-todC1-f_G8 ------------------------------------------------------------
Ni11-todC1-f_E8 ------------------------------------------------------------
Co11-todC1-f_E7 ------------------------------------------------------------
Sb01-todC1-f_F8 ------------------------------------------------------------
```

```
Cu12-todC1-f_F7 ------------------------------------------------------------
Fe10-todC1-f_G7 ------------------------------------------------------------
Ba11-todC1-f_D7 ------------------------------------------------------------
Hg11-todC1-f_A8 ------------------------------------------------------------
Li12-todC1-f_B8 ------------------------------------------------------------
Mn11-todC1-f_C8 AGCGAATTGAGTATGCACATGCCGCTGAGCTCCCGACCCCGGGGTGCCGGCGATCTACCG
Mn12-todC1-f_D8 ------------------------------------------------------------
Ag11-todC1-f_B7 ------------------------------------------------------------
Hg10-todC1-f_H7 GTTTCCGTGG--------TTGGTTGTA---TTCTTCGGTAGGGATTCCTCAGTTGCACCT
Sr11-todC1-f_H8 ------------------------------------------------------------
Sn11-todC1-f_G8 ------------------------------------------------------------
Ni11-todC1-f_E8 ------------------------------------------------------------
Co11-todC1-f_E7 ------------------------------------------------------------
Sb01-todC1-f_F8 ------------------------------------------------------------

Cu12-todC1-f_F7 -------------
Fe10-todC1-f_G7 -------------
Ba11-todC1-f_D7 -------------
Hg11-todC1-f_A8 -------------
Li12-todC1-f_B8 -------------
Mn11-todC1-f_C8 CAATTTAGCTTGG
Mn12-todC1-f_D8 -------------
Ag11-todC1-f_B7 -------------
Hg10-todC1-f_H7 GT----AGATTTT
Sr11-todC1-f_H8 -------------
Sn11-todC1-f_G8 -------------
Ni11-todC1-f_E8 -------------
Co11-todC1-f_E7 -------------
Sb01-todC1-f_F8 -------------
```

Figure B.3 Multiple sequence alignment of partial *todC1* sequences by Clustal Omega. Asterisks denote identical nucleotides.

File Display Average Caption Help

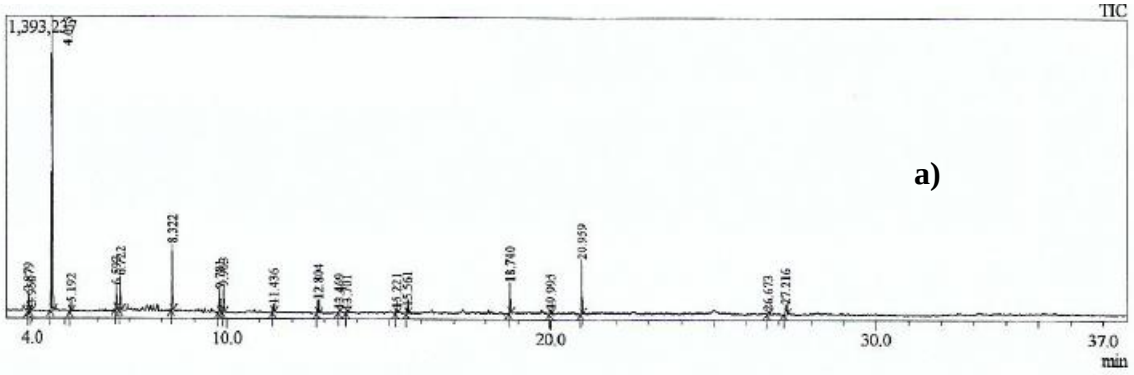
(A,B) 0.0 0.00 XL CSV MEGA TXT Caption

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. A. haemolyticus Mn12													
2. M. luteus Sr11	0.005												
3. P. agglomerans Sn11	0.019	0.014											
4. A. johnsonii Sb01	0.003	0.005	0.019										
5. C. testosteroni Ni11	0.095	0.090	0.087	0.095									
6. S. nematodiphila Mn11	0.008	0.008	0.019	0.011	0.095								
7. S. aureus Li12	0.172	0.166	0.166	0.172	0.101	0.169							
8. P. koreensis Hg11	0.213	0.210	0.210	0.213	0.202	0.210	0.169						
9. P. koreensis Hg10	0.044	0.046	0.054	0.041	0.128	0.049	0.202	0.245					
10. P. koreensis Cu12	0.322	0.324	0.330	0.324	0.343	0.327	0.316	0.330	0.349				
11. A. calcoaceticus Fe10	0.283	0.281	0.292	0.281	0.330	0.286	0.373	0.387	0.292	0.452			
12. S. warnerii Co11	0.003	0.008	0.022	0.005	0.093	0.011	0.169	0.213	0.046	0.322	0.283		
13. S. rhizophila Ba11	0.158	0.158	0.163	0.158	0.213	0.163	0.267	0.283	0.188	0.294	0.357	0.158	
14. R. plancticola Ag11	0.065	0.063	0.060	0.065	0.090	0.065	0.166	0.207	0.104	0.341	0.322	0.068	0.169

Figure B.4 Pairwise distance matrix of partial *todC1* sequences among bacterial strains

APPENDIX C

INTERMEDIATES OF BTEX DEGRADATION



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%
1	3.879	112720	2.36	90224	3.06
2	3.950	34862	0.73	23149	0.78
3	4.613	2225480	46.58	1357514	46.02
4	5.192	72352	1.51	39922	1.35
5	6.593	169290	3.54	112077	3.80
6	6.722	235067	4.92	157087	5.33
7	8.322	479904	10.04	305265	10.35
8	9.781	162610	3.40	106496	3.61
9	9.903	195242	4.09	117403	3.98
10	11.436	53040	1.11	34086	1.16
11	12.804	108826	2.28	62915	2.13
12	13.469	20298	0.42	11684	0.40
13	13.701	17335	0.36	11256	0.38
14	15.221	36785	0.77	19912	0.68
15	15.561	51926	1.09	29208	0.99
16	18.740	221896	4.64	141301	4.79
17	19.995	37410	0.78	20530	0.70
18	20.959	371783	7.78	249777	8.47
19	26.673	35769	0.75	15247	0.52
20	27.216	135492	2.84	44835	1.52
		4778087	100.00		

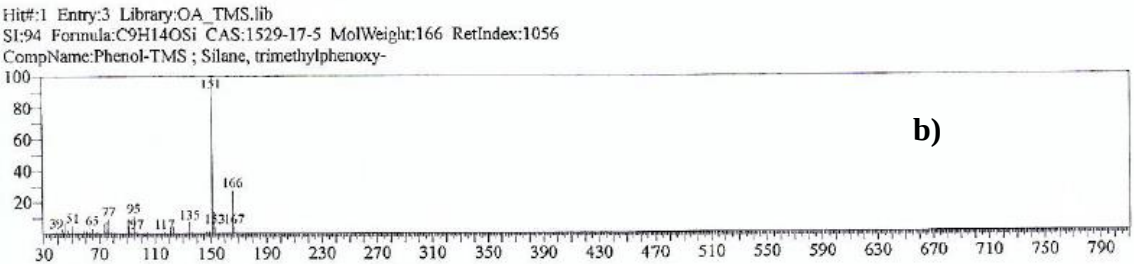
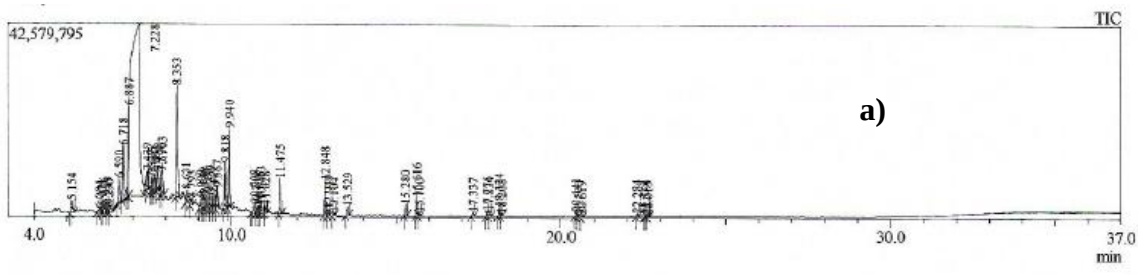


Figure C.1 GC chromatogram of intermediates detected (a), MS ionization dendogram of phenol (b) produced during benzene degradation by *R. plancticola* Ag11.



Peak Report TIC

Peak#	R.Time	Area	Area%
1	5.154	9203602	0.76
2	6.004	2472585	0.21
3	6.071	2654614	0.22
4	6.136	5210532	0.43
5	6.245	4185738	0.35
6	6.290	5220080	0.43
7	6.590	15529377	1.29
8	6.718	36498470	3.03
9	6.887	39690039	3.30
10	7.228	720898787	59.91
11	7.439	9804775	0.81
12	7.577	5763478	0.48
13	7.605	8010197	0.67
14	7.683	7981694	0.66
15	7.759	9715990	0.81

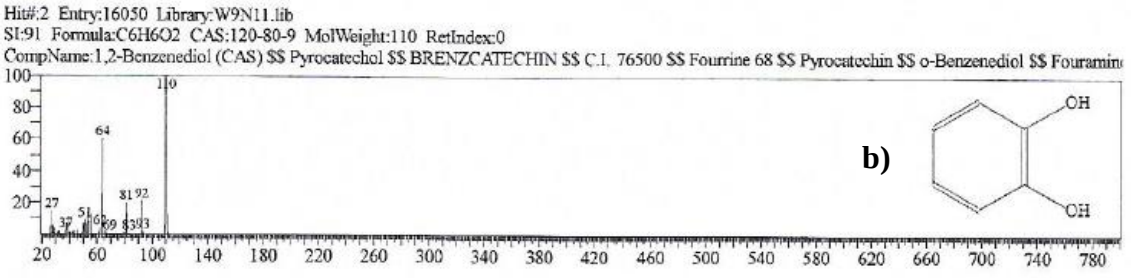
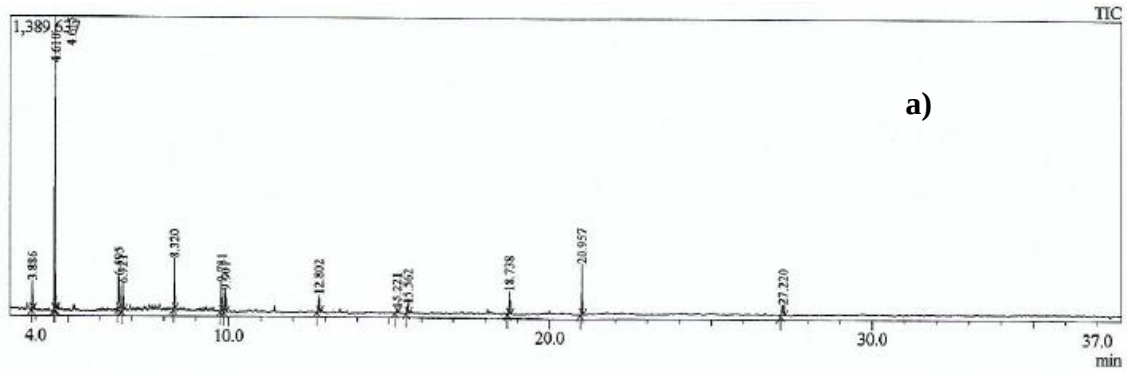


Figure C.2 GC chromatogram of intermediates detected (a), MS ionization dendogram of catechol (b) produced during benzene degradation by *S. nematodiphila* Mn11.



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%
1	3.886	150976	3.42	130692	3.36
2	4.610	1121613	25.38	1139705	29.32
3	4.615	1097223	24.83	1355348	34.87
4	6.595	210032	4.75	149509	3.85
5	6.721	186781	4.23	121097	3.12
6	8.320	351117	7.94	231039	5.94
7	9.781	216422	4.90	132997	3.42
8	9.901	167876	3.80	98997	2.55
9	12.802	128643	2.91	76921	1.98
10	15.221	37119	0.84	19801	0.51
11	15.562	60719	1.37	38707	1.00
12	18.738	177821	4.02	105394	2.71
13	20.957	368513	8.34	235033	6.05
14	27.220	144771	3.28	51311	1.32

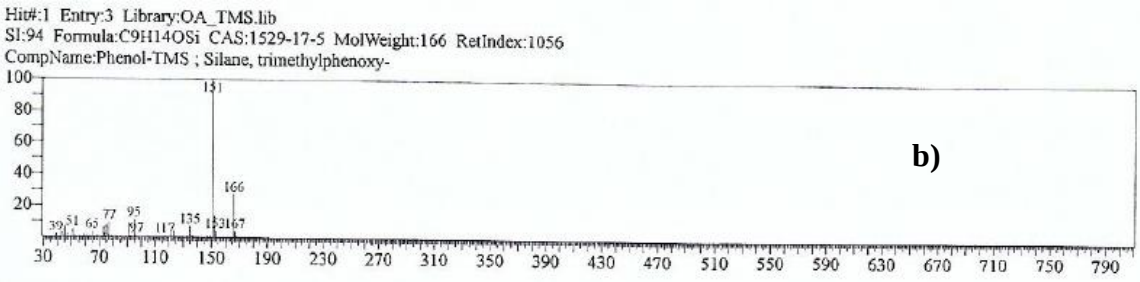
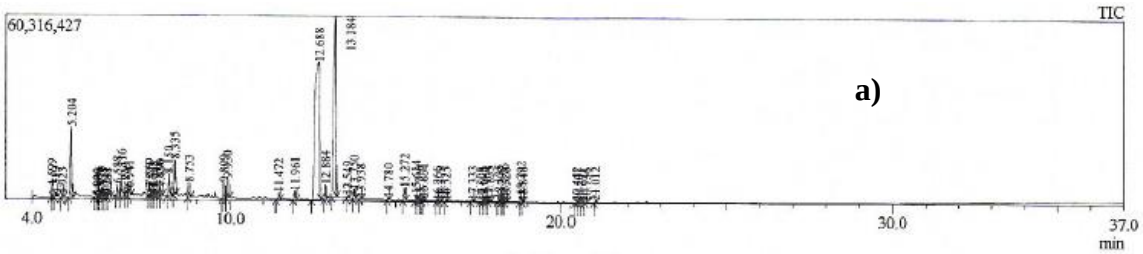


Figure C.3 GC chromatogram of intermediates detected (a), MS ionization dendogram of phenol (b) produced during benzene degradation by *M. luteus* Sr11.



Peak Report TIC

Peak#	R.Time	Area	Area%
1	4.599	5336423	0.55
2	4.719	1682326	0.17
3	4.923	1388637	0.14
4	5.204	70104157	7.24
5	5.980	437452	0.05
6	6.008	947836	0.10
7	6.076	2049165	0.21
8	6.137	1896876	0.20
9	6.247	2206498	0.23
10	6.288	965663	0.10
11	6.588	9743149	1.01
12	6.716	16548378	1.71
13	6.849	4591638	0.47
14	6.941	6862354	0.71
15	7.559	5511302	0.57

Hit#:1 Entry:131040 Library:W9N11.lib
SI:74 Formula:C10H16OSi CAS:14642-79-6 MolWeight:180 RetIndex:1078
CompName:Silane, trimethyl(phenylmethoxy)- $$ Silane, (benzyloxy)trimethyl- $$ (Benzyloxy)trimethylsilane $$ Benzyl alcohol, TMS $$ Benzyl alcohol, t

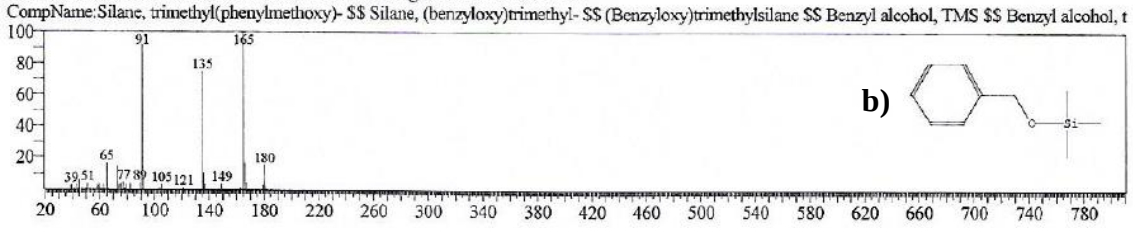
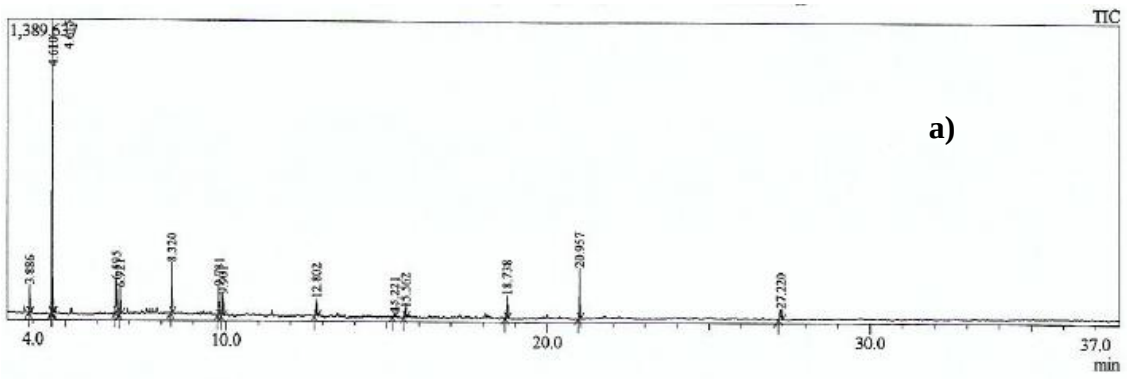


Figure C.4 GC chromatogram of intermediates detected (a), and MS ionization dendogram of benzyl alcohol (b) produced during toluene degradation by *A. calcoaceticus* Fe10.



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%
1	3.886	150976	3.42	130692	3.36
2	4.610	1121613	25.38	1139705	29.32
3	4.615	1097223	24.83	1355348	34.87
4	6.595	210032	4.75	149509	3.85
5	6.721	186781	4.23	121097	3.12
6	8.320	351117	7.94	231039	5.94
7	9.781	216422	4.90	132997	3.42
8	9.901	167876	3.80	98997	2.55
9	12.802	128643	2.91	76921	1.98
10	15.221	37119	0.84	19801	0.51
11	15.562	60719	1.37	38707	1.00
12	18.738	177821	4.02	105394	2.71
13	20.957	368513	8.34	235033	6.05

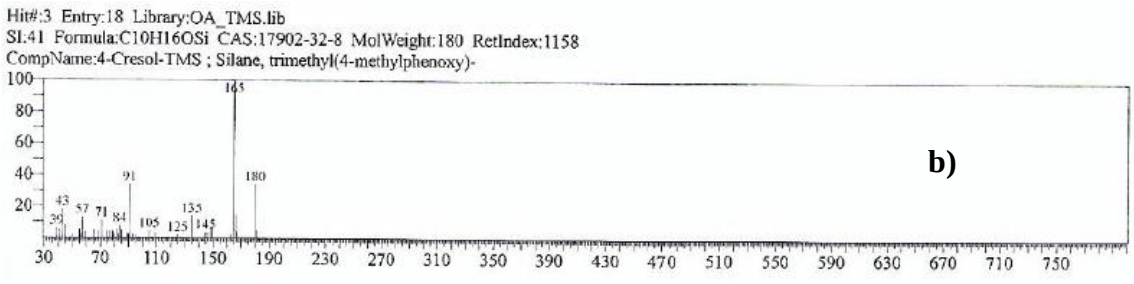
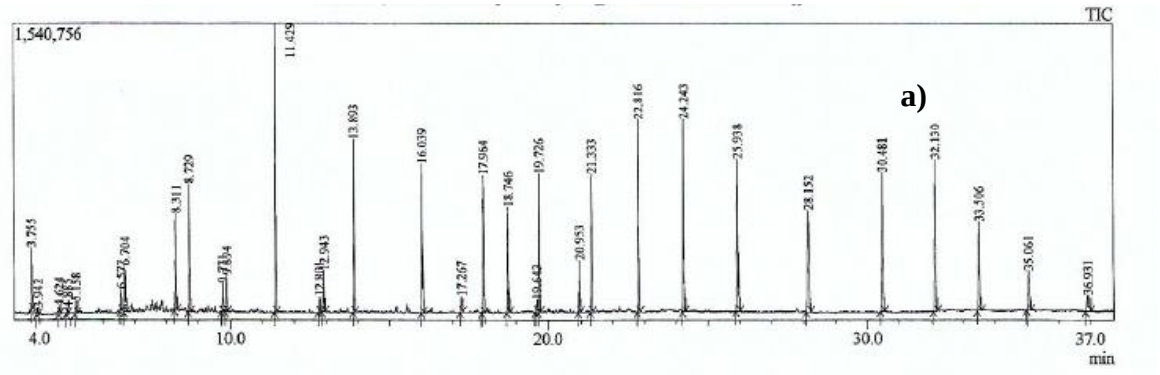


Figure C.5 GC chromatogram of intermediates detected (a), and MS ionization dendogram of 4-cresol (b) produced during toluene degradation by *M. luteus* Sr11.



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%
1	3.755	532504	1.97	317549	2.19
2	3.942	55231	0.20	35922	0.25
3	4.624	75544	0.28	39965	0.28
4	4.885	58295	0.22	32833	0.23
5	5.158	147354	0.55	61243	0.42
6	6.577	202286	0.75	112065	0.77
7	6.704	417456	1.54	237633	1.64
8	8.311	812995	3.01	499417	3.45
9	8.729	970057	3.59	661462	4.56
10	9.771	243921	0.90	148292	1.02
11	9.894	302484	1.12	190559	1.31
12	11.429	2177527	8.06	1501484	10.36
13	12.801	146375	0.54	82606	0.57
14	12.943	419810	1.55	212420	1.47
15	13.893	1340265	4.96	897527	6.19

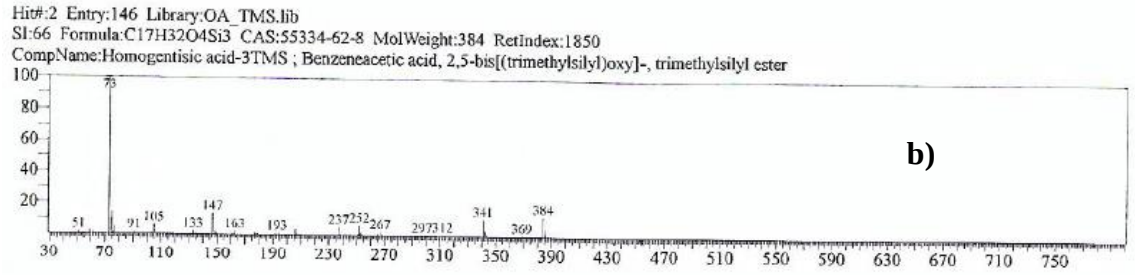
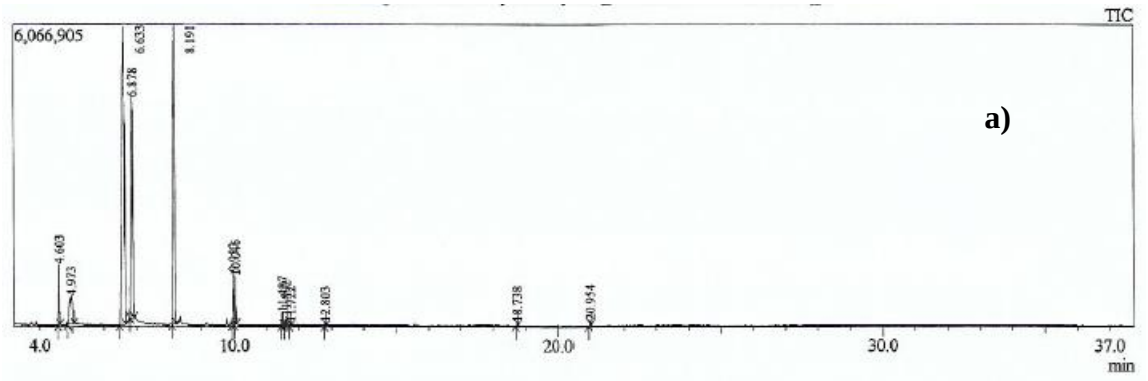
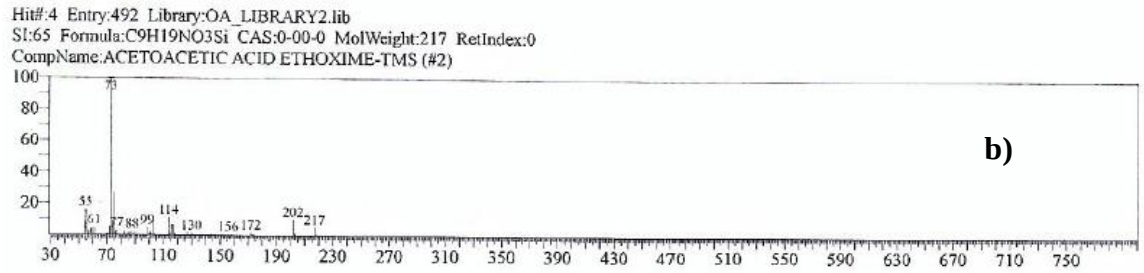


Figure C.6 GC chromatogram of intermediates detected (a), and MS ionization dendogram of homogentisic acid (b) produced during ethylbenzene degradation by *R. plancticola* Ag11.



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%
1	4.603	1978794	2.36	1218220	5.79
2	4.973	3389691	4.04	578290	2.75
3	6.633	31753471	37.89	5977600	28.39
4	6.878	15102479	18.02	4428557	21.03
5	8.191	27038921	32.26	6016712	28.57
6	9.983	1741553	2.08	1057244	5.02
7	10.046	1489538	1.78	985111	4.68
8	11.487	418199	0.50	270057	1.28
9	11.592	340519	0.41	186803	0.89
10	11.722	123805	0.15	71310	0.34
11	12.803	113064	0.13	70258	0.33
12	18.738	126391	0.15	79380	0.38
13	20.954	188171	0.22	117901	0.56



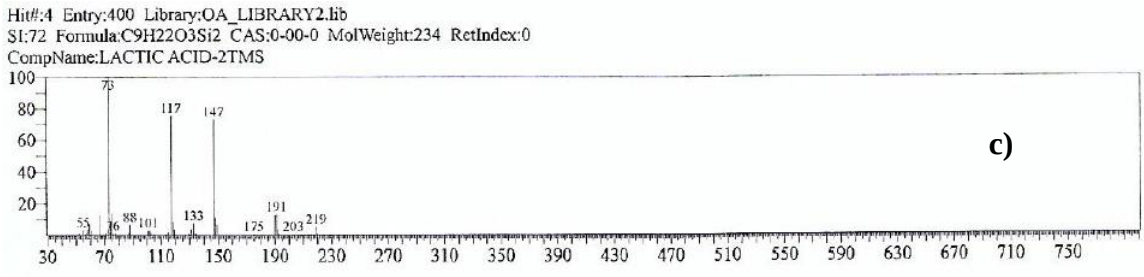
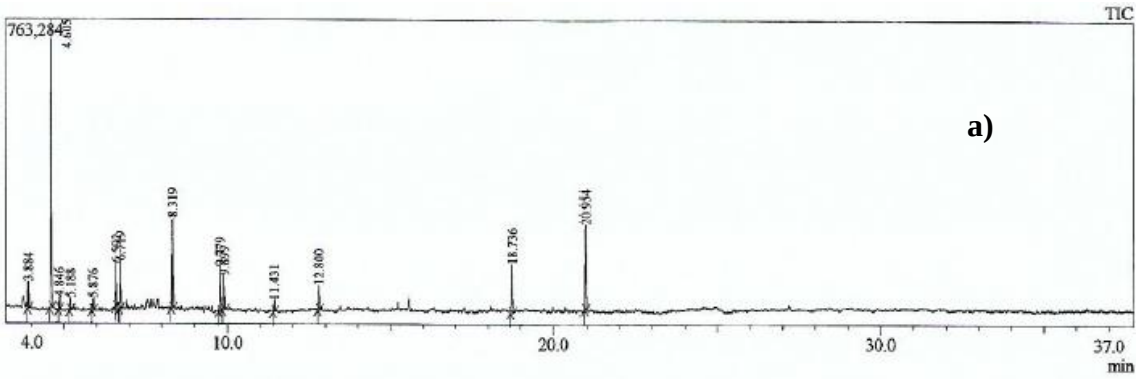


Figure C.7 GC chromatogram of intermediates detected (a), and MS ionization dendogram of acetoacetic acid (b) and lactic acid (c) produced during ethylbenzene degradation by *P. koreensis* Hg11.



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%
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3	4.846	43248	1.44	31265	1.59
4	5.188	50464	1.68	29664	1.51
5	5.876	48282	1.61	30645	1.56
6	6.592	162954	5.43	109631	5.58
7	6.719	181486	6.05	119669	6.09
8	8.319	360652	12.02	227516	11.59
9	9.779	188070	6.27	111898	5.70
10	9.899	142261	4.74	86499	4.40
11	11.431	48155	1.60	28110	1.43
12	12.800	102319	3.41	62054	3.16
13	18.736	209552	6.98	122371	6.23
14	20.954	317634	10.58	212565	10.82

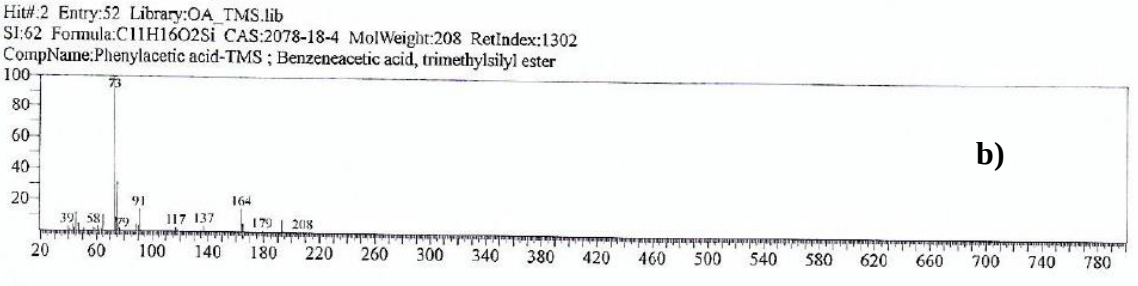
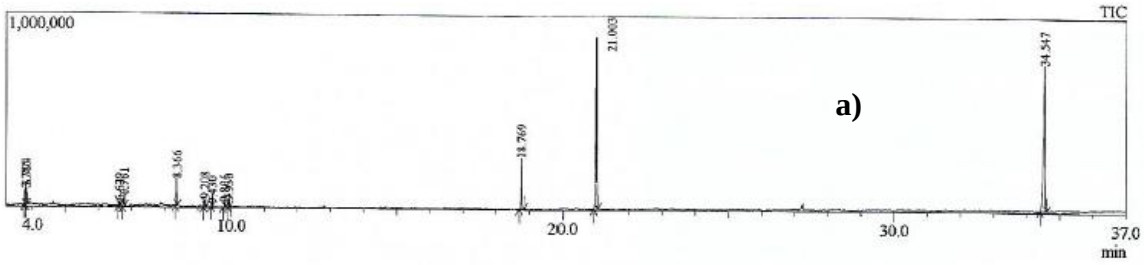
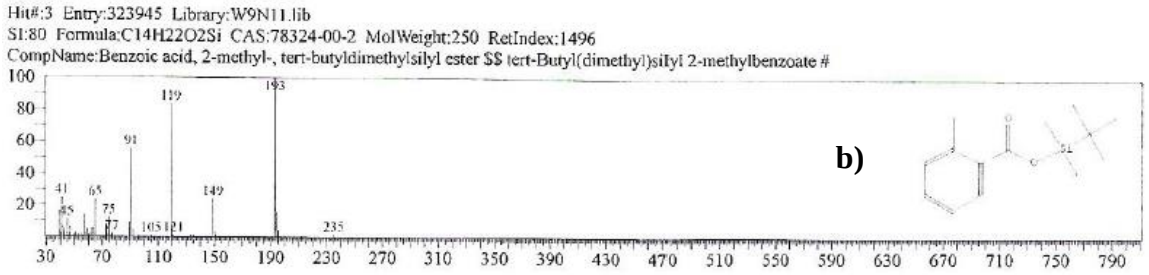


Figure C.8 GC chromatogram of intermediates detected (a), and MS ionization dendogram of phenylacetic acid (b) produced during ethylbenzene degradation by *S. nematodiphila* Mn11.



Peak Report TIC

Peak#	R.Time	Area	Area%
1	3.785	151584	2.95
2	3.794	120671	2.35
3	6.629	32946	0.64
4	6.761	81847	1.59
5	8.366	195239	3.80
6	9.208	62754	1.22
7	9.430	12822	0.25
8	9.806	46033	0.90
9	9.930	37426	0.73
10	18.769	323931	6.31
11	21.003	1495613	29.14
12	34.547	2571363	50.10



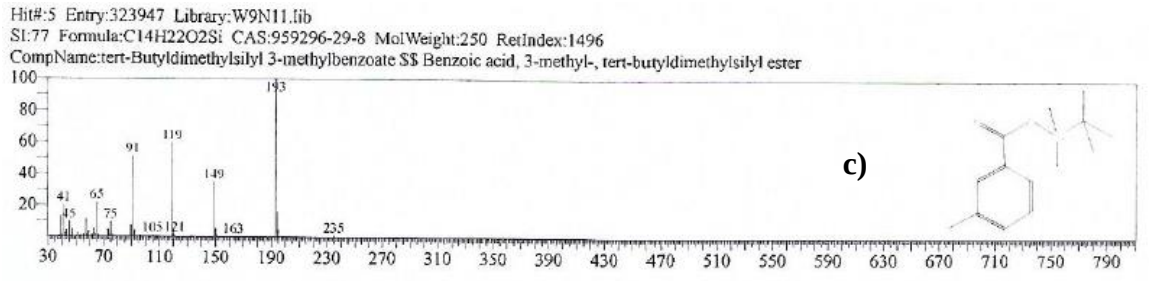


Figure C.9 GC chromatogram of intermediates detected (a), MS ionization dendogram of 2-methylbenzoate (b), 3-methylbenzoate (c) and 4-methylbenzoate (d) produced during xylene degradation by *A. calcoaceticus* Fe10.

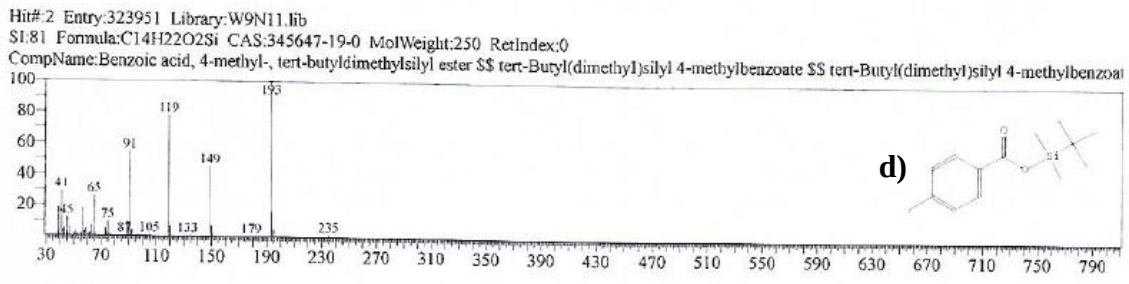


Figure C.9 cont'd