CLONING AND CHARACTERIZATION OF A GENE CLUSTER ENCODING TOLUENE MONOOXYGENASE FROM *Bradyrhizobium* sp. BTAi1

by

Kübra Cansu YANIK

June 2012

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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Prof. Dr. Halil Rıdvan ÖZ
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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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M. S. Thesis – Genetics and Bioengineering
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ABSTRACT

Bacterial toluene monooxygenases are known for their ability to oxidize a wide range of aromatics, degrade a variety of organic pollutants, and produce industrially-important compounds. These enzymes include toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4, toluene-*para*-monooxygenase (T*p*MO) of *Ralstonia pickettii* PKO1, toluene-4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1, and toluene *o*-xylene monooxygenase (ToMO) of *Pseudomonas* sp. OX1. Although they have great potential in bioremediation and green chemistry applications, their ability of oxidation is still have limitations and discovery of the new toluene monooxygenases are needed. *Bradyrhizobium* sp. BTAi1 is a photosynthetic rhizosphere bacterium that lives in the root of leguminous plant and the 5100 bp length region in its genome shows 38.5% amino acid similarity with ToMO, 37.7% with T4MO, 40.6% with T*p*MO and 17.2% with TOM. In this study, a new toluene monooxygenase from *Bradyrhizobium* sp. BTAi1 was successfully cloned and expressed in *Escherichia coli* TG1 and characterized for its ability to hydroxylate aromatic hydrocarbons such as toluene, benzene, phenol, nitrobenzene, *p*-nitrophenol and naphthalene and to degrade trichloroethylene.

Keywords: Bacterial toluene monooxygenases, *Bradyrhizobium* sp. BTAi1, aromatic hydrocarbons, trichloroethylene, green chemistry, bioremediation, rhizoremediation.

Bradyrhizobium sp. BTAi1 SUŞUNDA TOLUEN MONOOKSIJENAZ GENLERININ KLONLANMASI VE KARAKTERİZASYONU

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ÖZ

Bakteri kökenli toluen monooksijenaz enzimleri, çeşitli aromatik hidrokarbonları okside etmeleri, bir çok klorlu etenleri indirgemeleri ve önemli endüstriyel bileşikleri üretmeleri yönünden bilinirler. Bu enzimler; *Burkholderia cepacia* G4 suşundan izole edilen toluen-*orto*-monooksijenaz (TOM), *Ralstonia pickettii* PKO1 suşundan izole edilen toluen-*para*-monooksijenaz (T*p*MO), *Pseudomonas mendocina* KR1 suşundan izole edilen toluene-4-monooksijenaz ve *Pseudomonas* sporium OX1 suşundan elde edilen toluen/oksilen monooksijenazdır. Biyoremidasyon ve yeşil kimya alanlarındaki önemlerine rağmen, oksidasyon kapasiteleri çevreyle sınırlıdır. *Bradyrhizobium* sp. BTAi1 suşu, fotosentetik rizosfer bakteridir ve baklagillerin köklerinde yaşarlar. Genomunda bulunan 5100 bazlık bir bölge amino asit yönünden, ToMO ile %38.5, T4MO ile %37.7, T*p*MO ile %40.6 ve TOM ile %17.2 benzerlik göstermektedir. Bu çalışmada, 5100 bazlık bölgeyi oluşturan yeni enzim başarıyla *Escherichia coli* TG1 suşuna klonlanmış olup, aromatic hidrokarbonları oxidasyonu ve klorlu etenleri indirgemeleri karakterize edilmiştir. Sonuçlar, pBS(Kan)Tmo.BTAi1 enziminin *para*-hidroksile enzimi olduğunu göstermektedir.

Anahtar Kelimeler: Toluen monooksijenazlar, *Bradyrhizobium* sp. BTAi1, aromatik hidrokarbonlar, TCE, yeşil kimya, biyoremidasyon, rizoremidaston.

DEDICATION

To my beloved grandparents, Şenol and Coşkun Yıldırım

"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less."

Marie Curie

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SYMBOL/ABBREVIATION

∑	Summation
µl	microliter
µM	Micromolar
4NC	4-nitrocatechol
A_{570}	Absorbance at 570 nm
AMO	Alkene monooxygenase
BMMs	Bacterial multicomponent monooxygenases
bp	Base pair
BSA	Bovine serum albumin
BTAi1	*Bradyrhizobium* sp.
cis-DCE	*cis*-dichloroethylene
ddH_2O	Double distilled water
dH_2O	Distilled water
dNTPs	Deoxyribonucleotide triphosphate
DOE	U.S. Department of Energy
E. coli	*Escherichia coli*
EtOH	Ethanol
FAD	Flavin Adenine Dinucleotide
H_2O	Water
HPLC	High performance liquid chromatography
HQ	Hydroquinone
hr	Hour
IARC	International Agency for Research on Cancer
IPTG	Isopropyl β-D-1-thiogalactopyranoside
i	insert

kDa	Kilodalton
LB	Luria-Bertani
mg	Miligram
min	Minute
ml	Mililiter
mNP	*m*-nitrophenol
n	Number of values
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NaOAc	Sodium acetate
NB	Nitrobenzene
NCBI	National Center fpr Biotechnology Information
ng	Nanogram
nm	Nanometer
O_2	Oxygen
OD	Optical density
PCE	Tetrachloroethylene
PCR	Polymerase chain reaction
PHs	Phenol hydroxylases
pNP	*p*-nitrophenol
rpm	Revolutions per minute
RT	Retention time
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sMMO	Soluble methane monooxygenase
T4MO	Toluene 4-monooxygenase
TAE	Tris-acetate EDTA
TCE	Trichloroethylene
Tmo	Toluene monooxygenase
TmoA	α-hydroxylase
TmoB	γ –hydroxylase
TmoC	Rieske type ferredoxin
TmoD	Regulatory/Mediating/Effector protein
TmoE	β -hydroxylase

TmoF	NADH ferredoxin oxidoreductase
TOM	Toluene *ortho*-monooxygenase
ToMO	Toluene *o*-xylene monooxygenase
T*p*MO	Toluene *para*-monooxygenase
trans-DCE	*trans*-dichloroethylene
UV	Ultra violet
v	Vector
VC	Vinyl chloride
χi	Number of total values

CHAPTER 1

INTRODUCTION

Bacterial toluene monooxygenases are group of enzymes that catalyze the addition of hydroxyl groups on substrates (Wood et al, 2004; Vardar and Wood, 2004; Tao et al, 2004). These enzymes are able to oxidize a wide range of aromatics, degrade a variety of organic pollutants, and produce industrially-important compounds. There are basically four types of toluene monooxygenases that have been studied so far: toluene *o*-xylene monooxygenase (ToMO) from *Pseudomonas sporium* OX1 (Bertoni et al, 1996.), toluene *ortho*-monooxygenase (TOM) from *Burkholderia cepacia* G4 (Newman et al, 1995), toluene *para*-monooxygenase (T*p*MO) from *Ralstonia pickettii* PKO1 (Fishman et al, 2004), and toluene 4-monooxygenase (T4MO) from *Pseudomonas mendocina* KR1 (Yen et al, 1991.). These multicomponent enzymes involve six subunits: three hydroxylase ($\alpha_2\beta_2\gamma_2$) subunits with two non-heme, carboxylate-bridged, diiron center in α-subunit, a reductase, a mediating protein, and a ferrodoxin (Vardar and Wood, 2004). These enzymes differ in their regiospecific oxidation of toluene and ToMO is the only one that oxidizes *o*-xylene.

Toluene monooxygenases are a group of bacterial oxygenases that have importance in green chemistry and bioremediation applications (Tao et al, 2004, Vardar et al, 2005a; Vardar et al, 2005b). For green chemistry, they can produce pharmaceutical and industrial compounds such as catechol, which is used as a chemical in food, pharmaceutical and agrochemical industries; resorcinol, that is used to regulate plant growth and as a capacitor electrolytes and naphthols which are used in herbicides, insecticides, drugs and dyes. Toluene monooxygenases can also oxidize nitrobenzene and nitrophenols to produce nitrocatechols, an inhibitor of nitric oxide syntase and nitrohydroquinone, which is used as a precursor for Alzheimer's and Parkinson's diseases. For bioremediation, they can degrade all chlorinated aliphatics, including

tetrachloroethylene (PCE), trichloroethylene (TCE), *cis*-dichloroethylene (*cis*-DCE), *trans*-DCE, 1,1-DCE, vinyl chloride and chloroform which are health-threatening pollutants and persistent chemicals (Vardar and Wood, 2005b). In addition, TOM has been successfully used for rhizoremediation, which is a more beneficial bioremediation method due to the use of root colonizing bacteria (rhizobacteria) (Yee et al, 1998). However, the extents of degradation with these enzymes are still low in order to be used for bioremediation of the contaminated sites. Despite the fruitful history of monooxygenases and directed evolution, there is still an extreme need in the discovery and creation of new and more powerful toluene monooxygenases able to degrade chlorinated ethenes more efficiently and produce industrially important compounds much faster.

Bradyrhizobium sp. BTAi1 is a photosynthetic rhizosphere bacterium that lives in the root of leguminous plants and its whole genome was completed in 2007 (Giraud et al, 2007). It consists of 8.264.689 bp length and only 5100 bp region of this genome shows 38.5% amino acid similarity with ToMO, 37.7% with T4MO, 40.6% with T*p*MO and 17.2% with TOM. The predicted hydroxylase alpha subunit of BTAi1 shows 54% protein identity with ToMO, T4MO, and T*p*MO.

The purpose of this study is to clone and characterize a new toluene monooxygenase from *Bradyrhizobium* sp. BTAi1 for green chemistry and bioremediation applications. The anticipated result of this research is having a novel toluene monooxygenase to be used in the fields of bioremediation, biotechnology and organic synthesis. This is the first toluene monooxygenase characterized from a rhizobacterium strain. In addition, it is the first recombinant *Escherichia coli* TG1 expressing a toluene monooxygenase from *Bradyrhizobium* sp. BTAi1.

1.1 TOLUENE MONOOXYGENASES

1.1.1 Classification of Toluene Monooxygenases

Oxidoreductases are class of enzymes that are capable of transferring electrons from one molecule to the other in biochemical reactions. (Torres et al, 2010). These enzymes require cofactors which are tightly bound to the enzyme component. A class of

oxidoreductase is monooxygenases or hydroxylases that catalyze the formation of hydroxyl group on substrate by incorporation of one oxygen atom. The other oxygen atom is reduced by NAD(H) to water (H_2O). One of the families of monooxygenases is non-heme iron dependent monooxygenase which contains two iron atoms as cofactors in its active site for catalysis. Due to having more than one component, three or four, in their structure, these monooxygenases are member of bacterial multicomponent monooxygenases (BMMs) which also include, soluble methane monooxygenases (sMMOs), alkene monooxygenases (AMOs), and phenol hydroxylases (PHs) (Bailey, et al, 2008b). These enzymes have similar substrate hydroxylation, except, sMMO is able to catalyze the oxidation of methane, which is a difficult task to do. The diiron centers in active site of hydroxylase component are found to be structurally similar, making them belonging to the same ancestor. sMMOs, AMOs and PHs have a NADH oxidoreductase subunit with ferredoxin and FAD domain for supplying electrons by NADH. On the contrary, toluene monooxygenases have a separate Rieske-type ferredoxin subunit to facilitate electron transfer from reductase to hydroxylase component. Although, the best structurally characterized BMMs are soluble methane monooxygenases (sMMOs), phenol hydroxylases and toluene monooxygenases have a great potential for environmental and industrial applications since their expression can be accomplised in recombinant *Escherichia coli* strain (Leahy et al, 2003; Sazinsky et al, 2004; Sambrook et al, 1989).

Toluene monooxygenases oxidize many aromatic hydrocarbons, degrade organic pollutants and produce industrially-important compounds which make them useful as biocatalysts (Vardar and Wood, 2005a). There are four toluene monooxygenases that have been isolated since today. These are ToMO from *Pseudomonas* sp. OX1 (Bertoni et al, 1996), TOM from *Burkholderia cepacia* G4 (Newman et al, 1995), T*p*MO from *Ralstonia pickettii* PKO1 (Fishman et al, 2004), and T4MO from *Pseudomonas mendocina* KR1 (Yen et al, 1991.). They use toluene as a sole carbon and energy but the regiospecific oxidations of toluene and substrate range are different (Vardar et al, 2005b).

Toluene monooxygenases are multicomponent enzymes; contain six subunits encoded by six genes, *tmoABCDEF* (Cafaro et al, 2004). They have a hydroxylase component with a quaternary structure, consist of two copies of three subunits; TmoA,

TmoE and TmoB that are α-, β- and γ-subunits ($\alpha_2\beta_2\gamma_2$), respectively. In the α-subunit, there are two non-heme, carboxylate-bridged, diiron centers are present and they build the active site. Besides, these enzymes include a NADH-ferredoxin oxidoreductase (TmoF), a mediating (regulatory/effector) protein (TmoD), and a Rieske-type ferredoxin (TmoC). Electrons are transferred from NADH-ferredoxin oxidoreductase, TmoF to Rieske-type ferrodoxin, TmoC that contains Rieske cluster [2Fe-2S] and then TmoC sends electrons to hydroxylase subunit. (Cafaro et al, 2004). Finally, the last component is mediating or regulatory protein, TmoD which is proposed for its need in active enzyme and found to be effective in steady-state activity for substrate oxidation by its interactions with hydroxylase component. (Song, 2011). Each gene is essential for fully active toluene monooxygenase activity (Bertoni et al, 1998).

These four enzymes, ToMO, T4MO, T*p*MO and TOM have been characterized via a constructed vector pBS(Kan) by Prof. Thomas K. Wood et al, including toluene monooxygenase sites, for avoiding of background enzymatic reactions which are made by original strains. The plasmid pBS(Kan) has a kanamycin resistant gene as selective marker and also at the beginning of toluene monooxygenase encoding genes, there is a lac promoter which provides expression of toluene monooxygenase genes, constitutively. *E. coli* TG1 have been preferred as the host for constructed vector because of not having any oxygenase activity, having high expression of multicomponent toluene monooxygenases and producing NADH cofactor for enzyme activity (Canada et al, 2002).

1.1.2 Mechanism of Hydroxylation of Toluene Monooxygenases

Toluene monooxygenases are a group of enzymes with multiple components, containing non-heme diiron centers in their active site and using oxygen in their catabolic reactions of hydrocarbons (Figure 1.1) (Bailey et al, 2008a).

Figure 1.1 Toluene monooxygenase activities on an aromatic compound. Toluene

monooxygenases catalyzes the reaction of adding a hydroxyl (OH) group on substrate by using oxygen and produces water (Song, 2011).

They all are carboxylate-bridged diiron enzymes and include six proteins encoded by six genes, *tmoABCDEF* (Fishman et al, 2004b). The best characterized member is T4MO from *P. mendocina* KR1 and it is composed of TmoABE $(\alpha\beta\gamma)_2$ (212 kDa); a multisubunit hydroxylase with two of each subunit for catalysis , TmoF (36 kDa); a NADH ferredoxin-oxidoreductase for NADH oxidation and electron transport, TmoC (12.5 kDa); a Rieske-type [2Fe-2S] ferredoxin, for shifting electrons from TmoF to TmoABE and finally TmoD (11.6 kDa); a mediator or regulatory protein which is known to be responsible for catalysis because of its high affinity component of hydroxylase. Although it was demonstrated that without TmoD, a fully active enzyme cannot be provided, the importance of TmoD was not discovered until recently. (Bertoni et al, 1998) But now on, TmoD is found to be required for more active of hydroxylase by increasing the rate of catalysis and for regiospecific effects via changing interactions in hydroxylase subunit (Bailey et al, 2008a) (Figure 1.2).

In the active site, there are two iron atoms, bound to four glutamate and two histidine residues. Enzyme catalysis is performed by molecular oxygen (O_2). The oxygen has to enter the active site where diiron atoms are placed and then initiates the hydroxylase reaction on aromatic substrate (Figure 1.3).

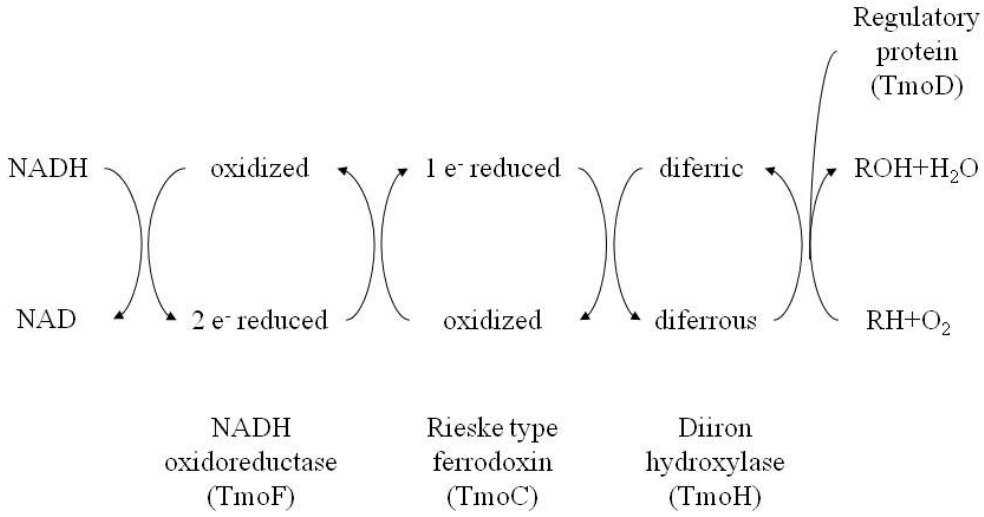


Figure 1.2 Toluene monooxygenase enzyme complex (Lountes, 2005).

There are three different hydrophobic cavities found in a region between protein exterior and the diiron center in the active site (Song et al, 2011; McCormick and Lippard, 2011). Oxygen migrates through these cavities and reaches to the diiron center. The diiron (III) resting state is reduced by two electrons coming from NADH-oxidoreductase and Rieske type ferredoxin correlation. The result is a reduced diiron (II) state which activates molecular oxygen to form oxygenated diiron (III) or peroxodiiron (III) intermediate. This highly active intermediate directly hydroxylates aromatic compound that migrates through a channel. The channel is linked to the active site diiron center and is only found in toluene monooxygenases of BMMs family. It contains hydrophobic residues for aromatic substrate entrance and product outgoing. At the end of the reaction, oxygenated intermediate regenerates to its resting state (Song, 2011; Torres et al, 2010).

The three cavities conveying for O_2 are not linked together in resting state of diiron center. In recent study done by Song W.J *et al*, the first two cavities are found to be linked to each other when TmoD regulatory subunit bound to the TmoH hydroxylase subunit. In addition to this, the third cavity is connected to the first two via conformational changes occuring in the α-subunit. This adjoining is proposed due to the oxygen entrance inside the first two cavities (Song et al, 2011).

As a summary, O_2 migrates through three adjacent cavities and activates the diiron center for substrate oxidation. Substrate enters the active site through a long hydrophobic channel found in hydroxylase subunit. Binding of regulatory subunit, TmoD to hydroxylase, TmoH, triggers a conformational change by opening the channel and connecting cavities to each other and then provides catalysis.

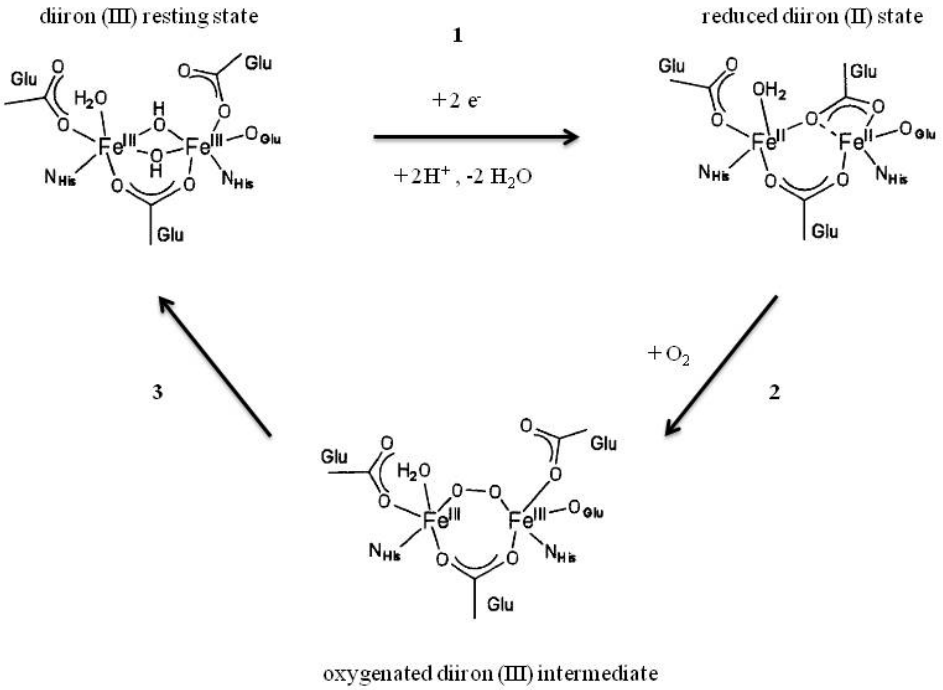


Figure 1.3 Catalytic cycles of toluene monooxygenases. Diiron (III) resting state is reduced by two electrons (1). Then oxygen is activated by reduced diiron (II) state (2) and generates an intermediate called oxygenated diiron (III). This intermediate catalysis the reaction of hydroxylation and yields the product (3) (Song, 2011).

1.1.3 3-D Structure of Toluene 4-Monooxygenase Hydroxylase Subunit

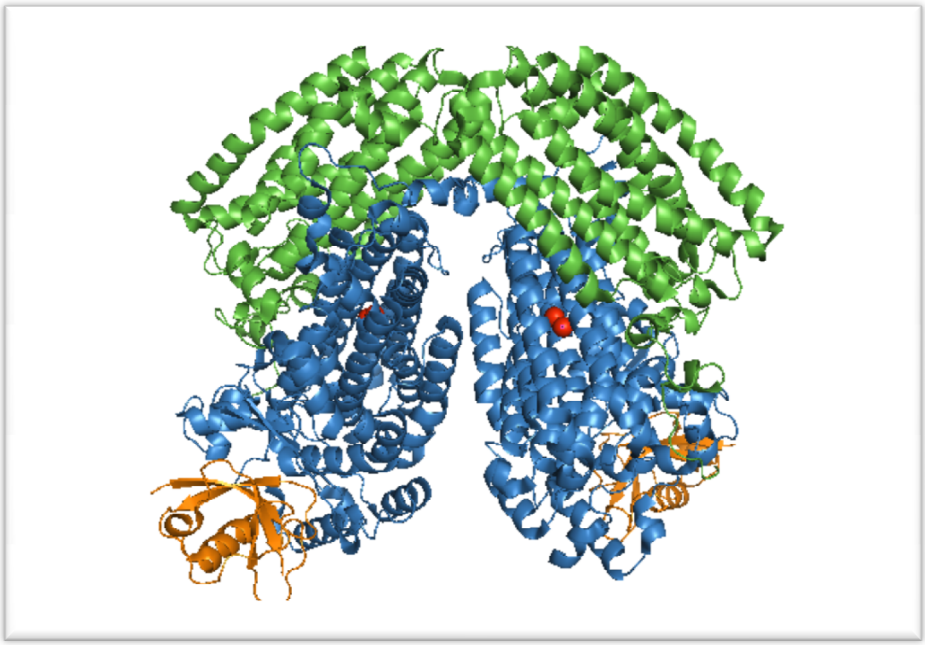


Figure 1.4 T4MO hydroxylase subunit with diiron center . T4moH contains two α-subunits (blue), β-subunits (green), and two γ-subunits (orange). In the α-subunits active site, there are two iron atoms (red) present (Pymol, Schrödinger; Protein Data Bank (PDB) Entry: 3DHG; doi: 10.2210/pdb3dhg/pdb; Bailey et al, 2008a).

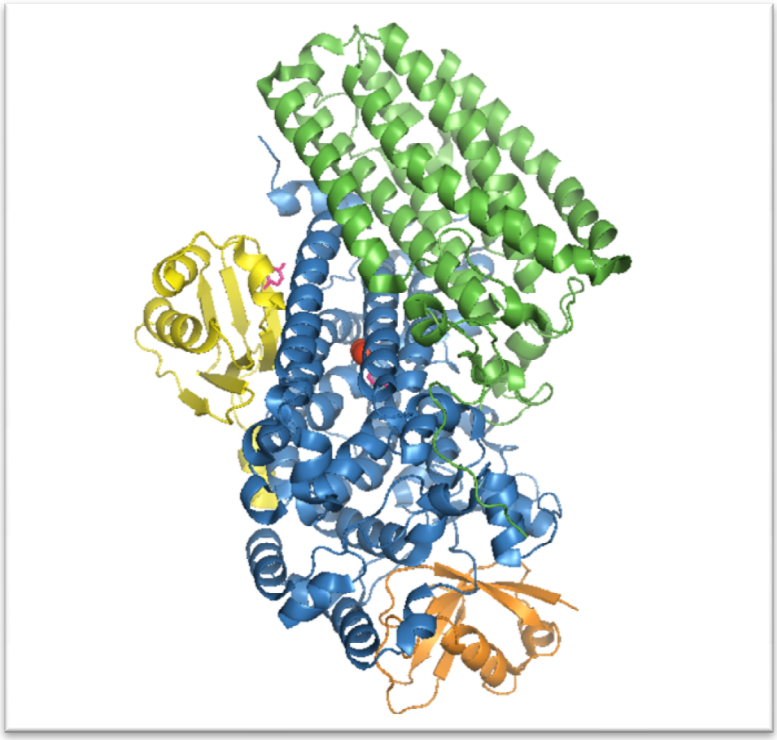


Figure 1.5 T4moD component bound to T4moH with *p*-cresol substrate (Pymol, Schrödinger; PDB Entry: 3Q14; doi: 10.2210/pdb3q14/pdb; Bailey et al, 2012). Effector T4moD protein is bound to T4moH complex and contributes substrate oxidation at the active site where diiron atoms (red) are found. *p*-cresol is represented with pink color.

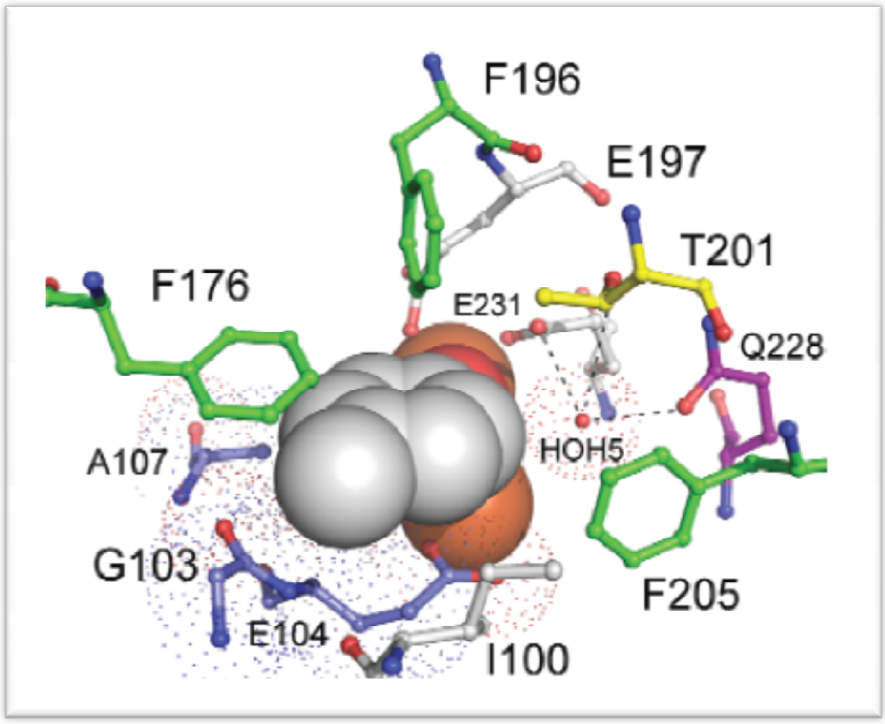


Figure 1.6 *p*-cresol substrate is bound to T4moH active site residues in diiron center (Pymol, Schrödinger; PDB Entry: 3Q14; doi: 10.2210/pdb3q14/pdb; Bailey et al, 2012). The image was taken from Bailey et al, 2012. *p*-cresol and diiron atoms are represented as gray and orange colors, respectively.

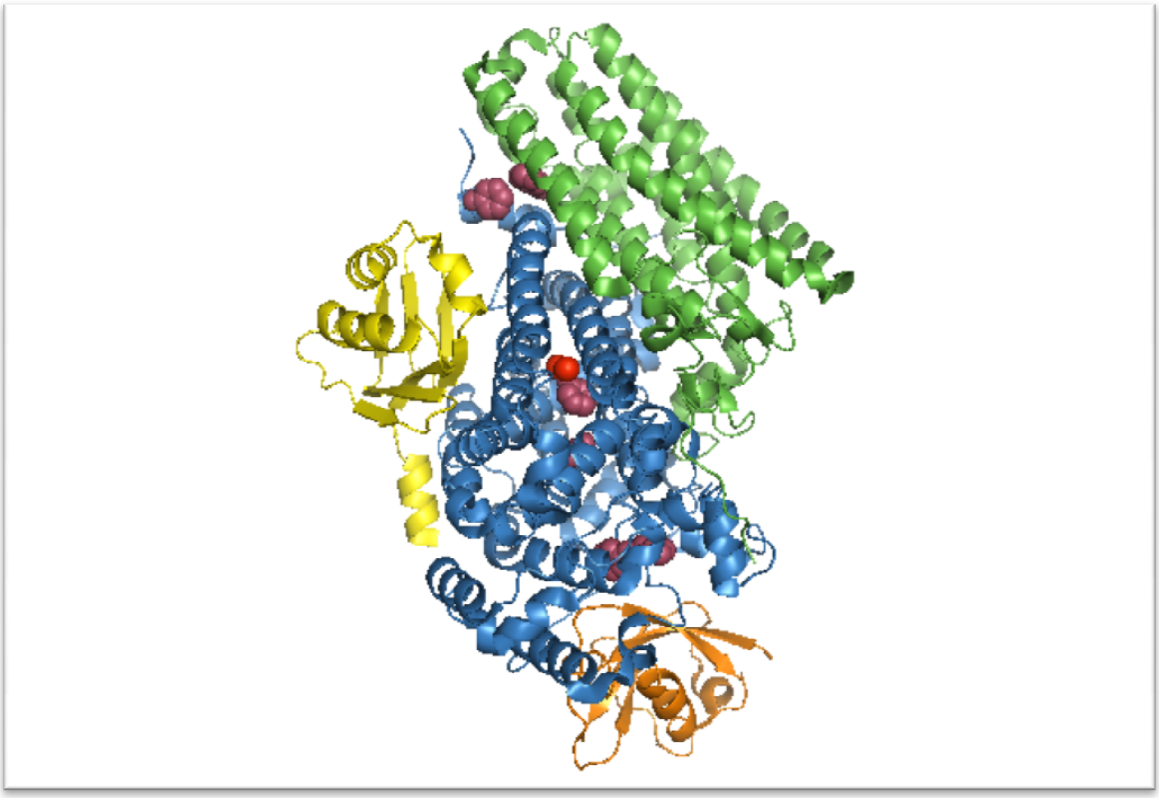


Figure 1.7 Phenol substrate inside the T4moHD complex (Pymol, Schrödinger; PDB Entry: 3Q3O; doi: 10.2210/pdb3q3o/pdb; Bailey et al, 2012). Phenol compounds are shown with purple color.

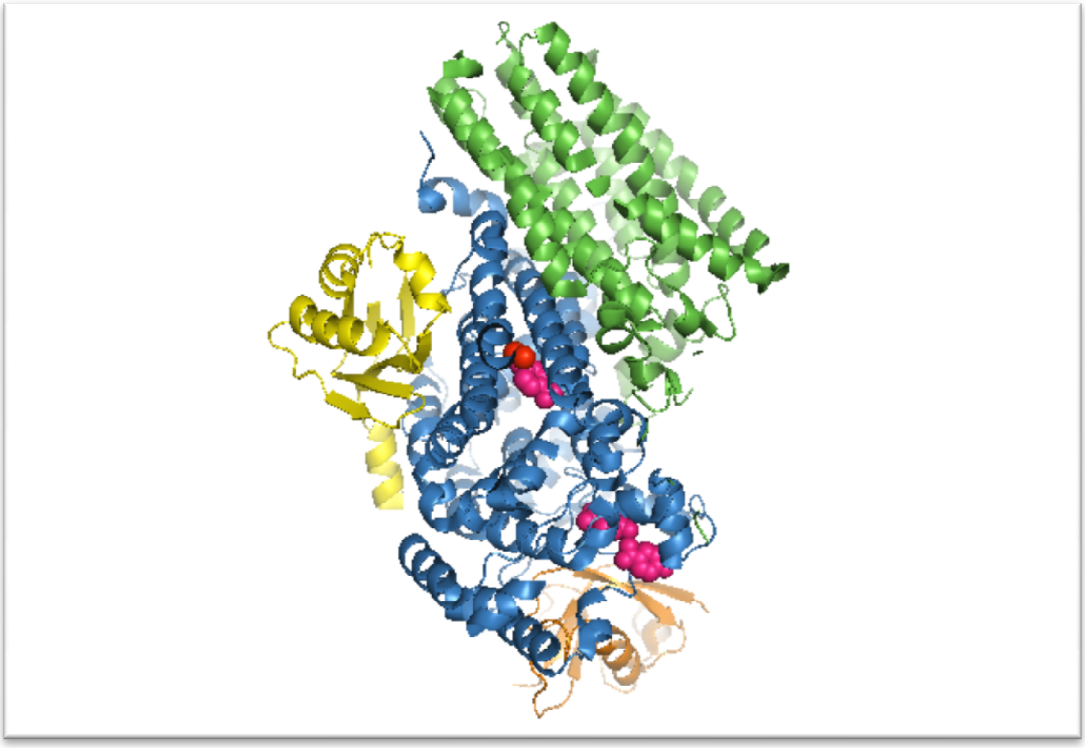


Figure 1.8 *p*-Nitrophenol substrate inside the T4moH complex (Pymol, Schrödinger; PDB Entry: 3Q3N; doi: 10.2210/pdb3q3n/pdb; Bailey et al, 2012). *p*-NP compounds are shown with pink color.

1.1.4 Applications of Toluene Monooxygenases

Bacterial toluene monooxygenases have a great potential for industrial and environmental applications.

1.1.4.1 Industrial

Aromatic hydrocarbons such as benzene, phenol, toluene, naphthalene, *o*-xylene are petroleum produced chemicals and they have wide range of usage in the industrial area as intermediates, solvents, precursor of plastics and pesticides (Fritsche and Hofrichter, 2007). They are volatile and persistent chemicals. Over consumption and incautiously release of these aromatic structure chemicals contaminate ground water and cause environmental pollution. These chemicals have been reported as possible human carcinogen by the International Agency for Research on Cancer (IARC).

Toluene monooxygenases are key enzymes for oxidizing these compounds. Furthermore, the catalysis reaction yields important compounds that are hardly synthesized by chemical reactions, require expensive methods and are used in crucial applications (Tao et al, 2004).

Benzene has a single aromatic structure and widely used as a solvent or an intermediate for the production of other chemicals (Tao et al, 2004). Over exposure of benzene can result with hematological disorders like leukemia (Smith, 1999). Benzene can be oxidized by monooxygenases, especially toluene monooxygenases are good choice for catalysis due to the formation of catechol. Benzene is oxidized into phenol, and then catechol is obtained (Tao et al, 2004). Catechol is a crucial chemical, it is rarely found in nature and its production is very demanding. It is an agent for photographic developers and synthetic flavors as well as a precursor of dyes and pharmaceuticals. (Wang et al, 2006) Hydroquinone is another compound produced by oxidation of benzene and has an antioxidant property and protective effect to genotoxic effects (O'Donoghue et al, 1999). It is also used as an agent for photographic developers, as stabilizer for industrial polymers, an ingredient of hair dyes and in cosmetics (Jagetia and Aruna, 1997; Topping et al, 2007). Resorcinol, another derivative of phenol is found in capacitor electrolytes, and used as precursor for regulatory growth factor for plants (Vardar and Wood, 2004).

All four known toluene monooxygenases; ToMO, T4MO, T*p*MO, TOM are known to transform benzene to phenol and to catechol (Tao et al, 2004).

Toluene is a volatile, persistent chemical and found in household products, paints, polishes and in rotogravure printing. Humans are exposed to toluene in their daily life. However, long term exposure of toluene has neurotoxic effects (Neubert et al, 2001; Win-Shwe and Fujimaki, 2003). Several studies proved that, inhalation of toluene affects brain functions and causes autonomic and peripheral nervous dysfunction.

Toluene is used by toluene monooxygenases as sole carbon and energy. ToMO is able to oxidize toluene into three different forms of cresols; *o*-cresol, *m*-cresol and *p*-cresol. T4MO and T*p*MO produce both *m*-cresol and *p*-cresol. TOM has a unique feature that forms only *o*-cresol (Tao et al, 2004) Cresols are one of phenolic compounds and used in several industrial purposes such as herbicides and pharmaceuticals. Nevertheless, they threaten human life in long-term exposure (Kavitha and Palanivelu, 2005). Methylcatechols derived from cresols by toluene monooxygenase activity, are important compounds in pharmaceutical applications; for example 4-methylcatechol inhibits growth of melanoma cells in humans (Payton et al, 2011).

Nitroaromatic compounds are produced by the oxidation of nitrobenzene. They are widely used in industrial area as pesticides, solvents, dyes and precursors of some drugs in the pharmaceutical area (Vardar et al, 2005a). Nitrocatechols are mainly used in drugs as therapeutic agents for certain diseases such as Alzheimer's and Parkinson's disease (Fishman et al, 2006). ToMO is able to oxidize nitrobenzene and produces 3-nitrocatechol which is a very expensive compound due to its rare presence in the environment.

Naphthalene is formed from two aromatic rings and toluene monooxygenases are able to oxidize it. It is mainly used for the production of other compounds and found in household products (Kang et al, 2012). Oxidation of naphthalene is achieved by four known toluene monooxygenases and yielding products are used in herbicides, insecticides, drugs and dyes (Canada et al, 2002).

Indigo is a dye with blue color, found to be produced by bacterial strains and mainly used in textile industry, especially as color of the blue-jeans, since the early

1980's (Murdock et al, 1993). The first plant produced indigo structure was discovered by Adolf von Baeyer and he became the recipient of the fifth Noble Prize in Chemistry (Rui, Reardon and Wood, 2005). Although the blue dye, indigo, is rarely found, the usage of this dye is extremely high in the worldwide textile industry. The extra need for indigo is undeniable. Chemical synthesis of indigo is a hard process and consequences are found to be dangerous due to production of extra toxic compounds (Frost and Lievense, 1994). As an alternative way, enzymes give great opportunity for the production of indigo since their biocatalyst reactions are safer than chemical processes. Today, a set of oxygenases expressed in *E. coli* strain are found to be producers of indigo and indigoid compounds (Ensley et al, 1983). Some of these are naphthalene dioxygenases, styrene monooxygenases, cytochromes P450, and toluene monooxygenases (O'Connor et al, 1997; Bernhardt, 2006; McClay et al, 2005).

Recombinant *E. coli* strains including toluene monooxygenases are known to be responsible of indole and indigo formation. Trypton is an ingredient of Luria Bertoni (LB) medium and used as nutrient source for *E. coli* strains. It contains high concentrations of tryptophan, an amino acid that is degradable by trypthophanase which is an enzyme produced by *E. coli* strain. The degradation of tryptophan results with indole formation which provides resistance to drugs, and stability of plasmid to the *E. coli* strain (Han et al, 2011). Indole is used by toluene monooxygenases and the intermediate product turns spontaneously into indigo, the blue dye (Figure 1.9) (Ensley et al, 1983).

ToMO, T*p*MO and T4MO are known for their ability to hydroxylate indole into indigo and produce blue colonies on LB medium. TOM produces another indigoid pigment which is isoindigo, and gives brown colonies on LB medium.

Several protein engineering approaches have been done for toluene monooxygenases to expand their regiospecificity for indole hydroxylation. In one study done by Rui et al, isoindigo produced TOM were subjected to saturation mutagenesis which is a directed evolution method to change the putative amino acids in a certain site of enzyme structure (Rui, Reardon and Wood, 2005). The result was promising: they identified different mutants able to produce indigo, indirubin and 4-hydroxyindole pigments. Indirubin has a pink color and useful in medical sciences. It has a potential

value as an inhibitor of certain kinases involved Alzheimer's diseases and some cancer diseases (Leclerc et al, 2001; Hoessel et al, 1999).

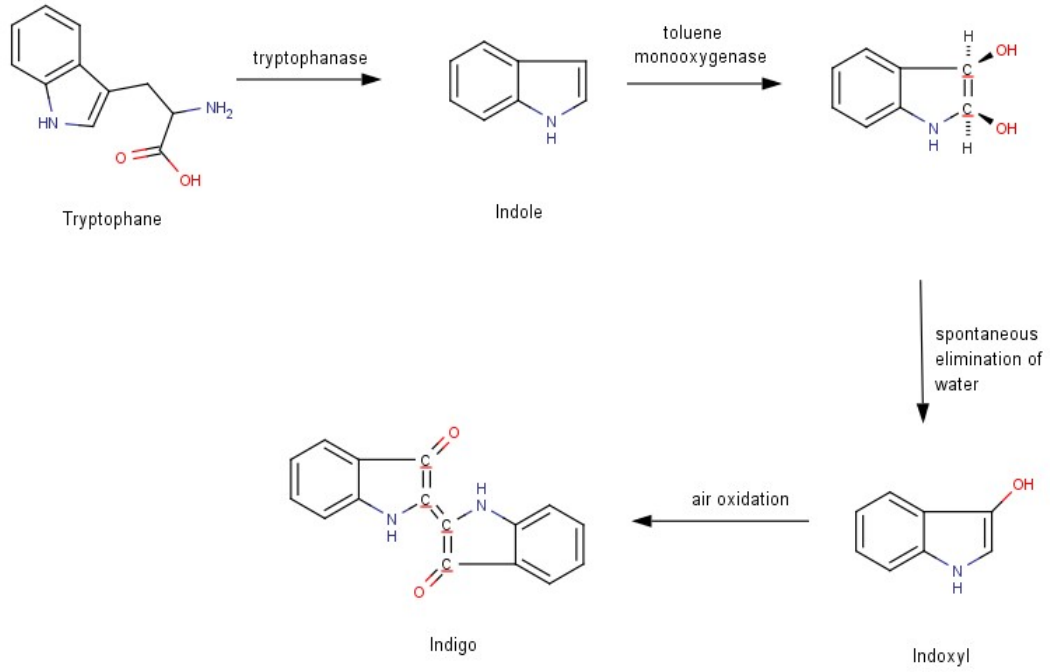


Figure 1.9 Possible pathway for indigo formation by toluene monooxygenases (Ensley et al, 1983).

In another study, site directed mutagenesis was applied to the α-subunit of T4MO, where the active site presents. Mutants were able to produce novel indigoid pigments; 7-hydroxyindole, 2-oxindole (McClay et al, 2005).

Consequently, toluene monooxygenases are great biocatalysts. Their ability to oxidize volatile, persistent, health-threatening aromatic compounds into useful chemicals makes them to have a significant role in industrial, green chemistry and pharmaceutical fields.

1.1.4.2 Bioremediation

Improvements in industrial area through human activities lead to a widespread pollution all over the world. Even in some countries, usage of persistent organic pollutants have been banned, there are still many countries spreading pollutants to the environment. Although there are some strict regulations by governments; these regulations are not enough to provide a clean environment. This deficiency requires a cleaning process which causes no harm to sites and completes decontamination (Ang, Zhao and Obbard, 2005).

Physical and chemical remediation techniques are not sufficient enough to clean all of the contaminated sites and wastes and also they need high costs to be successful. Bioremediation is an alternative and promising process to remedy or clean up all pollutants with low costs and provides permanent detoxification. Despite its relatively slow rate; it is a natural process and uses biocatalysts which are environmentally friendly and biodegradable molecules (Furukawa et al., 2001). Pollutants such as chlorinated ethenes are subjected to biodegradation processes which are achieved by naturally occurring microorganisms (Alcalde et al, 2006).

Chlorinated aliphatics including tetrachloroethylene (PCE), trichloroethylene (TCE), *cis*-dichloroethylene (*cis*-DCE), *trans*-DCE, 1,1-DCE, and vinyl chloride, are one of the groundwater contaminants and they are very toxic, carcinogenic, persistent (Winter al, 1989; Meza et al, 2003). Their widely use in industrial and textile area as agents for dry cleaning, metal cleaning, degreasing, chemical packaging and also as electronic components make them a significant cause of health-threatening compounds (Lipworth et al, 2011). These chemicals are low molecular weight, volatile solvents and they may contaminate drinking water supplies (Zylstra et al, 1989, Pant and Pant, 2010, Miller et al, 2011). This consideration brought the removal of these chemicals. In some countries, there are regulations applied for the usage of chlorinated ethenes, but these are not enough to eliminate the possible causes of those carcinogenic chemicals to human. Although their degradation is possible by anaerobically, vinyl chloride is produced as one of the final compound, which is more toxic than other chlorinated products and known to be human carcinogen (Figure 1.10). An optimistic pathway for those carcinogenic compounds is aerobically (Figure 1.11) (Zylstra et al, 1989).

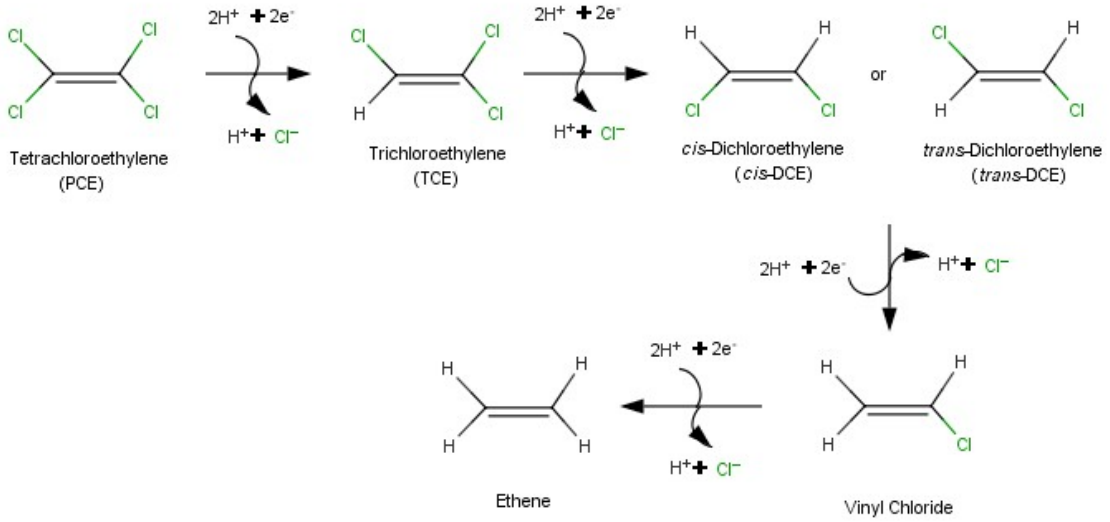


Figure 1.10 Anaerobic degradation of chlorinated aliphatics. In each cycle, one Cl-atom is taken off by the anaerobic enzyme. The final production is generally ethene, but is mostly end at vinyl chloride compound (Ernest, 2009).

Shields et al discovered that toluene monooxygenases are able to degrade chlorinated aliphatics and they determined the degradation of trichloroethylene is related with toluene degradation pathway (Shields et al, 1991). By this study, toluene monooxygenases became a major class of enzymes that can be involved in bioremediation applications for degradation of chlorinated compounds. These enzymes are able to degrade all chlorinated aliphatics which are very toxic, health-threatening, persistent chemicals and contaminate groundwater (Vardar and Wood, 2005b).

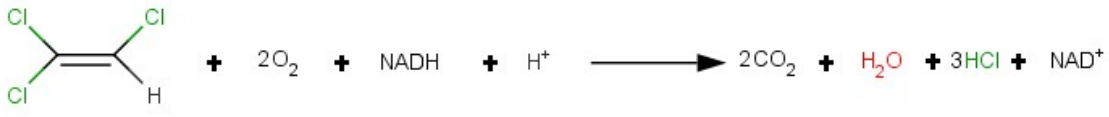


Figure 1.11 Aerobic degradation of TCE by toluene monooxygenases. The products are CO_2 and HCl which are not harmful substances (Shim and Wood, 2000).

ToMO is the only enzyme that is able to degrade PCE that has been thought as non-degradable via aerobic pathway. (Ryoo et al, 2000; Vardar and Wood, 2005b). One of directed evolution method, DNA shuffling was applied to ToMO to enhance the rate of degradation of chlorinated ethenes. TCE degradation was increased as two-fold, however no up-mutants were found for PCE degradation.

TOM was evolved via directed evolution methods, and one mutant, called TOM-Green due to its green colonies, was found to be able to mineralize chlorinated ethenes with 2-fold faster activity than its wild type (Canada et al, 2002). This limited activity increase needs more solutions. So, new toluene monooxygenases are needed for the degradation of certain recalcitrant compounds.

Despite the improvements in bioremediation, there is still a need for better environment. New approaches should be improved for the degradation of recalcitrant and persistent chemicals.

1.1.4.3 Rhizoremediation

Bioremediation is a cost-effective, environmentally friendly and often a permanent method since consumption and release of toxic organic chemicals are becoming a major problem of environmental pollution (Singh et al,2008; Wood, 2008). Several pathway engineering methods have been applied for removal of hazardous chemicals in polluted environments. However there is still a need for complete mineralization of those toxic chlorinated ethenes. In situ bioremediation is found to be most helpful and since chlorinated ethenes easily contaminate soil and migrate to groundwater, one promising method; rhizoremediation is more effective for its cost-effective approach and advantage of using correlation between naturally occurring microorganisms and plants (Gerhardt et al, 2009).

Rhizosphere is a large region in plant roots that creates a nutrient-rich environment and aeration, provides carbon and energy for growth of nitrogen fixing root-colonizing bacteria (*Rhizobium*), besides it delivers cofactors which are needed for enzyme activation of *Rhizobium* and bacteria supply nitrogen for plants' survival and maintenance (Megharaj et al, 2001; Molobela, 2004). Most of recalcitrant compounds

are found to be collected near the rhizosphere area which contributes efficiency for rhizoremediation approach.

Rhizoremediation can happen in both ways, either naturally occurring microorganisms that are present already in the contaminated soil or an introduction of natural microorganisms or optimizing engineered pollutant-degrading bacteria into the contaminated area (Megharaj et al, 2001). Rhizoremediation has many advantages; low-cost, safety, maintenance of soil structure, no need to supply additional nutrient sources or to remove of polluted soil, environmentally-friendly approach, usually results with complete mineralization and more acceptable by industrial field and governments (Gerhardt et al, 2009).

In 1990s, first rhizoremediation approach was applied to contaminated soil by herbicides and pesticides (Kuiper et al,2004). The result was promising; they have identified bacterial strains for degradation of those toxic compounds and figured out that bacterial strains have a protection effect on plants towards recalcitrant chemicals.

Kuiper et al suggests that introduction of suitable *Rhizobium* into a convenient plant provides an improvement in association between plant and microbe which enhances stimulation of rhizoremediation process of that certain polluted area (Kuiper et al, 2001).

Native *Rhizobium* may not be able to degrade all persistent, recalcitrant carcinogen compounds; consequently there is a potential need for protein engineering methods for enhanced rhizoremediation to be succeeded in the mineralization of wide range of chemicals (Gilbertson et al, 2007).

Recombinant techniques for rhizoremediation have been used for toxic, recalcitrant chemicals such as TCE and polycyclic aromatic hydrocarbons (PAHs) (Kavamura et al, 2010). In one study done by Yee et al, a recombinant *Pseudomonas fluorescens* strain including TOM genes was introduced to wheat plant root. Wild type *P. fluorescence* degrades TCE however; the rate of TCE degradation was increased in a short term with recombinant strain expressing TOM (Yee et al, 1998).

Introducing recombinant root-colonized bacteria in soil has many advantages to enhance elimination of toxic chemicals however; engineered microorganisms may need

to defeat some difficulties like survival and maintenance in rhizosphere area, and also expression of enzyme activity may be lost due to growth problems (Gilbertson et al, 2007). A natural *Rhizobium* with having already a toluene monooxygenase activity may overcome these problems and the enzyme activity of host bacteria may enhanced via engineering methods.

1.2 *Bradyrhizobium* sp. BTAi1

Bradyrhizobium was named for "slow growing" strains of rhizobacteria which are nitrogen-fixing, gram-negative, root-colonizing soil bacteria (Figure 1.12) (Berkum et al, 2006; Sudtachat et al, 2009).

Bradyrhizobium sp. BTAi1 is a rhizobacterium with a photosynthetic activity that is not found in most rhizobial strains and lives in the rhizosphere area of leguminous plants (peas and soybeans) (Figure 1.13) (Forquer, 2002).

Rhizosphere area allows a symbiotic association between bacterium and plant itself. Root of plant supplies nutrients and cofactors and provides an area for bacterial survival and maintenance, while it benefits from nitrogen fixation of bacteria (Yee et al, January 1998).

Bradyrhizobium sp. BTAi1 was isolated from the nodules of *Aeschynomene indica,* North America by Sadowsky *et al* in 2007 (Figure 1.12). The strain was sequenced by the U.S. Department of Energy (DOE) Joint Genome Institute (USA) (Giraud et al, 2007) (Figure 1.14). BTAi1 contains a large chromosome with 8.264.689 bp and a single, circular plasmid with 228.826 bp, called pBTAi1. The recent genome sequence analysis in large chromosome revealed the presence of toluene monooxygenase which is composed of six proteins encoded by *tmoABCDEF*. TmoA, TmoE and TmoB are similar to the α, β, γ subunits, respectively, of the hydroxylase component. TmoC is similar to ferrodoxin and TmoD to an effector protein. TmoF is found to be close to a NADH oxidoreductase which is responsible for the transfer of electrons to the terminal oxygenase (Table 1.1) (Cafaro et al, 2004).

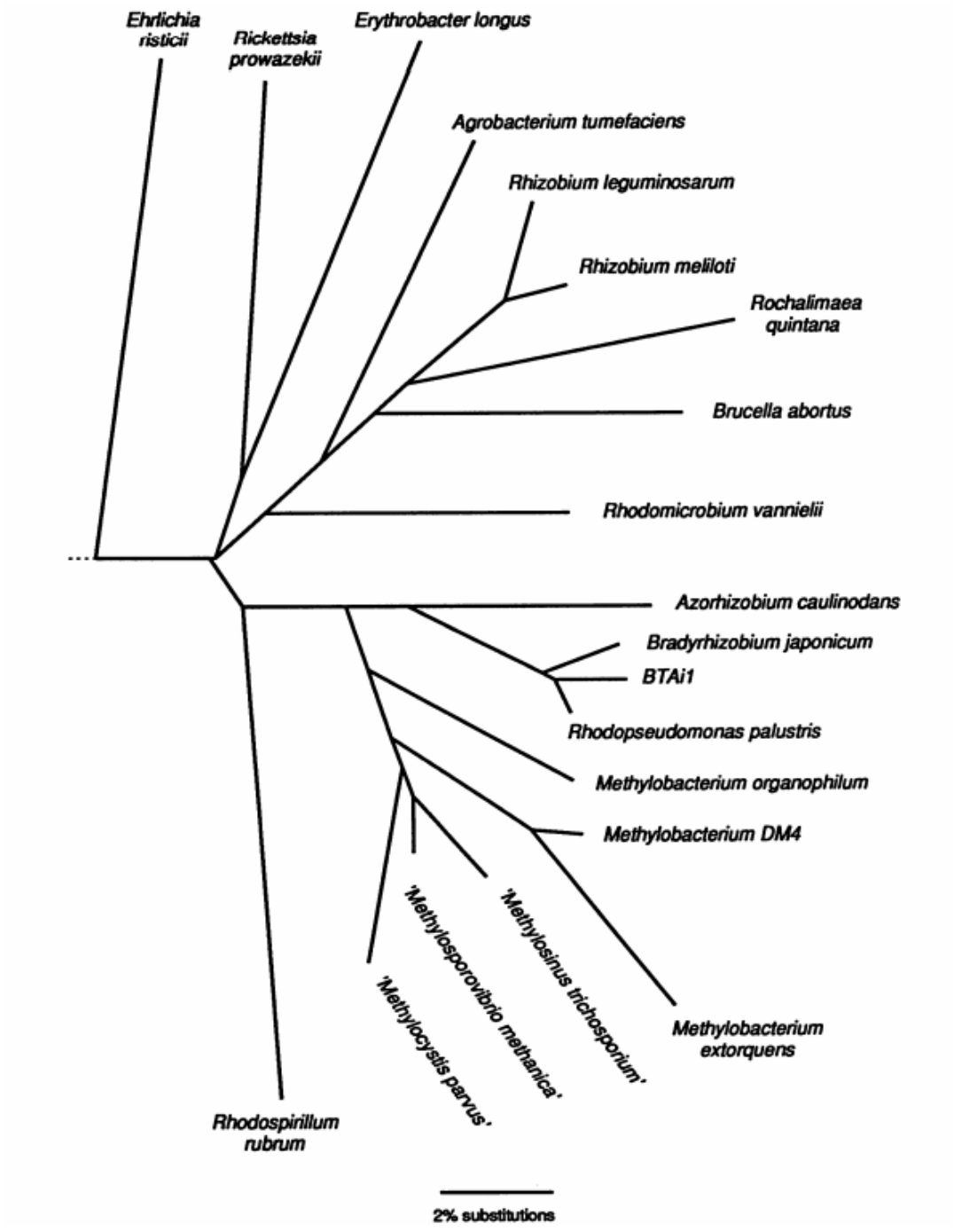


Figure 1.12 Phylogenetic tree of bacteria in the alpha subdivision of *Proteobacteria. Bradyrhizobium* sp. BTAi1 belongs to *Bradyrhizonium* genus. (Young et al, 1991)

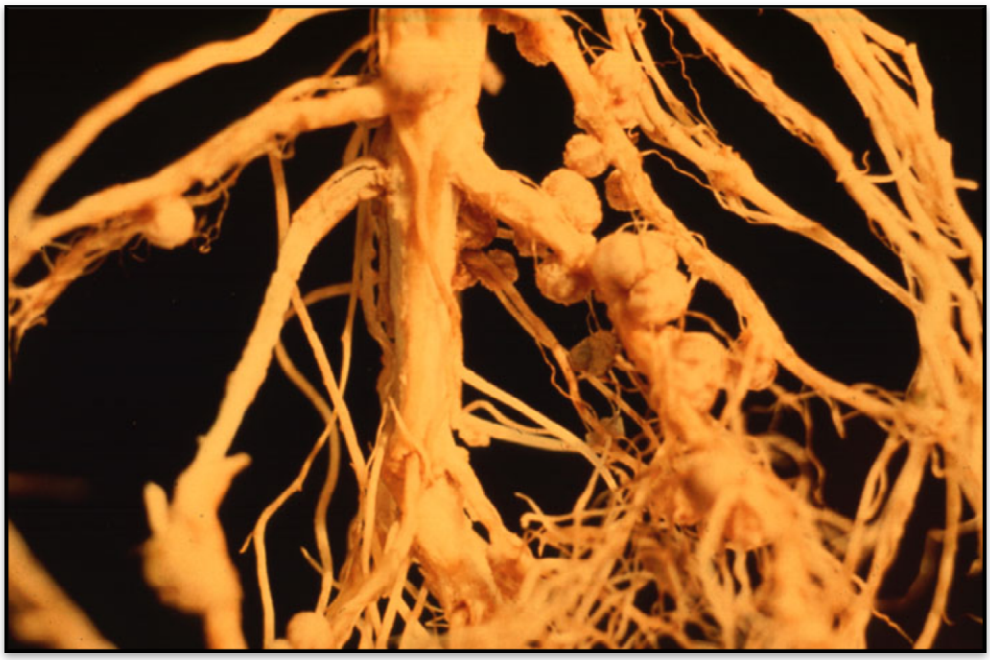


Figure 1.13 Nodules formed by root-colonizing Rhizobium bacteria on soybean roots. (Ingham, The Soil Biology Primer, Soil and Water Conservation Society, 2000, http://soils.usda.gov/sqi/concepts/soil_biology/bacteria.html)

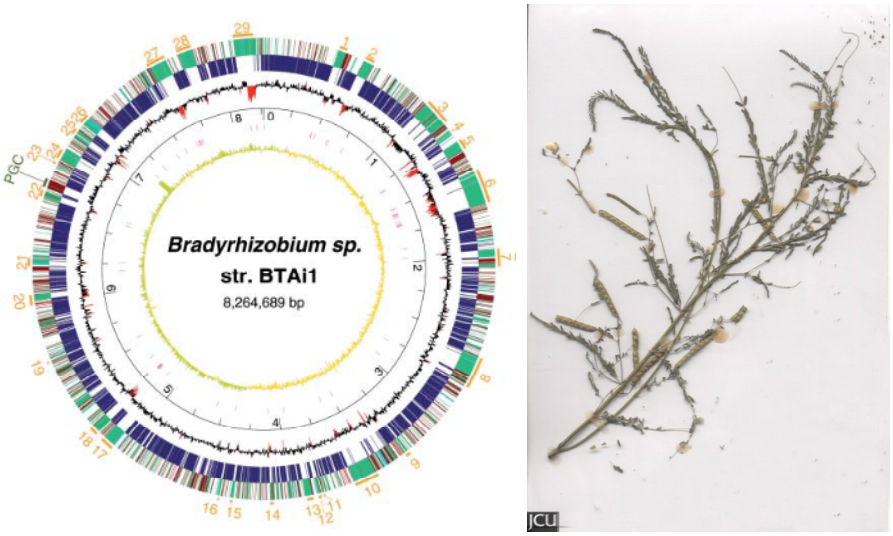


Figure 1.14 Circular representation of *Bradyrhizobium* sp. BTAi1 chromosome. U.S. Department of Energy (DOE) Joint Genome Institute (USA) (left). *A. indica* (Budda pea), photo was taken by James Cook University, Australia (right).

Table 1.1 *Bradyrhizobium* sp. BTAi1 putative toluene monooxygenase site, including six subunits and their locus on chromosome.

Bradyrhizobium sp. BTAi1[a]

Gene	Subunit	Gene locus
tmoA	α-hydroxylase	1670615-1672099
tmoB	γ-hydroxylase	1670273-1670584
tmoC	Rieske protein	1669900-1670253
tmoD	Regulatory protein	1669591-1669890
tmoE	β-hydroxylase	1668543-1669568
tmoF	Reductase	1667496-1668494

a(NCBI Reference Sequence: NC_009485.1, *National Center for Biotechnology Information,* USA)

Table 1.2 Percent amino acid sequence identity between BTAi1 and other subfamilies of toluene monooxygenases.

Percent Amino Acid Sequence Identity (%)

Subunit	Subfamilies			
	ToMO[a]	T4MO[b]	T*p*MO[c]	TOM[d]
α-hydroxylase	54.1	52.8	54.7	23.3
γ-hydroxylase	32.5	32.7	33.9	4.8
Rieske protein	27.8	30.2	28.7	ND[e]
Regulatory protein	31.4	27.5	36.9	26.7
β-hydroxylase	33.1	34.7	40.8	5.0
Reductase	31.3	35.2	34.9	19.2

[a] ToMO, toluene *o-xylene* monooxygenase from *Pseudomonas* sp. OX1;

[b] T4MO, toluene-4-monooxygenase from *Pseudomonas mendocina* KR1;

[c] T*p*MO, toluene-*para*-monooxygenase from *Ralstonia pickettii* PKO1;

[d] TOM, toluene-*ortho*-monooxygenase from *Burkholderia cepacia* G4

[e]ND: not determined. TOM does not have a Rieske protein.

Percent sequence analysis revealed that most similarities are in the α-subunit, which contains the active site (Figure 1.15-18). Also, the amino acid sequences are more related with ToMO, T4MO and T*p*MO (Table 1.2). Although TOM belongs to the toluene monooxygenases, it differs from the other subfamilies based on its amino acid sequences and the location of subunits inside its DNA sequence.

From this similarity, potential activity of a new toluene monooxygenase gene cluster from *Bradyrhizobium* sp. BTAi1 was investigated.

TMO.BTAi1 and ToMO α-subunits

Identity positions: 54.1%

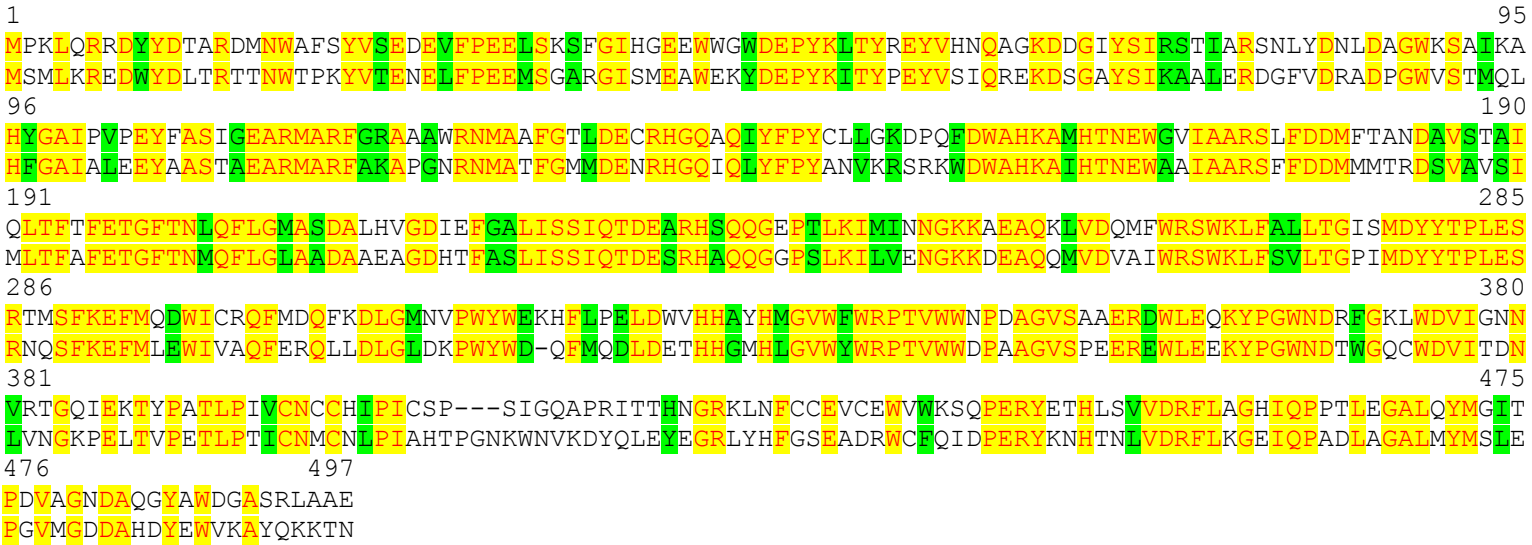


Figure 1.15 Comparison of α-subunit between BTAi1 putative of toluene monooxygenase site and ToMO. First and second lines represent BTAi1 and ToMO amino acid sequences, respectively.

TMO.BTAi1 and T4MO α-subunits

Identity positions: 52.8%

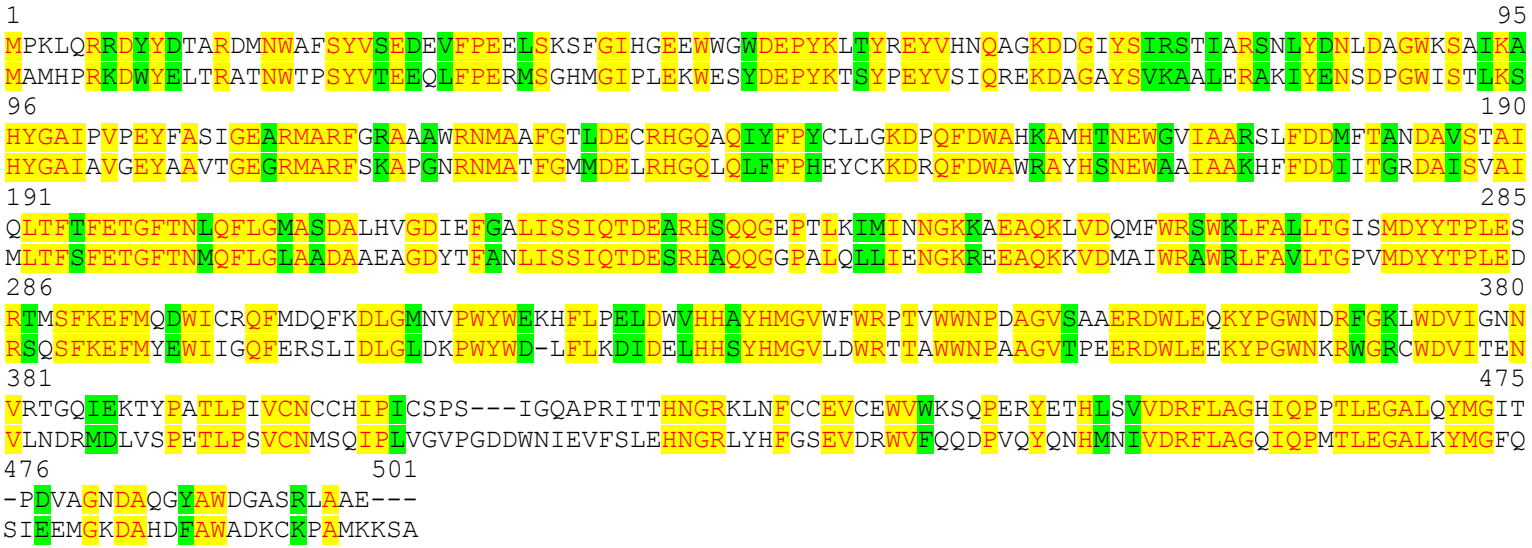


Figure 1.16 Comparison of α-subunit between BTAi1 putative of toluene monooxygenase site and T4MO. First and second lines represent BTAi1 and T4MO amino acid sequences, respectively.

TMO.BTAi1 and T*p*MO α-subunits

Identity positions: 54.7%

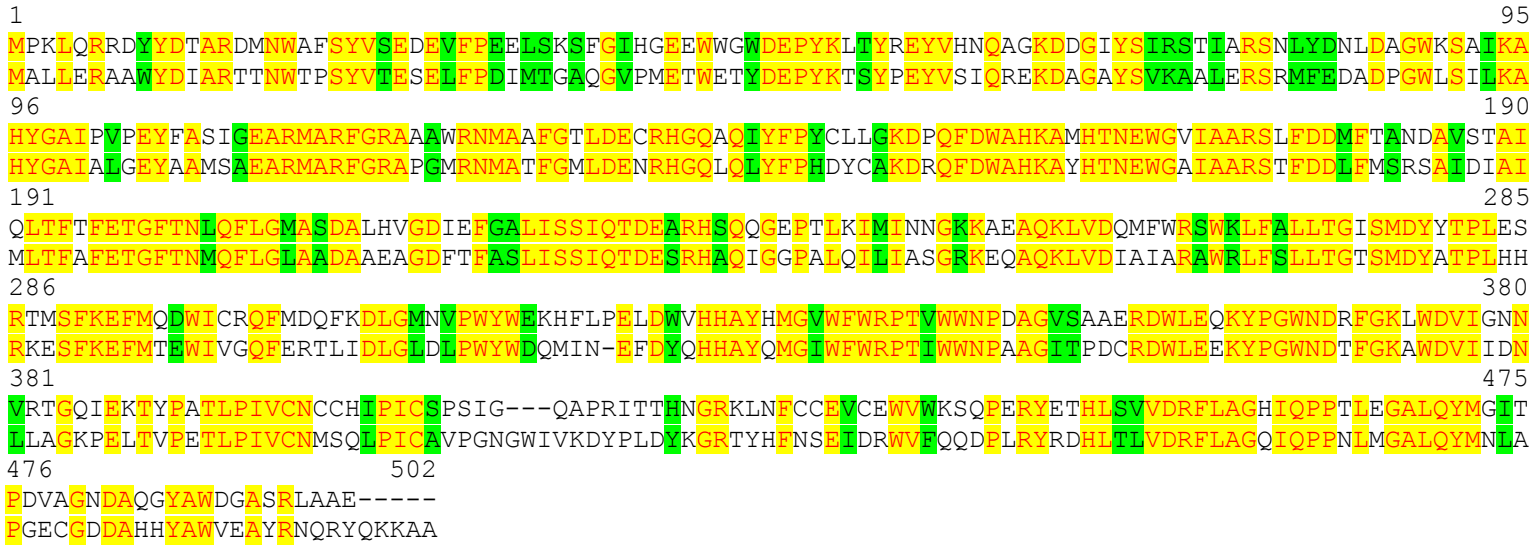


Figure 1.17 Comparison of α-subunit between BTAi1 putative of toluene monooxygenase site and T*p*MO. First and second lines represent BTAi1 and T*p*MO amino acid sequences, respectively.

TMO.BTAi1 and TOM α-subunits

Identity positions: 23.3%

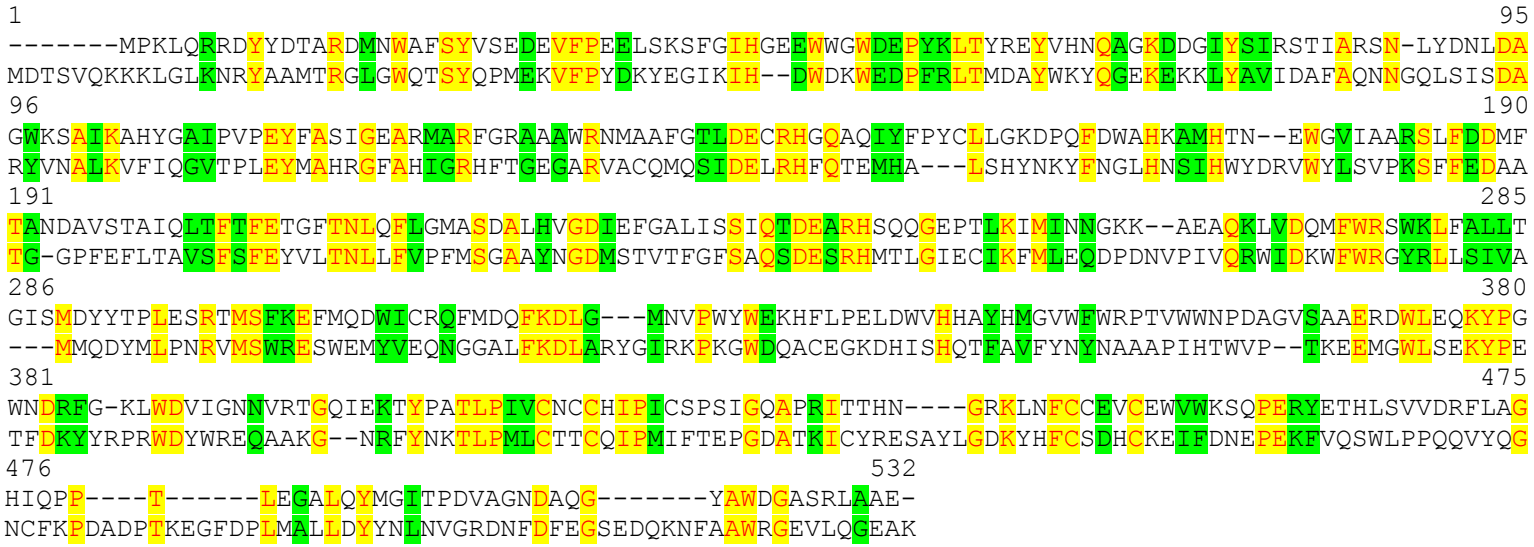


Figure 1.18 Comparison of α-subunit between BTAi1 putative of toluene monooxygenase site and TOM. First and second lines represent BTAi1 and TOM amino acid sequences, respectively.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and Enzymes

List of chemicals and enzymes are presented in Appendix A

2.1.2 Equipments

List of equipments are presented in Appendix B

2.1.3 Molecular Biology Kits

List of buffers and solutions are presented in Appendix C

2.1.4 Buffers and Solutions

List of molecular biology kits are presented in Appendix D

2.2 METHODS

2.2.1 Maintenance and Cultivation

Bradyrhizobium sp. BTAi1 was obtained from Prof. Michael Sadowsky, University of Minnesota, United States of America.

The culture was grown in a arabinose-glucose medium with HM salts medium with 0.1% yeast extract, 0.1% L-arabinose, and 0.1% glucose, instead of sodium gluconate (Cole and Elkan, 1973) at 28°C. HM included (grams per liter): Na_2HPO_4, 0.125; Na_2SO_4, 0.25; NH_4Cl, 0.32; $MgSO_4.7H_20$, 0.18; $FeCl_3$, 0.004; $CaCl_2.2H_20$, 0.013; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, 1.3; and 3-(N-

morpholino)propanesulfonic acid, 1.1. The pH was adjusted to 6.6 with 5 N NaOH prior to autoclaving.

pBS(Kan) plasmid was obtained from Prof. Thomas K. Wood, Texas A&M (*Patent* 20060051782). Luria-Bertani (LB) medium with 100 µg/ml kanamycin was used for routine growth of plasmid. Glycerol stock was prepared as 25%.

Escherichia coli strain TG1 was used as the host for gene cloning and expression. TG1 was cultivated in LB medium and 25% glycerol stocks were prepared, and then stored at -80°C.

2.2.2 Nucleic Acid Isolation Techniques

Total genomic DNA of BTAi1 was isolated by using GeneJet Genomic DNA Purification Kit with Gram Negative Bacteria Genomic DNA Purification Protocol.

pBSKan- plasmid was isolated by using Plasmid DNA Purification method of Nucleospin Plasmid Kit.

Quantification of DNA was measured with absorbance at 260 nm and 280 nm by using NanoDrop. The ratio of absorbance values of 260 and 280 nm was used for purity of DNA.

2.2.3 The Polymerase Chain Reaction

A polymerase chain reaction (PCR) was used for amplification of target gene from genomic DNA of BTAi1 Tmo.

Two oligonucleotide primers, KpnI front and BamHI rear, were designed by using Vector NTI Advance™ 11 (Invitrogen, USA) program and ordered from Elim Biopharmaceuticals, Inc. (Hayward, CA 94545). The designed primers have unique sites for KpnI and BamHI restriction enzymes (Table 1.2). Setting up a PCR with those designed primers provided additions of KpnI and BamHI restriction sites into target DNA, which is not found in the original genomic DNA. The amplification of target site was performed by using 30 pmole of each primer, 1 mM of $MgCl_2$, 200 µM of dNTPs, 1X *Pfu* Ultra II Reaction Buffer, 6 µl (optimal) QuickSolution, 2.5 U, *Taq* polymerase (NEB, UK) and 2.5 U, *Pfu* Ultra II polymerase and 20 ng of BTAi1 chromosomal DNA and sufficient dH_2O were added to make 100 µl of final volume. PCR conditions to

amplify target 4688 bp region from genomic DNA were, 2 minutes at 96ºC (first denaturing), 30 cycles included 45 seconds at 96ºC (denaturing), 45 seconds at 55ºC (annealing), 5 minutes at 72ºC (extension) and 10 minutes at 72ºC (final extension) (Table 2.2).

Table 2.1 Designed primers for amplification of target BTAi1 toluene monooxygenase site. Primers were designed with Vector NTI. KpnI site and BamHI site (shown in red with lines) were added into the designed primers.

Primer	Length bases	MW	GC%	Tm (°C)	Oligonucleotide Sequence
KpnI F	25	7822.3	68	72.78	5'- CAG CAG GGT ACC GGA GTT CGC CCG TTC-3'
BamHI R	28	8630.81	64.29	73.4	5´-GCA GCA GGA TCC TGT CGT CGA GCT GGA-3´

After running PCR program, samples were first hold at 4ºC then stored at -20ºC for storage. In the program *Taq* polymerase was added after first denaturing step and *Pfu* Ultra II polymerase was added at the beginning of the annealing step.

Ethanol participation protocol was applied to PCR product to obtain pure DNA. In this method, 1 volume from lower layer of TAE-saturated phenol/chloroform isoamylalcohol was added into the PCR product in a 1.5 ml microcentrifuge, mixture was vortexed and then centrifuged for 5 minutes at highest rpm in Microfuge.

Upper phase was pipetted carefully and transferred into a new 1.5 ml microcentrifuge tube. 1/10 volume of 3M NaOAc (pH 5.2) and 2 volume of 100% cold ethanol (EtOH) was added, respectively. Microcentrifuge tube was kept at -80ºC for 1 hour. Then it was centrifuged for 10 minutes at highest rpm in Microfuge. Supernatant was decanted quickly. 200 µl of cold 70% EtOH was added into the pellet, liquid was mixed via pipette. After 5 minutes and 1 minute centrifugations at highest rpm and

approximately 20 minutes vacuum dry (when no liquid remained in the tube) pellet was resuspended with 17 µl ddH_2O.

Table 2.2 PCR conditions for target BTAi1 Tmo site. Components are listed in the order of adding in a PCR tube.

PCR Mixture		**PCR Condition**		
Component	**Amount**	**Step**	**Temperature**	**Time**
dH2O	58 µl	Initial denaturation	96°C	2min
MgCl2	1 mM	Cycle 30x		
Primer mix	30 pmole each	denaturation	96°C	45sec
dNTPs	200 µM			
QuickSolution	6 µl	annealing	55°C	45sec
10x Buffer	10 µl			
Template DNA	20 ng	elongation	72°C	5min
Pfu Ultra II polymerase	2.5 U	Final elongation	72°C	10min
Taq polymerase	2.5 U			
Final volume	100 µl	Hold	4°C	

2.2.4 Restriction Enzyme Digestion of DNA

The amplified DNA was digested with BamHI (with 1x Buffer 3) for 4 hours at 37ºC in Dry Bath and then digested product was cleaned-up with PCR-Clean Up method of Nucleospin Extract II Kit, final volume was adjusted to 35 µl. The product was digested with KpnI (1x Buffer 1 was used), for 4 hours at 37ºC. Double digested product was loaded on 0.7% Agarose gel with 1x TAE buffer and Ethidium bromide (5 mg/ml) and run at 70V for 40 minutes. The gel was cut at 4688 bp under UV light and DNA was extracted by using Nucleospin Extract II Kit, final volume was adjusted to 35 µl.

Pure pBS(Kan) plasmid was digested both with BamHI and KpnI enzymes, respectively, including PCR clean-up step. Product was loaded on 0.7% Agarose gel and at 4076 bp the gel was cut and DNA was extracted, final volume was adjusted to 25 µl.

2.2.5 Ligation

Concentrations were measured by NanoDrop at 260 nm for both double digested pBS(Kan)Tmo.BTAi1 (insert) and digested pBSKan- (vector). Purity of samples was measured with dividing absorbance values of 260/230.

Ligation was set up according to following equation:

$$[vector] = (100ng\_insert) \times (\frac{1}{3}) \times (\frac{4076bp\_v}{4688bp\_i}) = 30ng \quad (2.1)$$

According to this equation the proportion of vector and insert was 3.3. The amount of insert and vector were loaded into 1.5 ml microcentrifuge tube by their ratio. T4 DNA Ligase and 1X Ligase Buffer were added next. Ligation mixture was incubated at 16ºC for overnight and 22ºC for 1 hour and then 65ºC for 20 minutes on Dry Bath.

2.2.6 Transformation of *E. coli* TG1 strain

TransformAid™ Bacterial Transformation Kit was preferred for cloning. Culture was spread on LB(30)Kan plate. For all cloning experiments *E. coli* TG1 was used. Four colonies were chosen randomly. Each colony was transferred to LB (30)Kan broth. Plasmid DNA of each four cultures was purified with Plasmid DNA Purification method of Nucleospin Plasmid Kit and tested for correct sizes with BamHI, XhoI, SacI and KpnI enzyme digestions for 2 hours at 37ºC. Then they were loaded into 1% agarose gel with 1x TAE buffer and Ethidium bromide (5 mg/ml), run at 70V for 40 minutes via Electrophoresis equipment.

Glycerol stocks of purified plasmids were prepared from their LB(30)Kan and LB(100)Kan overnight cultures at 37ºC shaker and stored at -80ºC.

Isopropyl-β-D-thiogalactopyranoside (IPTG) test was performed for two cultures incubated in LB(100)Kan broth by adding 1 mM IPTG into the laboratory tubes after three hours of incubation at 37°C shaker. Cultures were incubated at 37°C shaker for overnight.

2.2.7 DNA Sequencing

A dideoxy chain termination technique was applied to pBS(Kan)Tmo.BTAi1 products with M13 Front and M13 Rear primers by BIO BASIC Inc, Shanghai, China (Table 2.3). The sequencing data were analyzed with Vector NTI Advance™ 11 (Invitrogen, USA).

Table 2.3 Primers used for DNA sequencing of cloned pBS(Kan)Tmo.BTAi1.

Primer	Length Bases	MW	GC %	Tm (°C)	Oligonucleotide Sequence
M13R	19	5846.01	47.37	56	5'-GGA AAC AGC TAT GAC CAT G-3'
M13F	17	5228.58	52.94	52	5'-GTA AAA CGA CGG CCA GT-3'

2.2.8 Characterization of TMO

The cloned toluene monooxygenase characterization involves, first generating of a growth curve, then analysis for total protein and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and finally, whole cell analysis using reverse-phase High Performance Liquid Chromatography (HPLC).

2.2.8.1 Growth Curve of pBS(Kan)Tmo.BTAi1 *Expressed in E. coli TG1 Host*

Optical density measurements were done for fresh exponential growth pBS(Kan)Tmo.BTAi1 in LB(100)Kan broth with less than 1% inoculation of its overnight culture, at 600 nm, via Spectrophotometer (6300 Jenway, UK). Above 1.0

value, OD_{600} was quantified by 1:5 dilutions in cuvette, which is performed by mixing 800 µl of LB (blank) and 200 µl of fresh exponential growth culture.

2.2.8.2 Total Protein Analysis

From pBS(Kan)ToMO (ToMO) and pBS(Kan)Tmo.BTAi1 glycerol stocks, LB(100)Kan plates were prepared and incubated at 37°C incubator, overnight. Single colonies were inoculated into LB(100)Kan broth, respectively. When OD_{600} was equal to 2.50, cultures were taken and centrifuged for 10 minutes at 6000 rpm, at 4°C. For taking of remained supernatants, a dry cleaning was done for 2 minutes with same conditions. Pellets were dissolved in 1/10 Tris-EDTA (TE) buffer (pH 7.8) and OD_{600} contacts were measured by spectrophotometer. Then samples were sonicated with ultrasonic homogenizer for 30 seconds with 1 pulsation, for ten times at 100% amplitude. Between two sonication step, samples were rested for 3 minutes on ice. Both samples were centrifuged for 20 minutes, at 9500 rpm, at 4°C. Supernatants were taken into new 1.5 ml microcentrifuge tube for each sample.

Bradford assay was performed via Bradford Assay Kit for ToMO for three times by making measurements on spectrophotometer and Shimadzu UV-Vis 7100. Also, Protein Quantification Assay was applied with three different cultures of ToMO and pBS(Kan)Tmo.BTAi1 to measure total protein concentrations by spectrophotometer.

2.2.8.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

pBS(Kan), pBS(Kan)Tmo.BTAi1 and pBSKanToMO were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook et al, 1989). Strains were streaked from their glycerol stocks to LB(100)Kan plates. Single colonies from each were transferred into LB(100)Kan broth then tubes were incubated at 37°C shaker for overnight. From each overnight culture, less than 1% was inoculated into flasks with fresh LB (100)Kan broth, respectively; from pBS(Kan)Tmo.BTAi1 and ToMO cultures two for each were inoculated and then incubated at 37°C shaker. 1 mM IPTG was added into one of the flasks for both pBS(Kan)Tmo.BTAi1 and ToMO cultures at OD_{600} was equal to 0.6. When OD_{600} was reached to 2.42, bacterial cells were transferred to falcon tubes and centrifuged for 10 minutes at 6000 rpm at 4°C. Supernatant was decanted. Cells were washed with 1 volume of Tris-EDTA buffer (pH 7.8), centrifuged for 10 minutes at 6000 rpm at 4°C. Supernatant was discarded. Cell pellets were suspended in

1/10 volume of TE buffer. 2 volume of Protein Solving Buffer (PSB) taken from Protein Quantification Assay, of suspended cells was added into each five falcon. Samples were heated at 100°C boiling water in a large flask for 10 minutes, and then they were immediately placed on ice.

Table 2.4 Adding reagents are listed in the order for SDS-PAGE experiment.

	SDS-Polyacrylamide Gel Electrophoresis	
Reagent	**12% Resolving**	**5% Stacking**
ddH2O	5 ml	4.1 ml
30% Acrylamide/Bis	6 ml	1 ml
Tris Buffer	3.8 ml (1.5 M Tris-HCl, pH 8.8)	0.75 ml (1.0 M Tris-HCl, pH 6.8)
10% SDS	0.15 ml	0.06 ml
10% APS	0.15 ml	0.06 ml
TEMED	0.015 ml	0.006 ml
TOTAL	15 ml	6 ml

Gel was set up in vertical electrophoresis system (Table 2.4). Glass plates with integrated spacers were held together with casting frames. 12% Resolving gel was prepared and loaded into the space between glass plates. Isopropanol was added immediately via pipette to remove bubbles. Gel was waited for 1 hour to be frozen. 5% Stacking gel was loaded from the top of Resolving gel. Comb was then placed onto the Stacking gel at once. When gels were solidified, comb was taken off. In the order, 10 µl of ladder (Pierce), 15 µl of pBS(Kan-), pBS(Kan)Tmo.BTAi1, pBS(Kan)Tmo.BTAi1, pBS(Kan)Tmo.BTAi1 IPTG induced, pBS(Kan)Tmo.BTAi1 IPTG induced, ToMO, ToMO IPTG induced were loaded into wells, in the order. The glass with gel was placed in the apparatus, and gel was run at 90 V in the Stacking gel, and 180 V in the Resolving gel. Electrophoresis was stopped after 6 hours when blue bands were not seen. Gel was removed from the glass plates, stacking gel part was cut and remained gel

was moved into a small box. Coomassie Blue Staining solution was added onto gel, waited for 1 hour on orbital shaker (WiseMix, Korea). Gel was destained with Destaining Solution. In every 20 minutes, solution was replaced with new one. Then, gel was left for destaining, overnight. A day after, gel photo was taken.

2.2.8.4. High Performance Liquid Chromatography (HPLC) Product Analysis

pBS(Kan)Tmo.BTAi1 was streaked to LB(100)Kan plate from its glycerol stock, and incubated at 37°C incubator, overnight. The next day, from single colonies LB(100)Kan cultures were prepared and incubated at 250 rpm at 37°C shaker, overnight. At the day of characterization, less than 1% of culture was inoculated into LB(100)Kan in a flask. At OD_{600} was equal to 2.5, culture was centrifuged and washed with Tris-HNO_3 buffer (pH 7.0) for 5 minutes, at 6500 rpm, at 25°C. Then pellet was dissolved in Tris-HNO_3 buffer (pH 7.0). Product formation from toluene, benzene, phenol, nitrobenzene, *p*-nitrophenol and naphthalene were measured using reverse-phase HPLC.

2.2.8.4.1 Toluene

The cells dissolved in Tris-HNO_3 buffer (pH 7.0) were subjected to 0.250 mM toluene (91 μM according to Henry's Law constant) for several minutes at 250 rpm at 25°C. HPLC analysis was done with 80:20 water:methanol, Astec Cyclobond I2000 Column (SupelCo Analytical, USA).

2.2.8.4.2 Benzene

The cells dissolved in Tris-HNO_3 buffer (pH 7.0) were subjected to 0.800 mM (320 μM according to Henry's Law constant) benzene for several minutes at 250 rpm at 25°C. HPLC analysis was done with 90:10 water:acetonitrile, Inertsil C8-3 Column (GL Sciences Inc., Japan).

2.2.8.4.3 Phenol

The cells dissolved in Tris-HNO_3 buffer (pH 7.0) were subjected to 0.800 mM phenol for several minutes at 250 rpm at 25°C. HPLC analysis was done with 90:10 water:acetonitrile, Inertsil C8-3 Column (GL Sciences Inc., Japan).

2.2.8.4.4 Nitrobenzene

The cells dissolved in Tris-HNO_3 buffer (pH 7.0) were subjected to 0.200 mM nitrobenzene for several minutes at 250 rpm at 25°C. HPLC analysis was done with 70:30 water:acetonitrile, Nucleosil C18 Column (Agilent, USA).

2.2.8.4.5 p-Nitrophenol

The cells dissolved in Tris-HNO_3 buffer (pH 7.0) were subjected to 0.500 mM *p*-nitrophenol for several minutes at 250 rpm at 25°C. HPLC analysis was done with 70:30 water:acetonitrile, Nucleosil C18 Column (Agilent, USA).

2.2.8.4.6 Naphthalene

The cells dissolved in Tris-HNO_3 buffer (pH 7.0) were subjected to 5 mM naphthalene (0.27 mM according to Henry's Law constant) for several minutes at 250 rpm at 25°C. HPLC analysis was done with 70:30 water:acetonitrile, Nucleosil C18 Column (Agilent, USA).

2.2.8.5 Chloride Assay

Chloride assay was applied to determine trichloroethylene (TCE) degradation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1 cells. TCE degradation produces Cl^- ions. Two reagents were used: 0.25 M Ferric ammonium sulfate and Mercury (II) thiocynate. The assay is based on a chemical reaction:

$$2\ Cl^- + Hg(SCN)_2 + 2\ Fe^{3+} \leftrightarrow\ HgCl_2 + 2\ Fe(SCN)^{2+}$$

$Fe(SCN)^{2+}$ has an orange color and maximum absorbance at 460 nm was measured via spectrophotometer.

The assay was performed as follows: pBS(Kan)Tmo.BTAi1 was streaked to LB(100)Kan plate from its glycerol stock, and incubated at 37°C incubator, overnight. After a day, from single colonies LB(100)Kan liquid cultures were prepared from a single colony and incubated at 250 rpm at 37°C shaker, overnight. At the day of characterization, less than 1% of culture was inoculated into LB(100)Kan in a flask. At OD_{600} was equal to 2.5, culture was centrifuged and washed three times with Tris-HNO_3 buffer (pH 7.0) for 5 minutes, at 6500 rpm, at 25°C. Then pellet was dissolved in Tris-HNO_3 buffer (pH 7.0). OD_{600}contact was measured via spectrophotometer.

2.5 ml of cells were distributed into 10 ml serum vials that were closed with 20 mm crimp cap aluminum. Into first three vials, 0.200 mM DMF and into other three vials, 0.200 mM TCE solution dissolved in DMF were injected micro syringe. Vials were incubated at 250 rpm at 37°C for overnight.

Caps were opened in the fume hood. Cells were killed by putting vials into hot water (>90°C) for 90 seconds. After 15 minutes of waiting, 1 ml cells were loaded into microcentrifuge tubes, centrifuged for 4 minutes at 13000 rpm. 500 µl of supernatant was taken and examined for chloride assay.

Into 500 µl of supernatant, first 100 µl Ferric ammonium sulfate reagent and then 100 µl Mercury (II) thiocyanate reagent were added. Microcentrifuge tubes were made vortex and left at room temperature for 5 minutes. Absorbance at 460 nm was measured with spectrophotometer in cuvettes.

Standard measurements were performed by using NaCl solutions with different concentrations dissolved in Tris-HNO_3 buffer (pH 7.0); 10 µM, 25 µM, 50 µM, 100 µM, 125 µM, 250 µM.

2.2.8.6 Initial Formation Rate and Initial Degradation Rate Calculations

To calculate initial degradation or formation rates, following equation was used:

$$\sum_{i=1}^{n} \chi i \pm \frac{\sqrt{\frac{1}{n}\sum_{i}^{n}(\chi i - \bar{\chi})}}{\sqrt{n}} \qquad (2.2)$$

All calculations were done with *Microsoft Office Excel 2007* program as the average of all values plus/minus standard deviation over square root of number of values.

CHAPTER 3

RESULTS

3.1 MOLECULAR CLONING OF TOLUENE MONOOXYGENASE FROM *Bradyrhizobium* sp. BTAi1 INTO pBS(Kan) PLASMID

3.1.1 Molecular Cloning

Bradyrhizobium sp. BTAi1, which was obtained from Prof. M. Sadowsky, was first cultured in a arabinose-glucose medium containing HM salts.

Whole genome of *Bradyrhizobium* sp. BTAi1 was first analyzed on Vector NTI Advance 11 Software for its putative toluene monooxygenase site. The primers were designed according to the desired properties and two restriction sites; KpnI and BamHI were included. They contained approximately 60% GC content which provide good annealing.

A polymerase chain reaction (PCR) was needed to amplify 4668 bp region which corresponds to the putative toluene monooxygenase gene cluster from the genome of BTAi1 containing 8 billion-DNA base pair. After spending couple of months to optimize the PCR conditions such as different annealing temperatures, time, polymerase and even different thermal cyclers, the amplification was achieved. The best efficiency of PCR product was obtained with a program consisting of 2 minutes at 96°C, 30 cycles of 45 seconds at 96°C, 45 seconds at 55°C and 5 minutes at 72°C with a final extension at 72°C for 10 minutes. At the end, a large size of 4.7 kb PCR DNA containing the toluene monooxygenase gene cluster of BTAi1 was obtained. The purity of product was important for the efficiency of cloning. For this reason, ethanol participation method was used, and pure DNA was obtained with high efficiency.

Digestions for both PCR product and pBS(Kan) plasmid were done in two steps; because KpnI and BamHI digestions requires different reaction buffers. This may cause loss of product, which was prevented by setting up more than one digestion mixture for each PCR product and pBS(Kan) plasmid.

TransformAid™ Bacterial Transformation Kit, a chemical method, was chosen for transformation which is suitable for *E. coli* cells. From the obtained 30 colonies, only the one with correct clone was needed and searched.

The putative toluene monooxygenase site from BTAi1 was cloned into pBS(Kan) vector and the constructed plasmid was transformed into *E. coli* TG1 host. Randomly chosen four colonies obtained from transformation were analyzed for their possibility of carrying the correct plasmid. For this purpose, three procedures were followed. These are restriction enzyme digestions, IPTG induction and DNA sequencing.

These randomly chosen four colonies were first transferred into LB(30)Kan and incubated at 37°C shaker, overnight. After 24 hours, only two overnight cultures formed indigo which gives the blue color, but one of them produced more indigo which is the indication of toluene monooxygenase expression and activity by the gene belonging to BTAi1. After the correction of right clone, the following studies were done with the clone producing more blue color.

3.1.2 Restriction Enzyme Digestion

Four restriction enzymes were chosen for the digestion of purified constructed plasmid. These are BamHI, XhoI, SacI and KpnI. These enzymes were chosen according to their location on the plasmid (Figure 3.1). BamHI restriction site is placed at the end of *tmo*F gene, XhoI restriction sites are in the *tmo*A gene of BTAi1 and Kan^R, kanamycin resistance gene in pBS(Kan) site. SacI has four restriction sites which locate in between *tmo* genes and pBS(Kan) vector; in *tmo*A and *tmo*E genes. Finally KpnI restriction site is found at the beginning of *tmoA* gene where lac promoter ends.

Enzyme digestions were observed on 1% Agarose gel with 1x TAE buffer and 5 mg/ml Ethidium bromide under the UV light. The expected sizes were analyzed with Vector NTI Advance™ 11 (Invitrogen, USA) and were listed in Table 3.1.

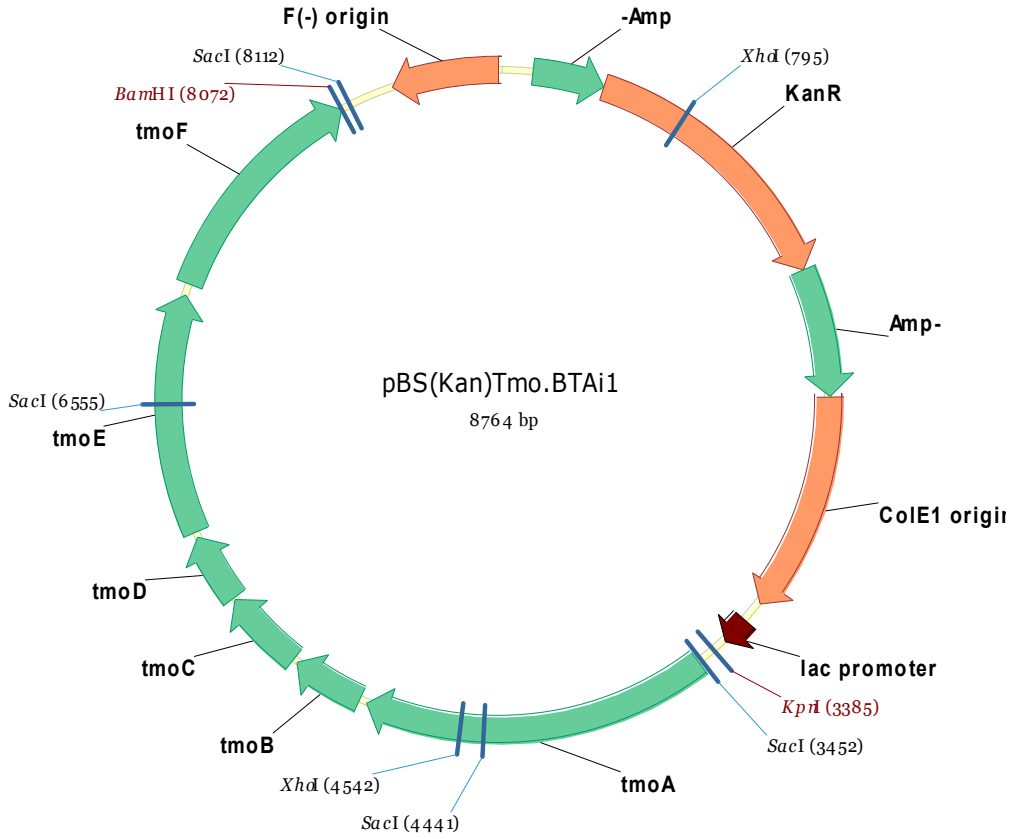


Figure 3.1 Constructed plasmid, Vector NTI Advance™ 11 (Invitrogen, USA). *tmo*ABCDEF are genes originated from putative toluene monooxygenase site of BTAi1 genomic DNA. Six genes coding are *tmo*AEB (hydroxylase), *tmo*C (ferrodoxin), *tmo*D (mediating protein), and *tmo*F (NADH-ferrodoxin oxidoreductase). Kan^R, kanamycin resistance gene and lac promoter are included in pBS(Kan) vector. Lac promoter provides constitutive expression of toluene monooxygenase genes. The figure also shows restriction sites for KpnI, BamHI, XhoI and SacI endonucleases.

Table 3.1 Expected band sizes (bp) after restriction enzyme digestions.

Restriction Enzyme	Expected Band Number	Expected Sizes (bp)
XhoI	2	5016
		3666
SacI	4	989
		1557
		2114
		4104
KpnI	1	8764
BamHI	1	8764

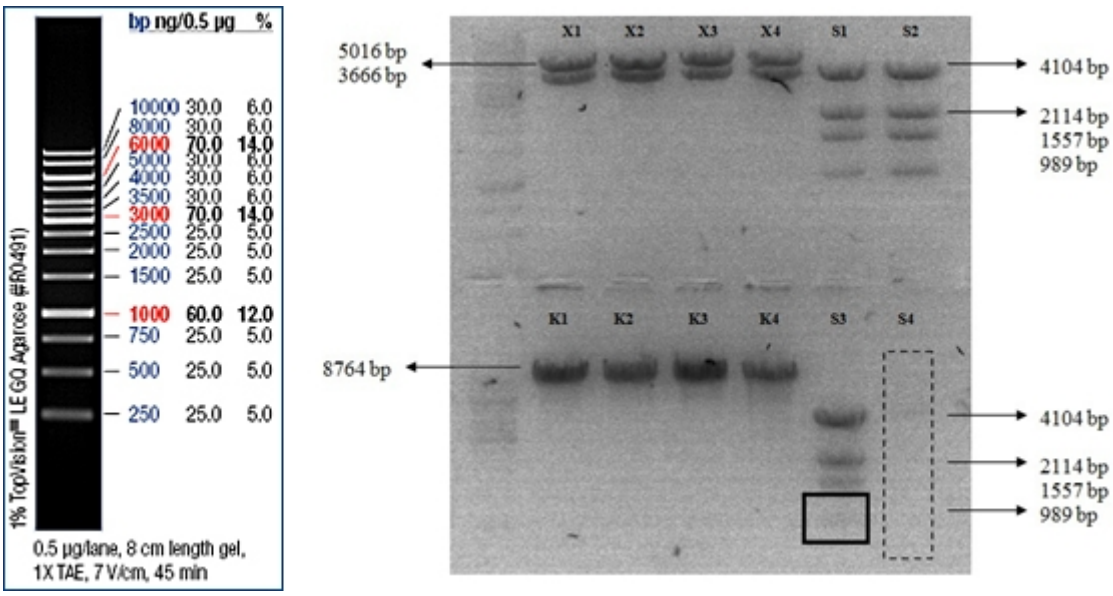


Figure 3.2 Gel electrophoresis result of restriction enzyme digestions for constructed plasmid. Expected bands with expected sizes were obtained for XhoI (X), SacI (S), KpnI (K) for four isolated plasmids. The band at 989 bp were actually there four S3 (SacI digested plasmid #3) but it was not clear in the photo. This is same with S4 result, which was the result of low concentration of DNA.

The result of agarose gel electrophoresis confirmed the expected sizes for restriction enzyme digestions (Figure 3.2).

3.1.3 IPTG Induction

E. coli TG1 strain with toluene monooxygenase from BTAi1 constructed plasmid produced more blue color with the induction of 1 mM IPTG which enhances the indigo formation by inducing the lac promoter. The blue color is the indication of indigo formation which is the product of tryptophan oxidation (Ensley et al, 1983). *E. coli* TG1 host expresses tryptophanase that degrades trypthophan into indole which is used by toluene monooxygenase as a substrate and oxidized into indigo (Figure 3.3).

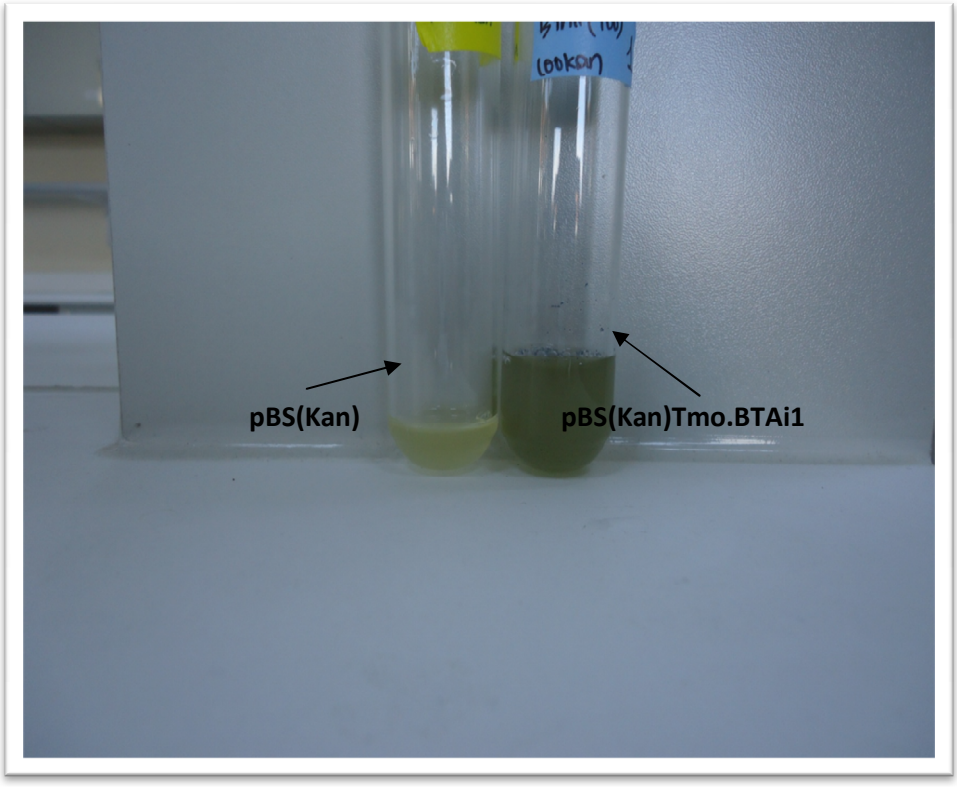


Figure 3.3 Indigo formation shows the expression of toluene monoocygenase from BTAi1. pBS(Kan) plasmid expressed in *E. coli* TG1 host (left) and pBS(Kan)Tmo.BTAi1 in *E. coli* TG1 host (right). pBS(Kan)Tmo.BTAi1 expression produces indigo which has a blue color.

3.1.3 DNA Sequencing

A dideoxy chain termination technique was used to determine the nucleotide sequence of pBS(Kan)Tmo.BTAi1 in *E. coli* TG1 host. M13 Rear primer was used to sequence the beginning of the BTAi1 Tmo region which includes the KpnI restriction site. M13 Front primer read the sequences from the last part of BTAi1 Tmo region and containes BamHI restriction site. The reason of sequencing with two primers, a forward and a reverse proves the gene cluster of BTAi1 Tmo is successfully cloned into pBS(Kan).

The results showed that constructed plasmid includes a KpnI and BamHI sites, which were added with the polymerase chain reaction, with designed primers, and [ATG] site that is known to be encoder nucleotides of methionine, the first amino acid encoded with protein expression (Figure 3.4-3.10)

By restriction enzyme digestions, indigo formation and DNA sequencing, the cloned plasmid is proved to be pBS(Kan)Tmo.BTAi1.

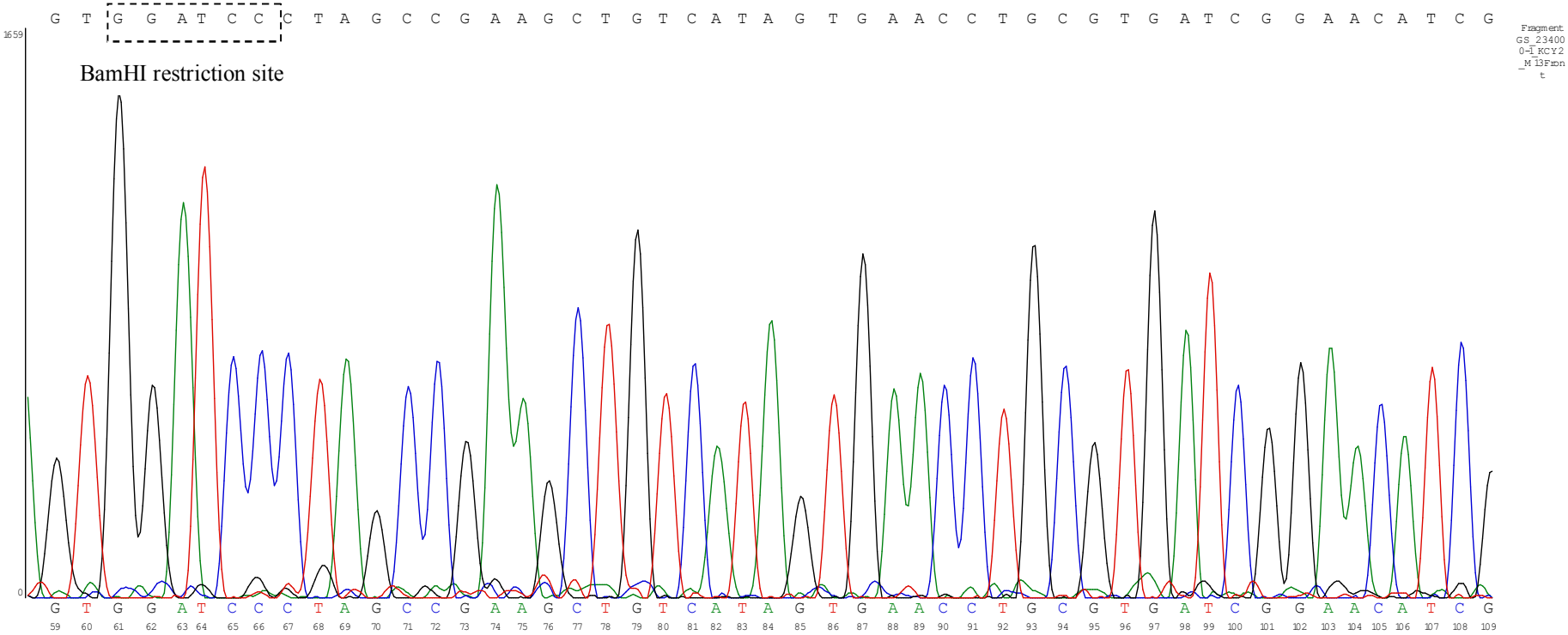


Figure 3.4 DNA sequencing results using M13F primer and pBS(Kan)Tmo.BTAi1 plasmid DNA. Chromatogram data includes 49 bases, showing BamHI restriction site which is located at the end of *tmoF* site. Read length: 836 bp (Vector NTI Advance™ 11).

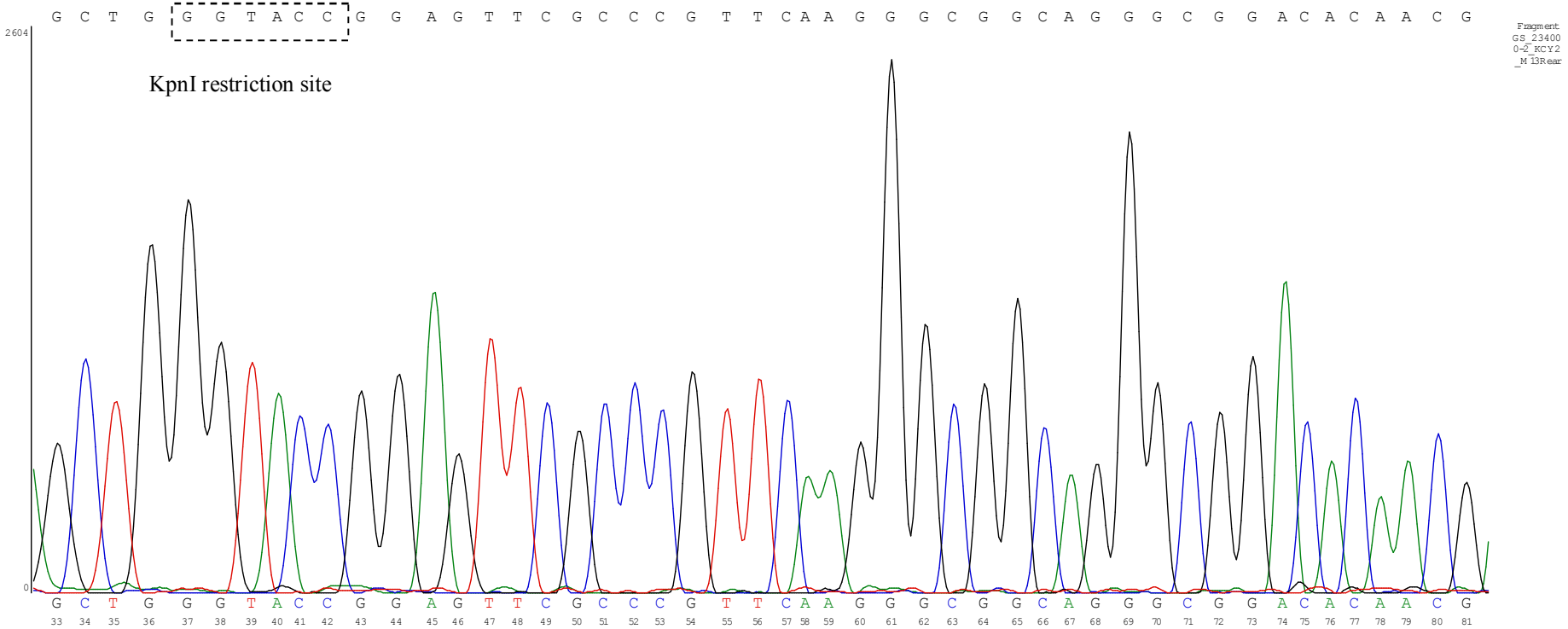


Figure 3.5 DNA sequencing results using M13R primer and pBS(Kan)Tmo.BTAi1 plasmid DNA. Chromatogram data includes 49 bases, showing KpnI restriction site which is located at the beginning of *tmo*A site. Read length: 1239 bp (Vector NTI Advance™ 11).

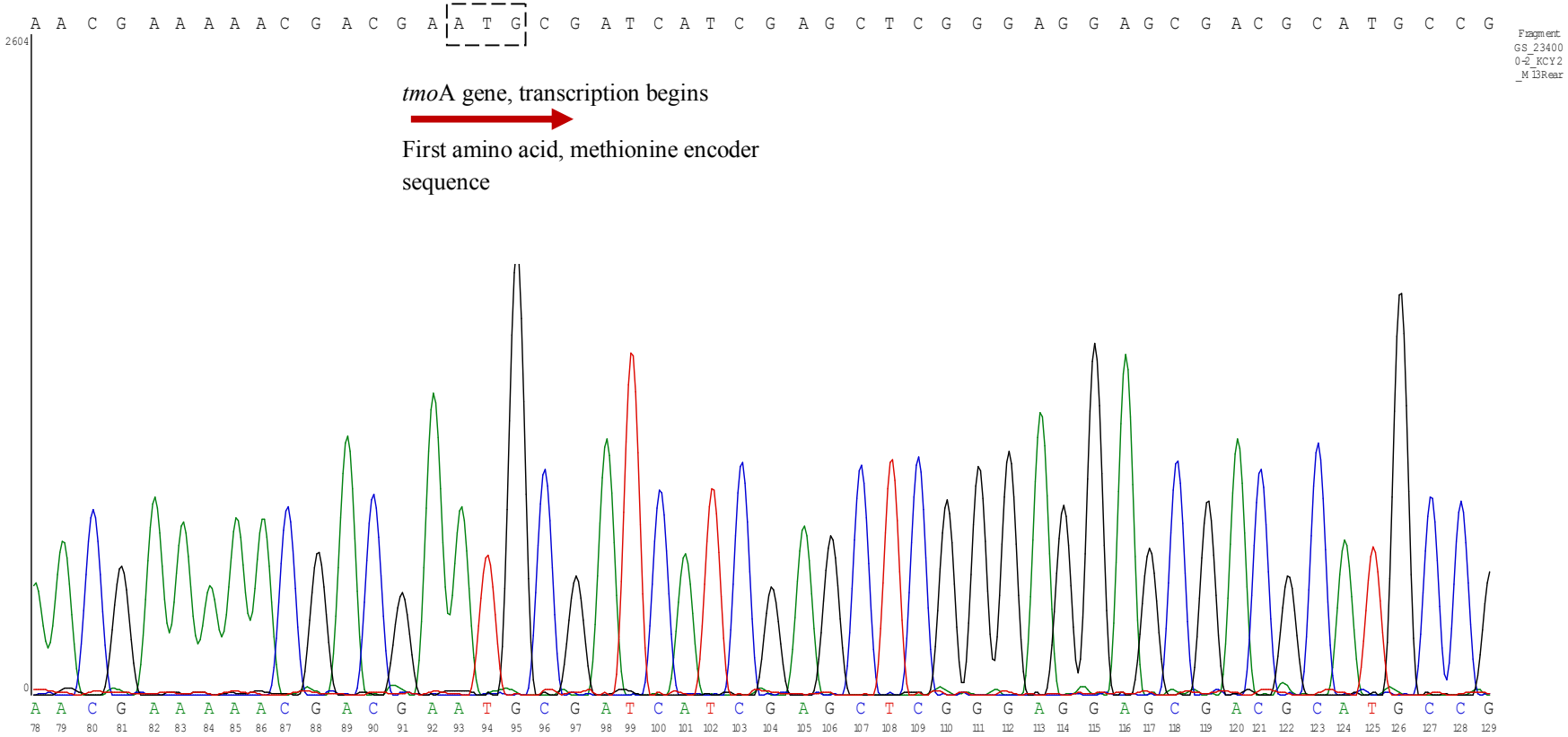


Figure 3.6 DNA sequencing results using M13R primer and pBS(Kan)Tmo.BTAi1 plasmid DNA. Chromatogram data includes 52 bases, showing the first amino acid of the *tmo*A gene. Read length: 1239 bp (Vector NTI Advance™ 11).

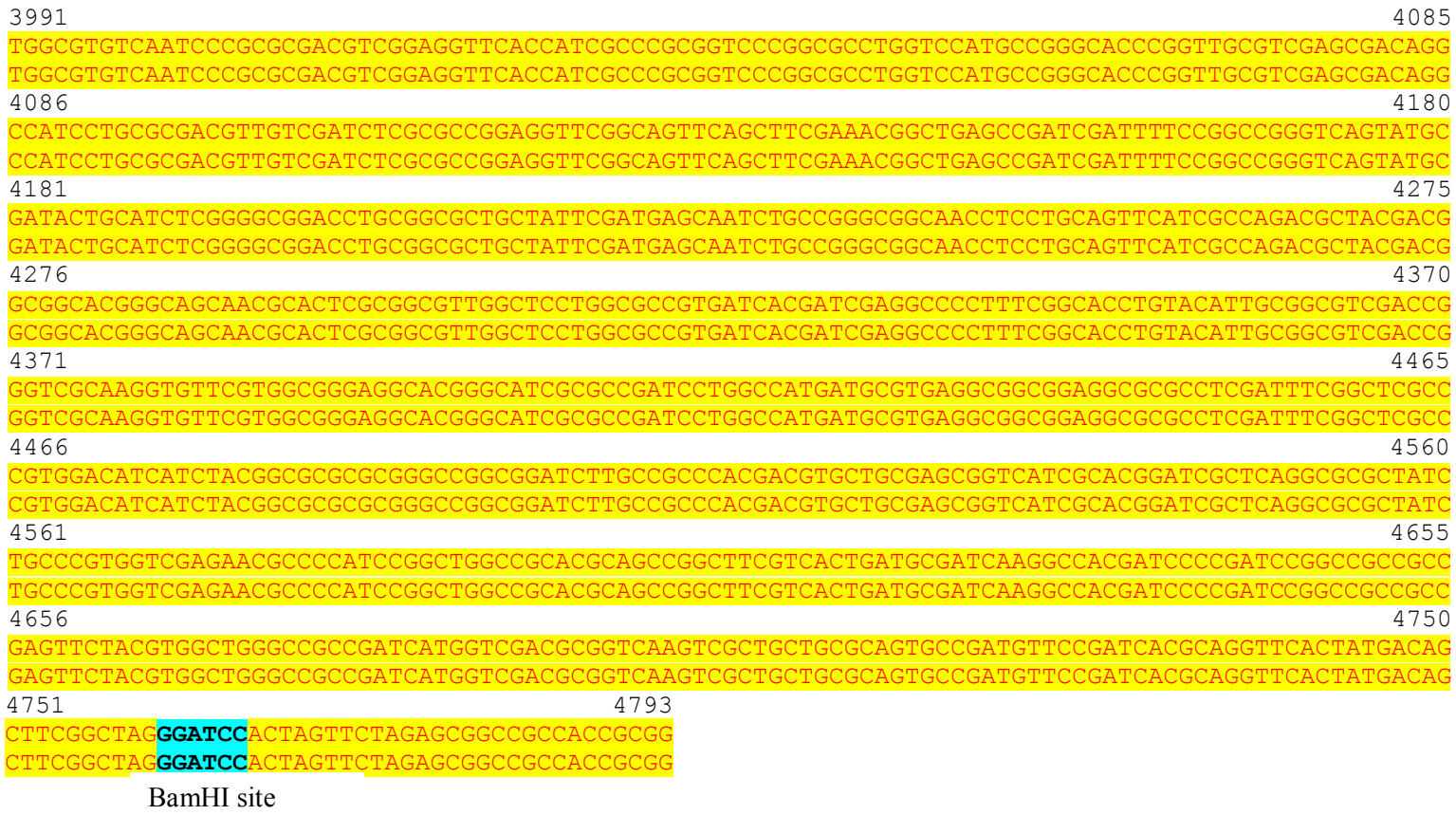


Figure 3.7 DNA sequence base pair comparison of sequenced site with M13F primer and proposed sequence site of pBS(Kan)Tmo.BTAi1 including BamHI restriction site, respectively (Vector NTI Advance™ 11).

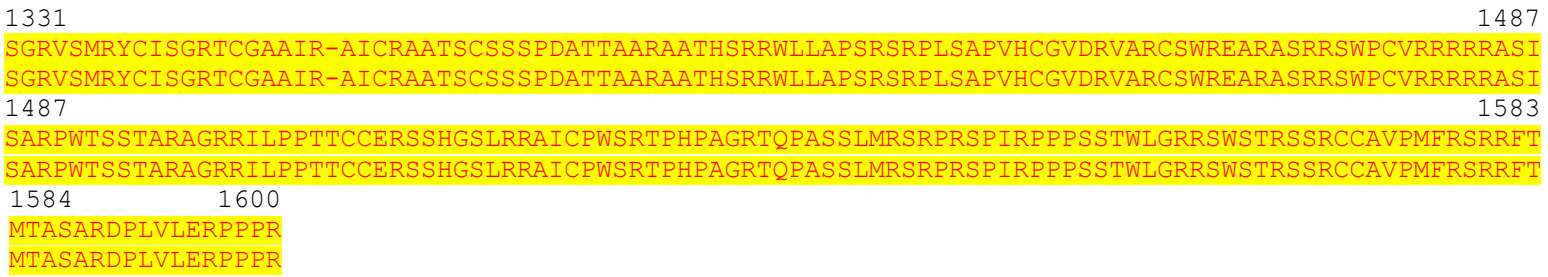


Figure 3.8 Amino acid sequence comparison of sequenced site with M13F primer and proposed sequence site of pBS(Kan)Tmo.BTAi1, respectively (Vector NTI Advance ™ 11).

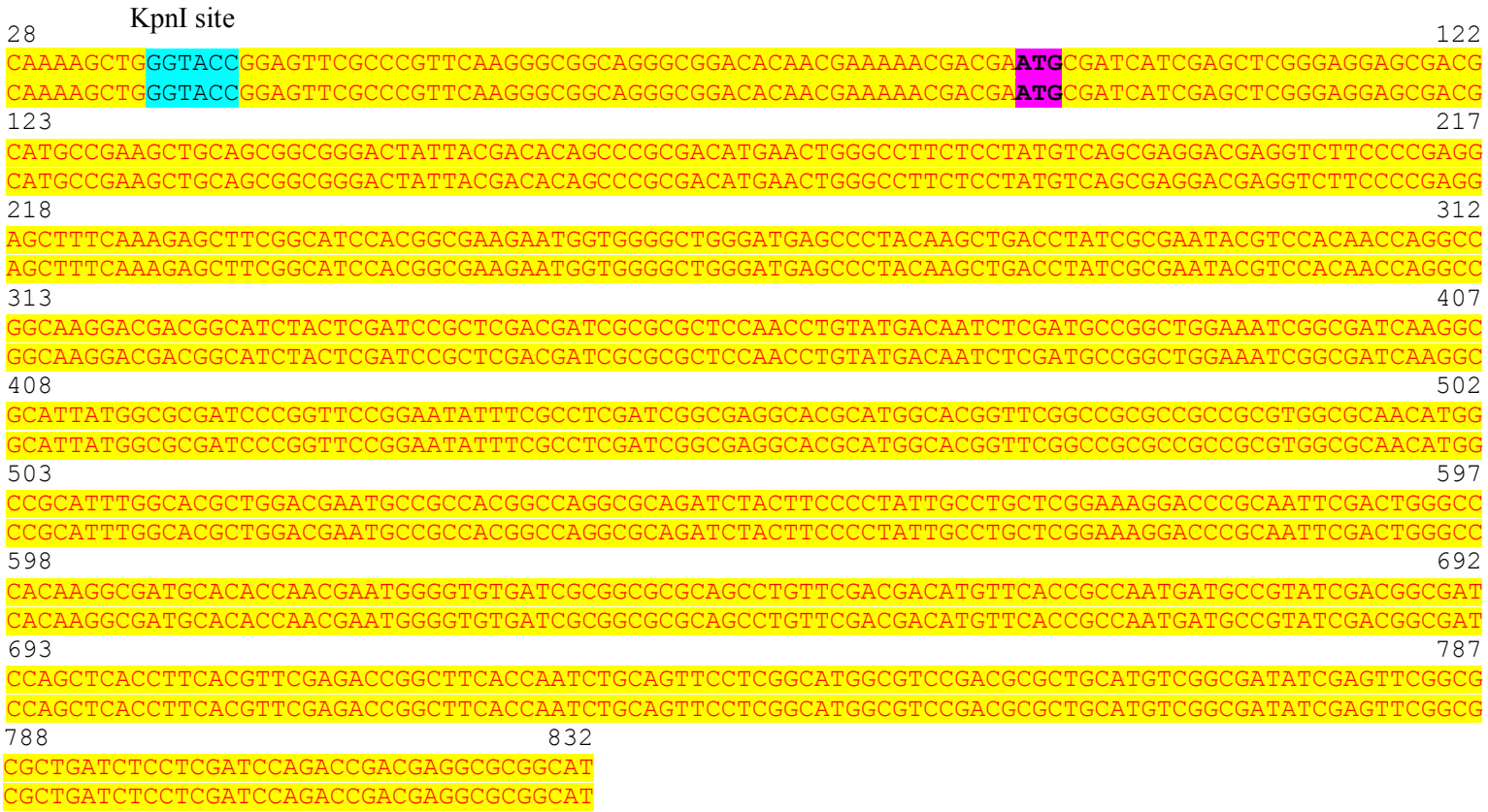


Figure 3.9 DNA sequence base pair comparison of sequenced site with M13R primer and proposed sequence site of pBS(Kan)Tmo.BTAi1 in the order; including KpnI restriction site and ATG sequences (Vector NTI Advance (TM) 11).

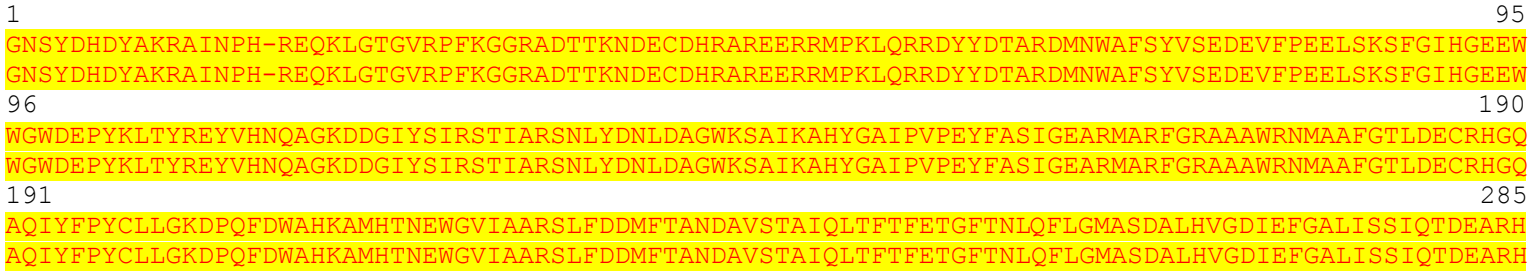


Figure 3.10 Amino acid sequence comparison of sequenced site with M13F primer and proposed sequence site of pBS(Kan)Tmo.BTAi1, respectively (Vector NTI Advance™ 11).

3.2 CHARACTERIZATION OF TOLUENE MONOOXYGENASE FROM *Bradyrhizobium* sp. BTAi1 EXPRESSED IN *E. coli* TG1 HOST

3.2.1 Growth Curve of *E. coli* TG1/pBS(Kan)Tmo.BTAi1

Optical density at 600 nm (OD_{600}) measurements were done with *E. coli* TG1/pBS(Kan)Tmo.BTAi1, via Spectrophotometer (6300 Jenway, UK). Above OD_{600}=1.0 value, measurements were done by 1:5 dilutions. The result of growth curve at 600 nm is shown in Figure 3.11.

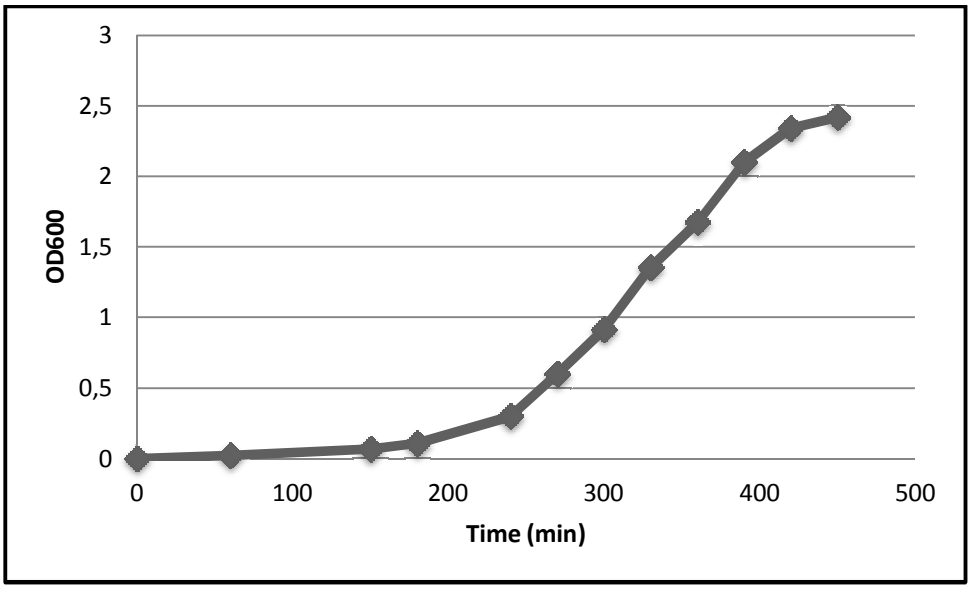


Figure 3.11 Growth curve of *E. coli* TG1/pBS(Kan)Tmo.BTAi1. Spectrophotometer results were taken at 600 nm by 6300 Jenway, UK.

Lag phase of *E. coli* TG1/ pBS(Kan)Tmo.BTAi1 which is the time for adaptation of bacteria into new growth conditions lasts about 4 hours. Cell doubling is happening in exponential (log) phase for approximately 3.5 hours. The mature bacterial cells are obtained at stationary phase where growth slows and OD_{600} values become constant, and cells are ready for substrate incubations.

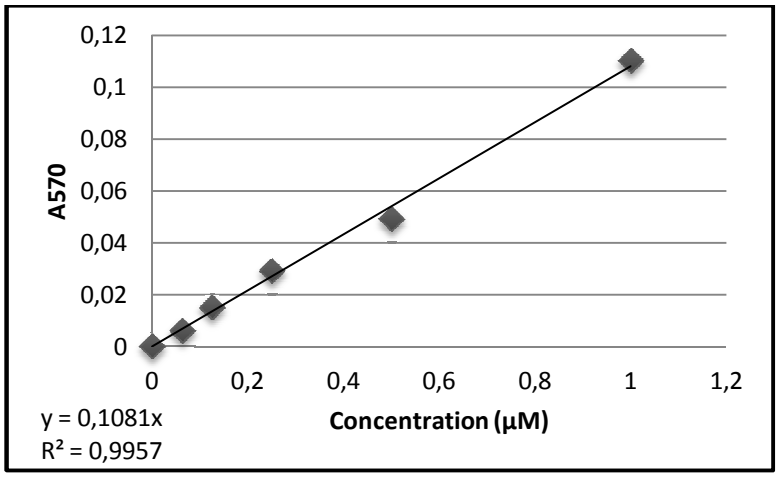
By evaluating these results, fresh exponential culture grew until OD_{600} reaches to 0.484 with 1:5 ratios which is actually 2.42 (Table 3.2). For all following experiments, this OD_{600} values were considered.

Table 3.2 OD_{600} measurements of *E. coli* TG1/pBS(Kan)Tmo.BTAi1 over time.

Time (min)	OD600	OD600 (1:5)
0	0	
60	0.023	
150	0.067	
180	0.106	
240	0.299	
270	0.600	
300	0.915	
330	1.355	
360	1.675	0.355
390	2.100	0.420
410	2.340	0.468
440	2.420	0.484

3.2.2 Total Protein Analysis

Total Protein Assay was done for several times by using Spectrophotometer (Jenway 6300), UV-Vis 3600 Spectrophotometer (Schimadzu), and NanoDrop (Thermo Scientific) for confirmation (Figure 3.12).



Concentration of BSA (µM)	A_{570}
0	0
0.063	0.006
0.125	0.015
0.250	0.029
0.500	0.049
1.000	0.110

Figure 3.12 Standard curve of BSA solutions at 570 nm by Spectrophotometer (Jenway, 6300). Data was obtained from Protein Quantification Assay Method.

Average of *E. coli* TG1/pBSKan(Tmo).BTAi1 absorbance at 570 nm (A_{570}) values was measured and applied to the data y=0.1081x; here y is the absorbance value and x is the concentration of protein (mg/ml). Result was found to be 2.478 mg protein/ml. The total concentration of protein is equal to 0.122 mg protein/ml OD and this value has been used for all the data calculations in this study.

According to spectrophotometer Jenway 6300:

OD_{600}: 2.47

$ODc_{600:}$ 20.3

$$\frac{2.478\ mg\ protein}{ml \times 20.3\ OD} = 0.122\ mg\ protein/ml.OD \qquad (3.1)$$

3.2.3 SDS-PAGE

The product of each *tmo* genes were analyzed and compared with ToMO by SDS-PAGE. Molecular masses of TouA, TouE and TouF were estimated at 55 kDa, 38 kDa and 37 kDa, respectively. TouB, TouC and TouD were not seen because of their molecular weights being lower than 15 kDa. 1 mM IPTG induction of lac promoter increased the thickness of protein band which means the expression of this protein is higher. IPTG induction increases the expression level of toluene monooxygenase which also enhances the enzyme activity (Figure 3.13 and Table 3.3).

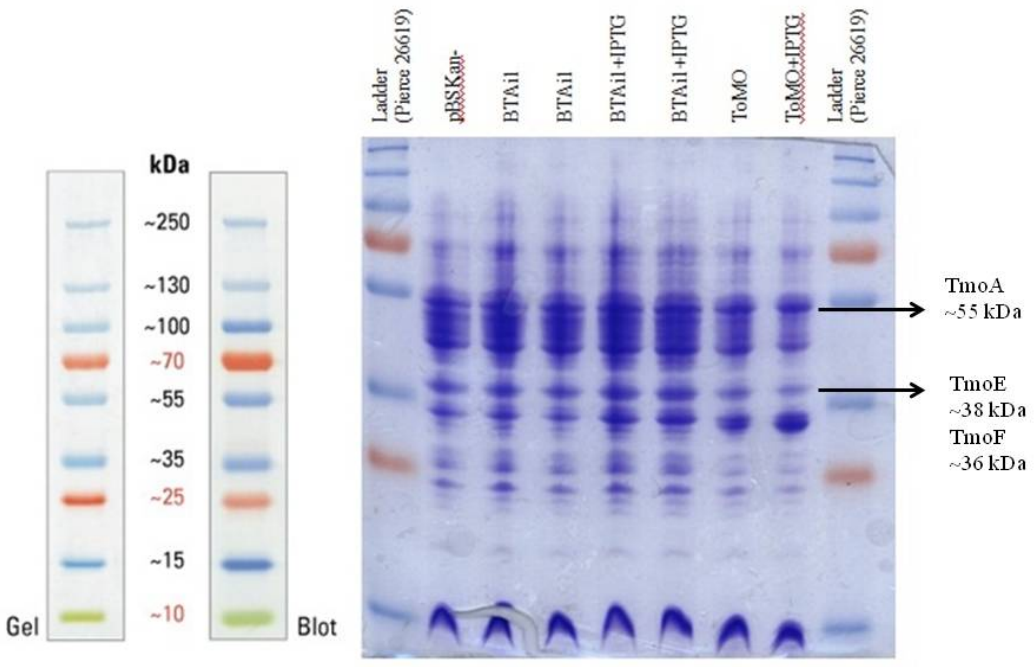


Figure 3.13 SDS-PAGE result of pBS(Kan)Tmo. The SDS gel contains pBS(Kan), pBS(Kan)Tmo.BTAi1, IPTG induced pBS(Kan)Tmo.BTAi1, ToMO, IPTG induced ToMO, respectively.

Table 3.3 Proteins including TmoA, TmoE and TmoF analysis of pBS(Kan)Tmo.BTAi1.

Coordinates	*tmo* *ORF*	Amino acid no.	Molecular mass (kDa) of the product		Molecular mass (kDa) of ToMO[b]
			Expected	Estimated[a]	Expected
3467-4951	*tmo*A	495	56	55	57.6
5998-7023	*tmo*E	342	38	38	38.3
7072-8070	*tmo*F	333	38	37	36.6

[a]SDS-PAGE.
[b](Bertoni et al, 1998)

3.2.4 HPLC Analysis

3.2.4.1 Toluene

Characterization of *E. coli* TG1 expressing the toluene monooxygenase gene cluster from *Bradyrhizobium* sp. BTAi1 was done for toluene, which is its natural substrate that is used as sole carbon and energy (Figure 3.14-16). Characterization was assayed in five different days with increasing incubations times; beginning from 5 minutes to 120 minutes. Analysis of more than twenty data revealed that; pBS(Kan)Tmo.BTAi1 is able to hydroxylase toluene and produces 100% *p*-cresol.

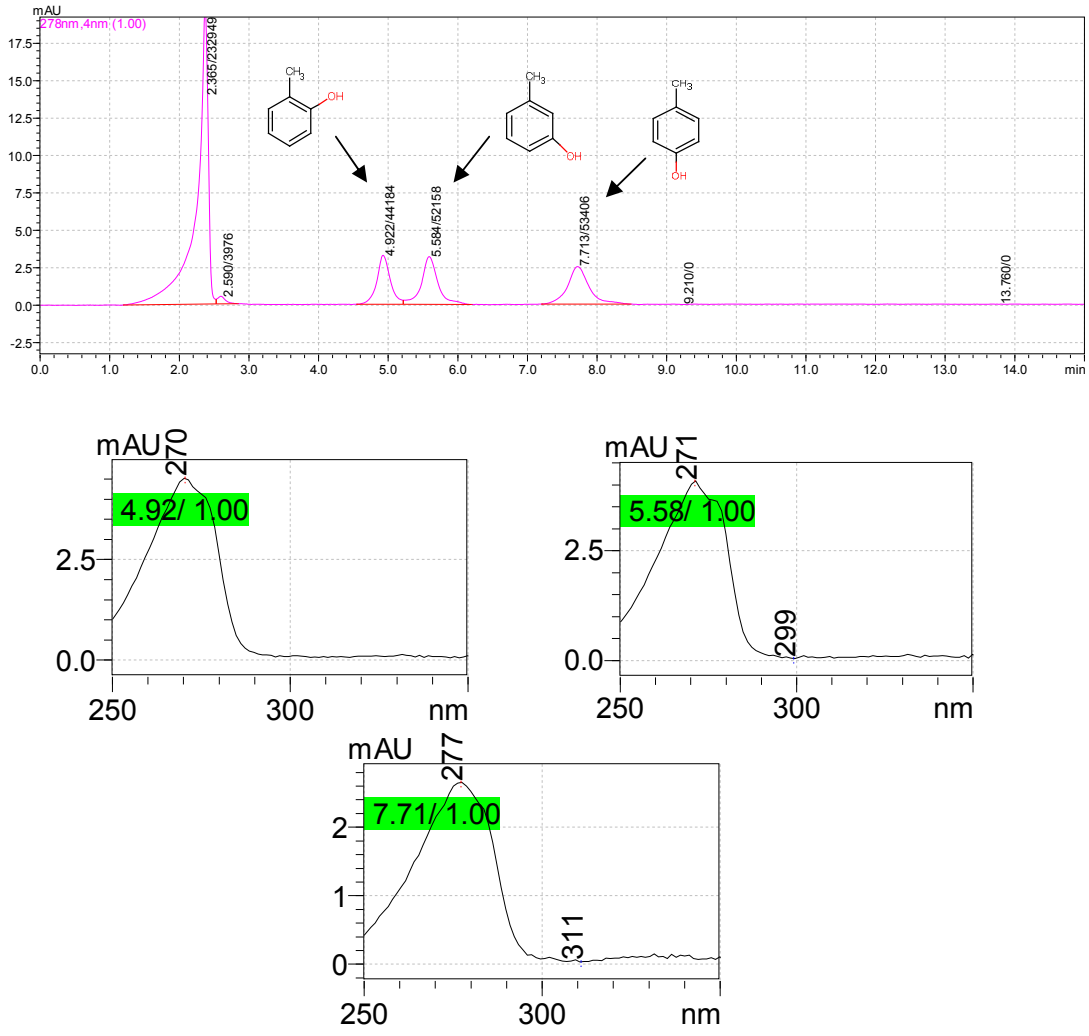
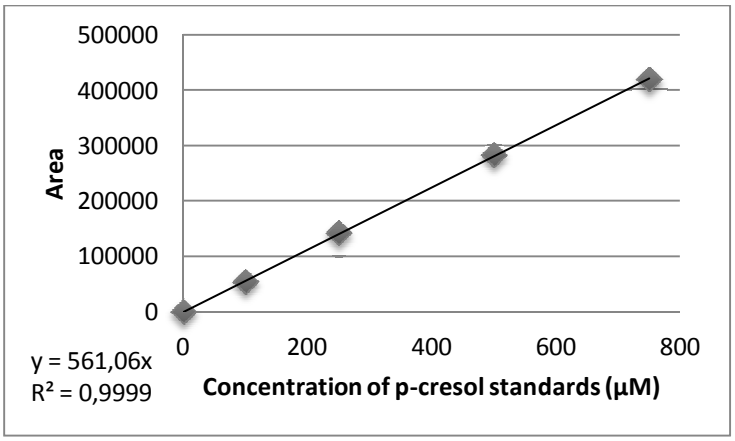
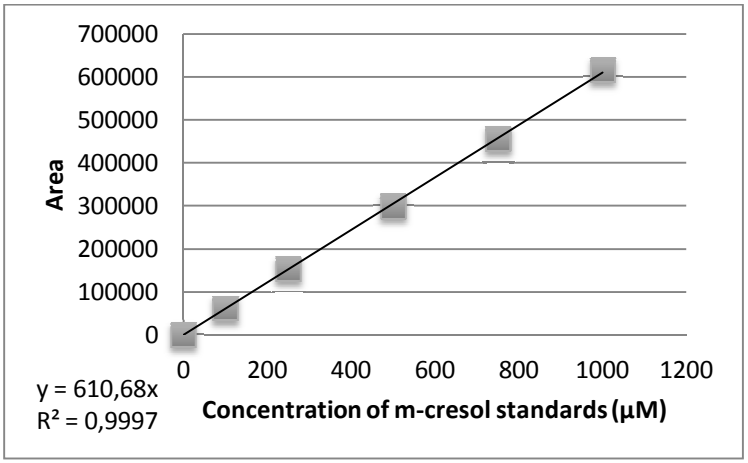


Figure 3.14 HPLC chromatogram data and graphs of cresol standards. *o*-Cresol at 4.9 min, 270 nm; *m*-cresol at 5.5 min, 270 nm and *p*-cresol at 7.7 min, 277 nm.

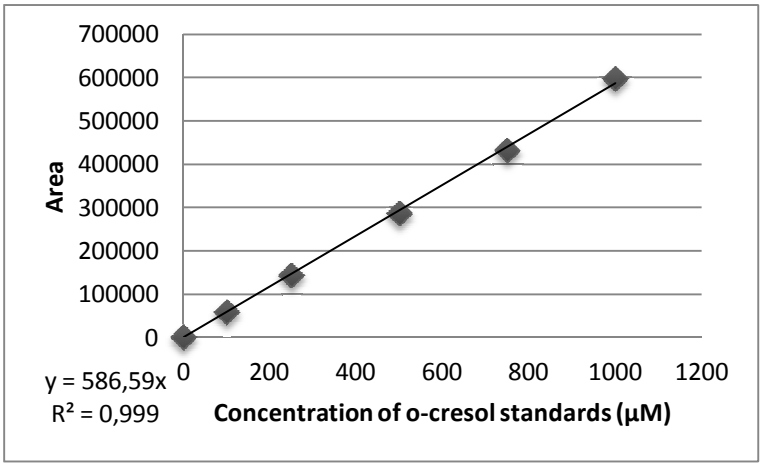


Concentration of *p*-cresol (µM)	Area
0	0
100	53956
250	141907
500	282687
750	419098

Figure 3.15 *p*-cresol standards obtained by HPLC analysis including standard concentrations of *p*-cresol and its corresponding area at 277 nm.



Concentration of *m*-cresol (µM)	Area
0	0
100	61171
250	152818
500	298862
750	455642
1000	615643



Concentration of *o*-cresol (µM)	Area
0	0
100	58091
250	142880
500	286125
750	431530

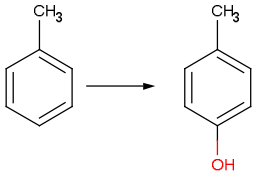
Figure 3.16 *m*-cresol (up) and *o*-cresol (down) standards obtained by HPLC analysis including standard concentrations of both and their corresponding area at 270 nm, from the chromatograms.

Co-elution method was applied to confirm the existence of the product. For this method, 10 µM pure *p*-cresol was added into the sample containing *E. coli* expressing pBS(Kan)Tmo.BTAi1 incubated with toluene for 120 minutes, which was run before the co-elution method.

Also maximum wavelengths were checked for each product peaks and confirmed with the standard data of those compounds.

IPTG induced culture produces *p*-cresol approximately three-fold more compated to the non-induced culture. This was expected because of the increased protein expression by the IPTG induction. Initial formation rates of the products were calculated and shown in Table 3.4.

Table 3.4 Initial formation rates of *p*-cresol from toluene oxidation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1.

Product: *p*-cresol	Initial formation rate (nmol/min/mg of protein)		Initial formation rate (nmol/hr/mg of protein)	
	non-induced	IPTG-induced	non-induced	IPTG-induced
	0.019±0.0037	0.036	1.254±0.219	2.135

3.2.4.2 Benzene

As in all toluene monooxygenases, benzene oxidation was resulted with 100% phenol formation (Figure 3.17-18). Up to 180 minutes incubations with benzene *E. coli* TG1 expressing pBS(Kan)Tmo.BTAi1 can also oxidize benzene and produces phenol (Figure 3.19 and Table 3.5).

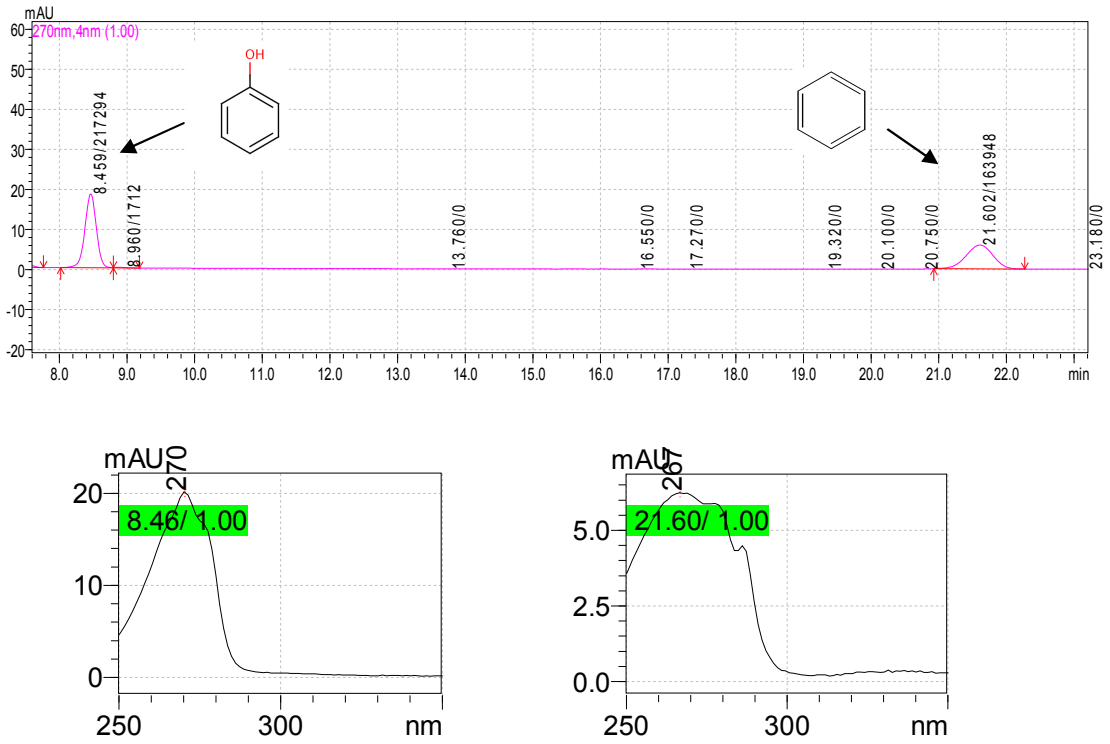
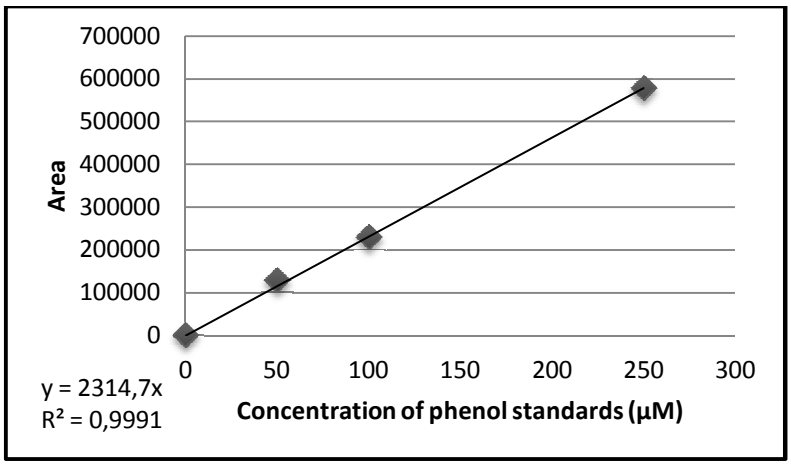


Figure 3.17 HPLC chromatogram of benzene degradation and phenol formation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1. At 270 nm, 8.46 min, and phenol and at 267 nm, 21.6 min, benzene pick are presented in graph.



Concentration of phenol (µM)	Area
0	0
50	128240
100	228841
250	577223

Figure 3.18 Standard graph curve of phenol product at 270 nm via HPLC analysis and in the table there are standard concentrations of phenol and its corresponding area.

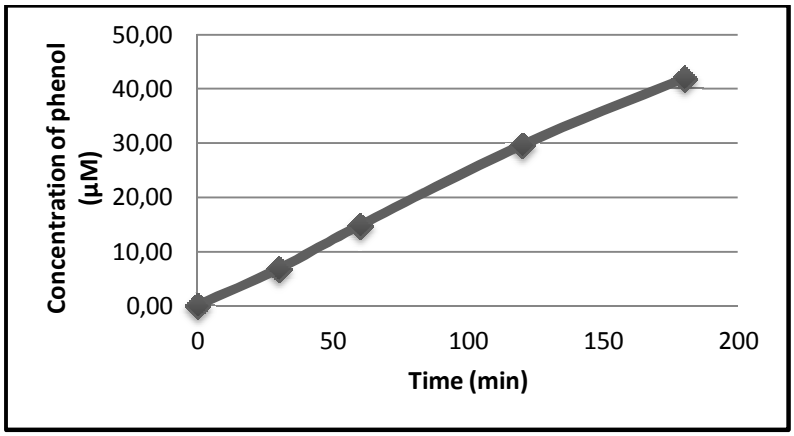
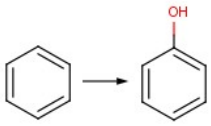


Figure 3.19 Phenol formation from benzene oxidation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1. Figure includes HPLC analysis for up to 180 minutes, at 270 nm.

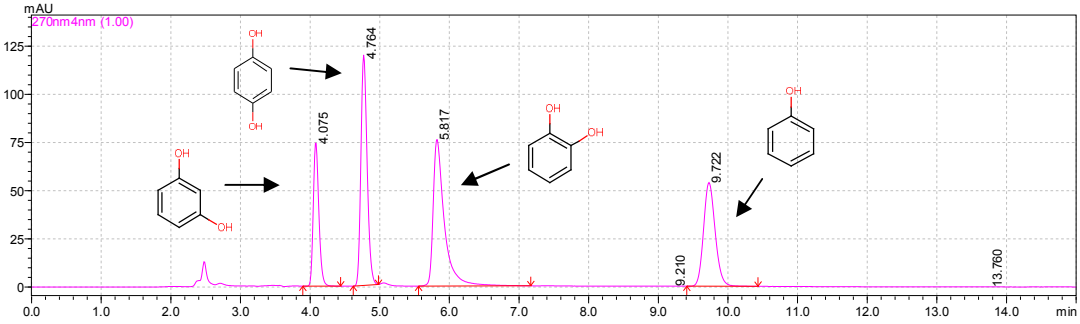
Table 3.5 Initial formation rates for phenol from benzene oxidation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1.

Product: Phenol	Initial formation rate (nmol/min/mg of protein)	Initial formation rate (nmol/hr/mg of protein)
	0.035	2.078

3.2.4.3 Phenol

Successive hydroxylation of phenol by *E. coli* cells expressing pBS(Kan)Tmo.BTAi1. Standard measurements are shown in Figure 3.20, 3.21 and 3.22 and Table 3.6. Analysis were done for both IPTG induced and non-induced culture and incubations; beginning from 30 minutes, up to 180 minutes revealed that, *E. coli* TG1/pBS(Kan)Tmo.BTAi1 oxidizes phenol into 88% catechol and 12% hydroquinone (Figure 3.22).

(a)



(b)

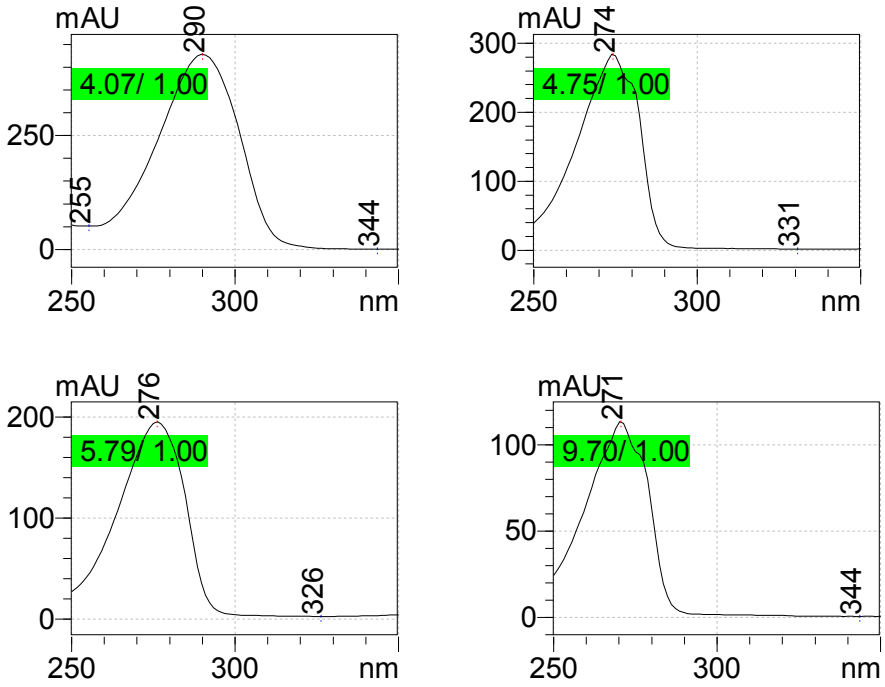
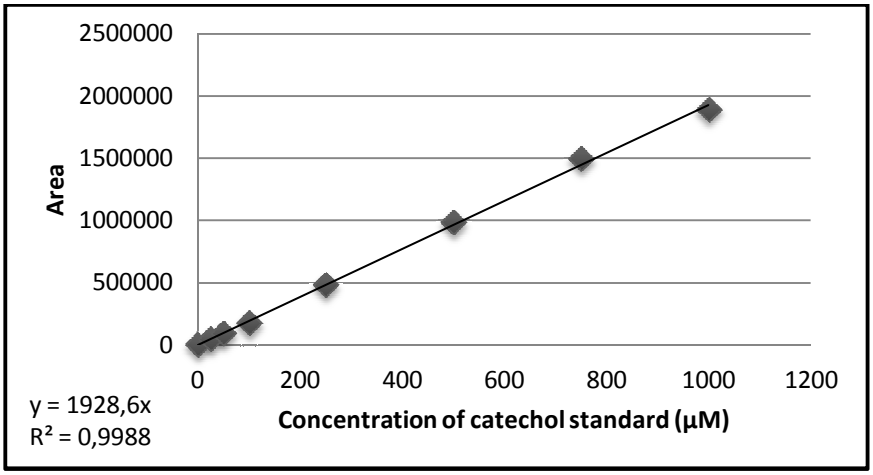


Figure 3.20 HPLC chromatogram of phenol (at 9.7 min, 270 nm) and its products; catechol at 5.79 min, 275 nm, hydroquinone at 4.75 min, 290 nm and resorcinol at 4.07 min, 275 nm (a). HPLC graphs give maximum wavelength of each compound (b).

(a)



(b)

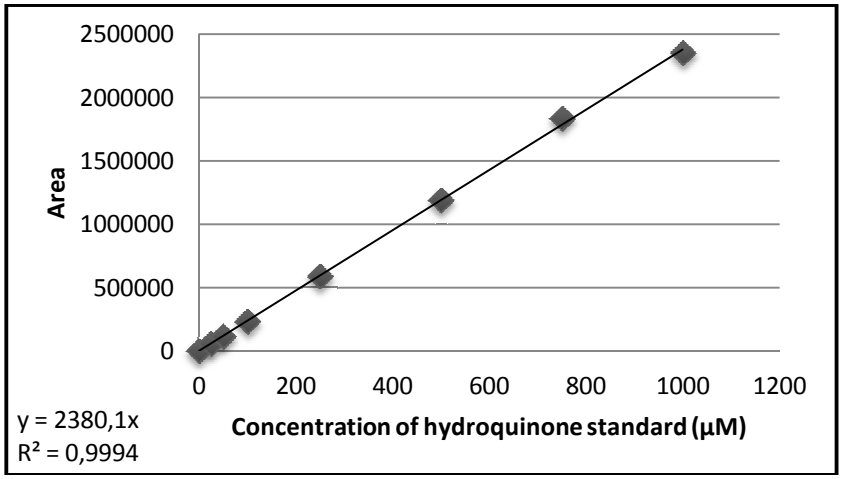
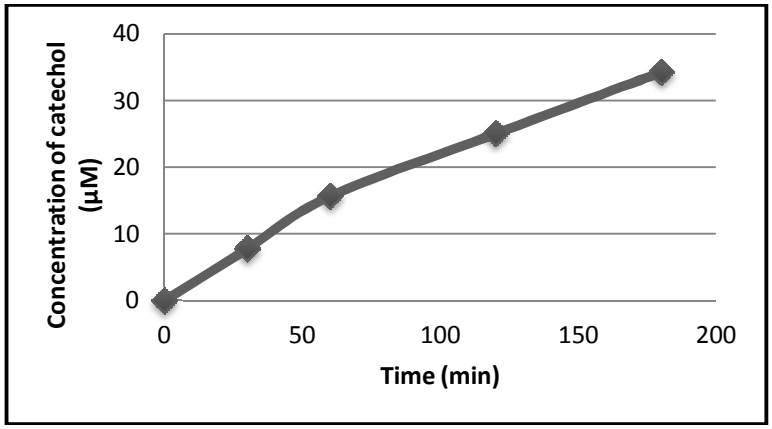


Figure 3.21 Standard curves for catechol (a) and hydroquinone (b) compounds. Catechol is at 5.9 min and 275 nm, hydroquinone is at 4.7 min and 290 nm.

Table 3.6 Standard concentrations of catechol and hydroquinone and their corresponding area on HPLC analysis.

Concentration (µM)	Area of Catechol [(a)]	Area of Hydroquinone [(b)]
0	0	0
25	45461.5	57579
50	904995	114845
100	174062.5	231313.5
250	479547	587294.5
500	981352	1187076
750	1492733	1831723
1000	1888276	2349453

(a)



(b)

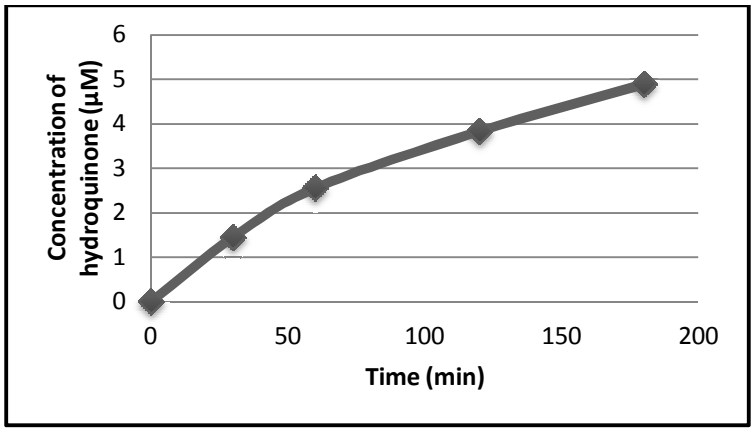


Figure 3.22 Catechol at 275 nm (a) and hydroquinone at 290 nm (b) formations from phenol oxidation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1. Data include up to 180 minutes incubation with phenol.

A co-elution method also proved the presence of hydroquinone as a result of phenol oxidation. 20 µM of hydroquinone was added into the sample which was loaded on HPLC before. The increase in peak at a specific retention time with a maximum wavelength at 290 nm shows the presence of hydroquinone (Figure 3.23).

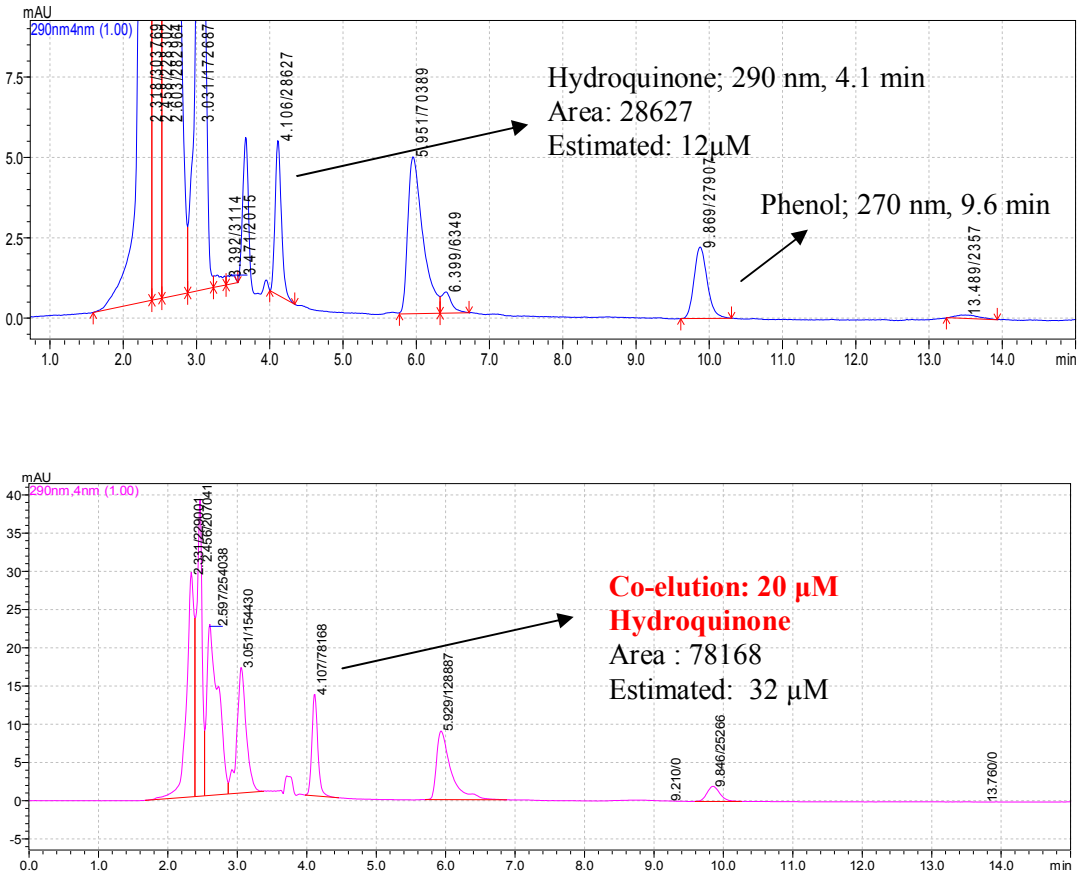
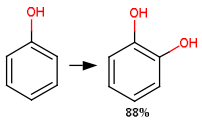
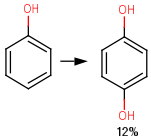


Figure 3.23 Co-elution of hydroquinone (290 nm, 4.1 min). Known amount of hydroquinone was added to prove the presence of hydroquinone as a result of phenol oxidation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1.

Table 3.7 Initial formation rates for catechol and hydroquinone from phenol oxidation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1.

Products: Catechol and Hydroquinone	Initial formation rate (nmol/min/mg of protein)		Initial formation rate (nmol/hr/mg of protein)	
	non-induced	IPTG-induced	non-induced	IPTG-induced
 88%	0.036	0.087	2.141	5.208
 12%	0.005	0.009	0.300	0.544

Phenol substrate incubations were done for both IPTG induced and non-induced cultures of *E. coli* TG1/pBS(Kan)Tmo.BTAi1. Results were consistent with each other. *E. coli* TG1/pBS(Kan)Tmo.BTAi1 is able to produce 88% catechol and 12% hydroquinone from phenol (Table 3.7).

3.2.4.4 Nitrobenzene

Analysis for nitrobenzene hydroxylation was tested in five different days, with total of twenty five time point analysis including IPTG induced and non-induced culture. Standard measurements are shown in Figure 3.24, 3.25 and 3.26. Results were consistent with each other and it is found that *E. coli* TG1/pBS(Kan)Tmo.BTAi1 produces 10% *m*-nitrophenol and 90% *p*-nitrophenol from nitrobenzene (Table 3.8).

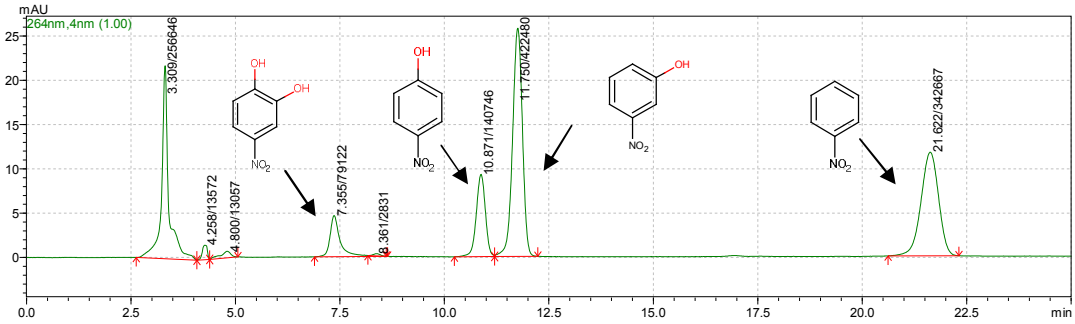
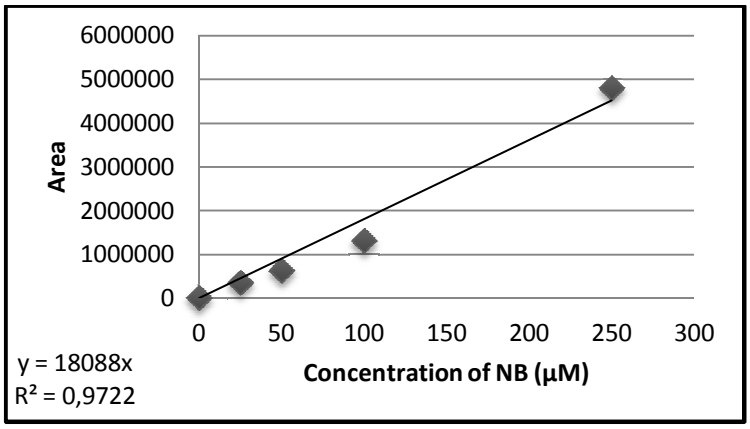


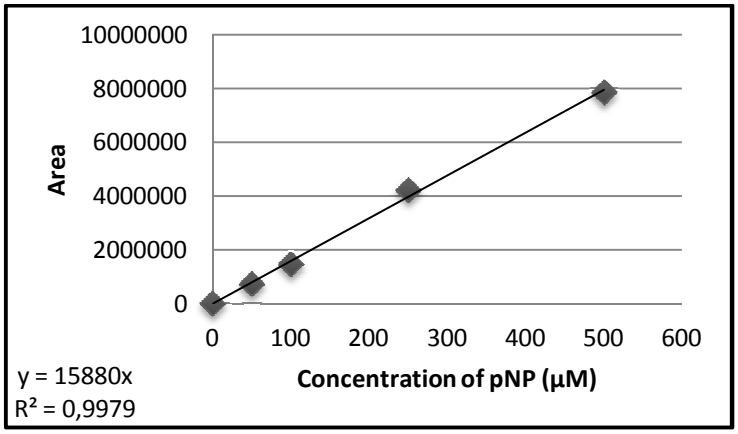
Figure 3.24 HPLC chromatogram data of nitrobenzene (21.6 min, 264 nm), *p*-nitrophenol (10.8 min, 317 nm), *m*-nitrophenol (11.7 min, 274 nm) and 4-nitrocatecol (7.3 min, 348 nm) standards.



Concentration of NB (µM)	Area
0	0
25	342667
50	619423
100	1301922
250	4792669

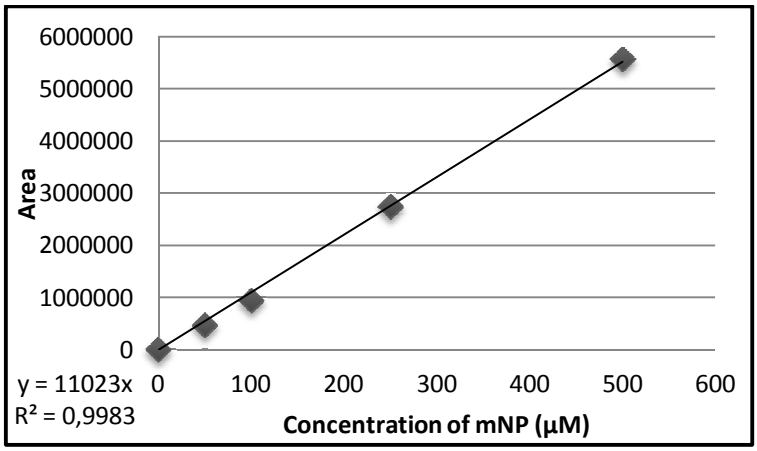
Figure 3.25 Standard curves for nitrobenzene (NB) at 264 nm, its corresponding table.

(a)



Concentration of *p*NP (µM)	Area
0	0
50	734701
100	1466033
250	4215823
500	7847633

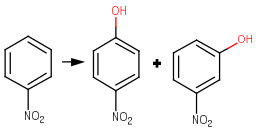
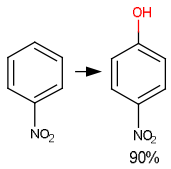
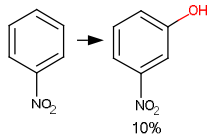
(b)



Concentration of *m*NP (µM)	Area
0	0
50	469815
100	942565
250	2738270
500	5560618

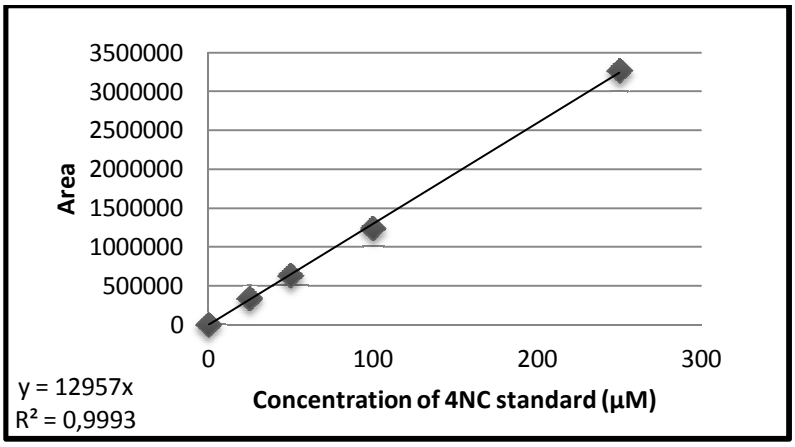
Figure 3.26 Standard curves for *p*-nitrophenol (pNP) at 317 nm (a) and *m*-nitrophenol (mNP) at 274 nm (b) and their corresponding tables.

Table 3.8 Initial formation rates for *p*-nitrophenol and *m*-nitrophenol and degradation rate for nitrobenzene oxidation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1.

Substrate: NB	Initial degradation rate (nmol/min/mg of protein)		Initial degradation rate (nmol/hr/mg of protein)	
	non-induced	IPTG-induced	non-induced	IPTG-induced
	0.066±0.0107	0.048	3.967±0.64	2.849
Products: *p*NP and *m*NP	**Initial formation rate (nmol/min/mg of protein)**		**Initial formation rate (nmol/hr/mg of protein)**	
	non-induced	IPTG-induced	non-induced	IPTG-induced
	0.005±0.0007	0.009	0.287±0.040	0.554
	0.0006±0.0002	0.0013	0.033±0.009	0.080

3.2.4.5 p-Nitrophenol

p-nitrophenol standard measurements are shown in Figure 3.27 and 3.28. The analysis; which were repeated three times in different days and included fifteen different data of five different time points beginning from 15 minutes to 120 minutes; are revealed that *E. coli* TG1/pBS(Kan)Tmo.BTAi1 oxidizes *p*-nitrophenol (*p*-NP) into 100% 4-nitrocatechol (4-NC) which can be performed by all other subunits of toluene monooxygenases (Table 3.9).



Concentration of 4NC (µM)	Area
0	0
25	338673
50	633713
100	1236364
250	3264299

Figure 3.27 Standard curve of 4-NC, at 4.9 min and 348 nm.

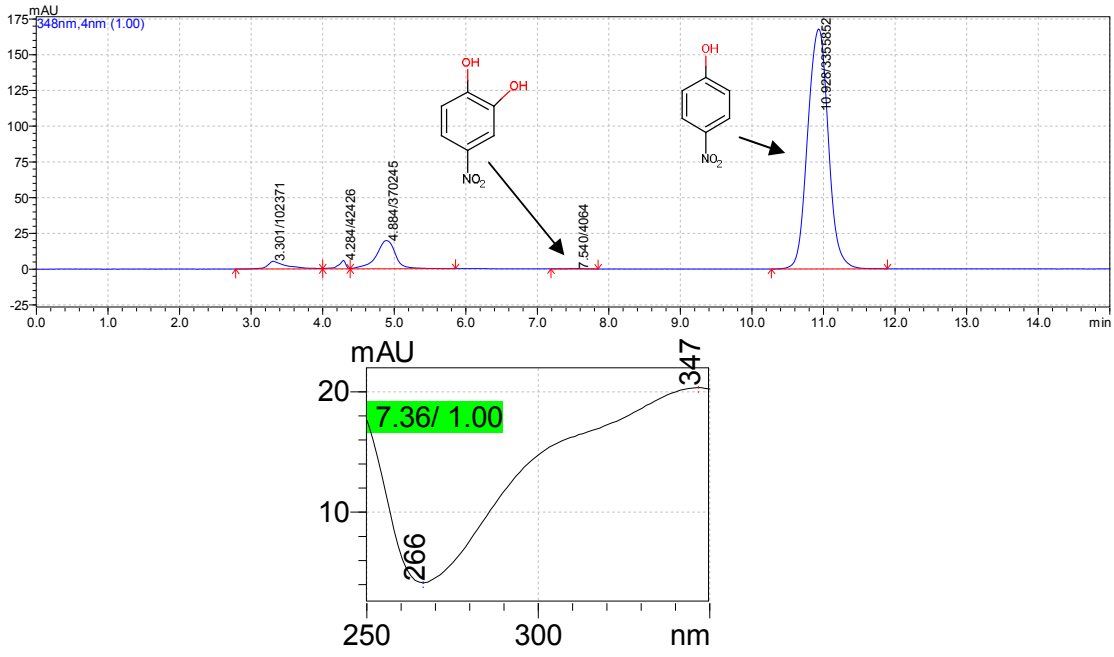
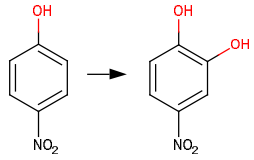
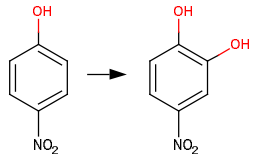


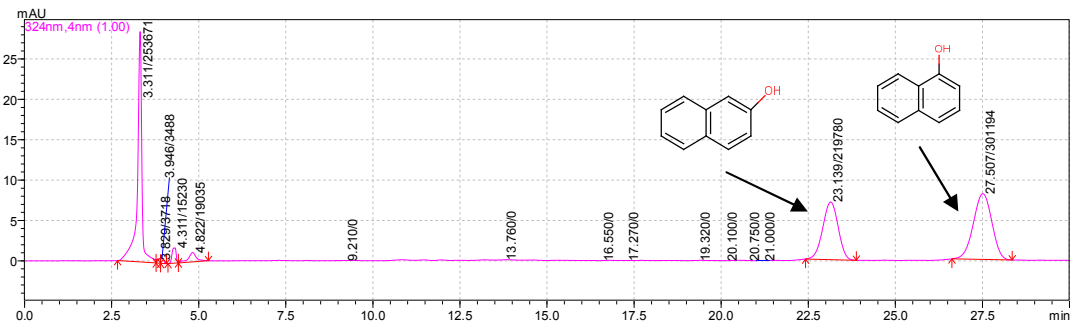
Figure 3.28 HPLC chromatogram data of 4-NC (7.5 min, 348 nm) formation from *p*NP (10.9 min, 317 nm).

Table 3.9 Initial formation rates for 4-nitrocatechol and degradation rates for *p*-nitrophenol oxidation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1.

Substrate: *p*-NP	Initial degradation rate (nmol/min/mg of protein)	Initial degradation rate (nmol/hr/mg of protein)
	0.073±0.0004	4.401±0.023
Product: 4-NC	Initial formation rate (nmol/min/mg of protein)	Initial formation rate (nmol/hr/mg of protein)
	0.0011±0.0001	0.045±0.015

3.2.4.6 Naphthalene

Naphthalene oxidation was done with both IPTG induced and non-induced pBS(Kan)Tmo.BTAi1 cultures including more than ten different time points. The analysis was sufficient and consistent with each other according to the regiospecificity results. *E. coli* TG1/pBS(Kan)Tmo.BTAi1 is able to produce 42% 1-naphthol and 58% 2-naphthol. Standards are shown in Figure 3.29 and 3.30.



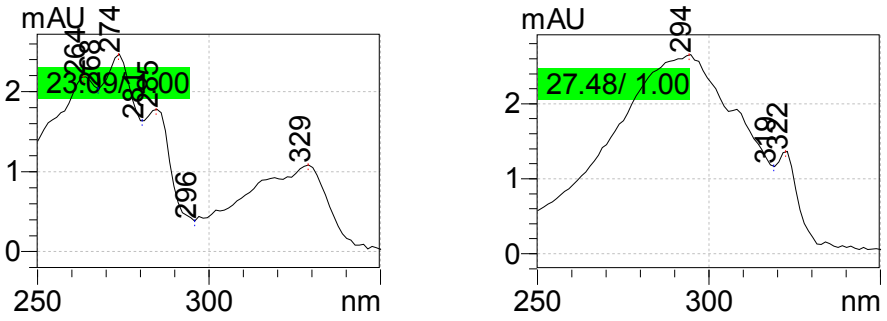
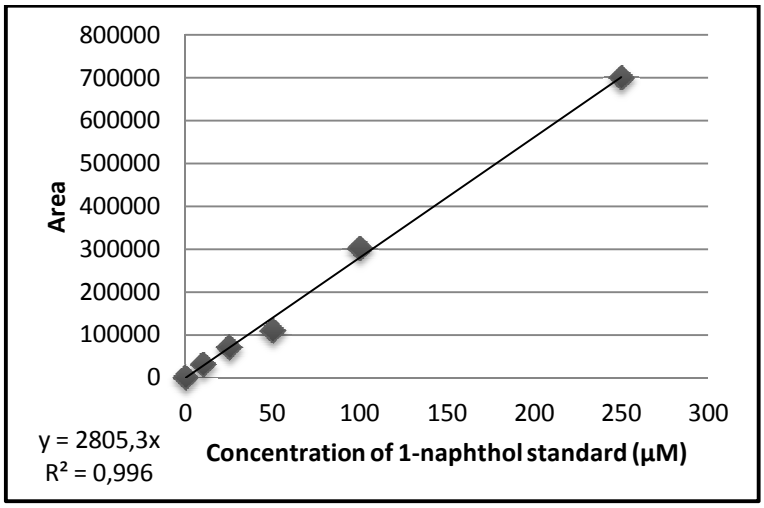


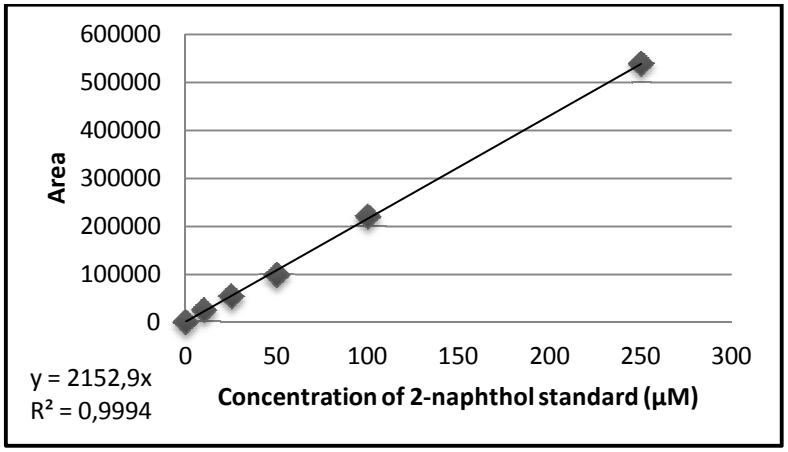
Figure 3.29 HPLC chromatogram for calibrations of 1-naphthol (27.5 min, 324 nm) and 2-naphthol (23.1 min, 324 nm).

(a)



Concentration of 1-naphthol (µM)	Area
0	0
10	30416
25	70331
50	109139
100	301194
250	699173

(b)



Concentration of 2-naphthol (µM)	Area
0	0
10	25039
25	53524
50	97901
100	219780
250	538256

Figure 3.30 HPLC analysis of 1-naphthol (a) and 2-naphthol (b) standard curves and their corresponding tables.

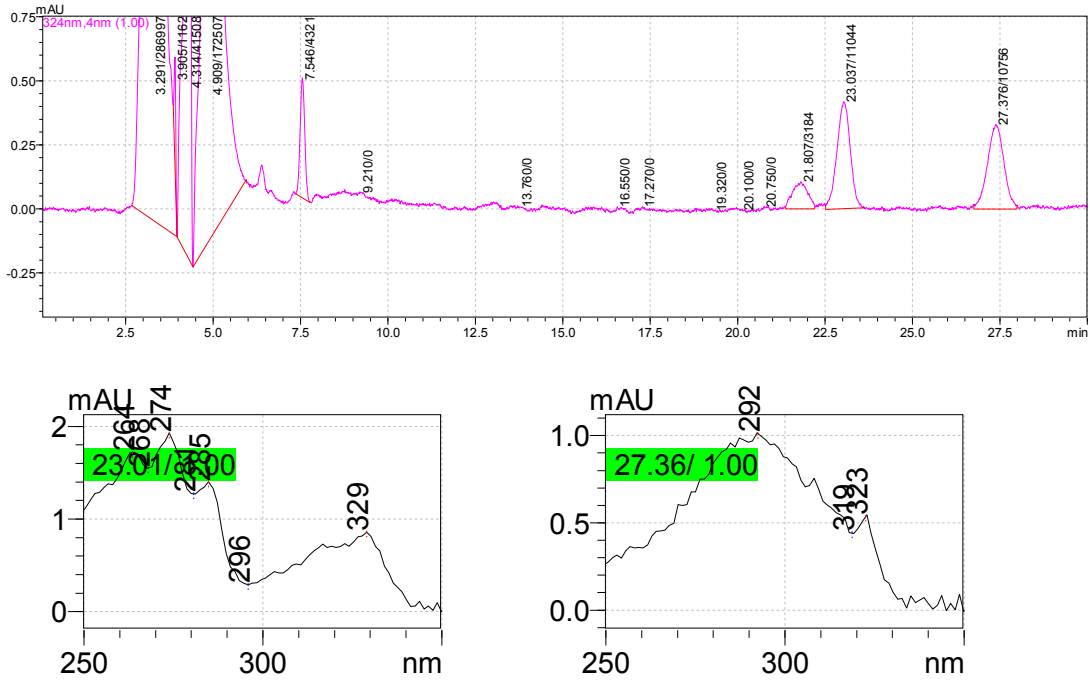
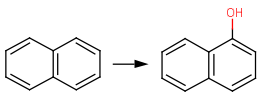
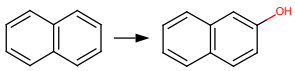


Figure 3.31 1-naphthol (27.3 min, 324 nm) and 2-naphthol (23.03 min, 324 nm) formations from naphthalene oxidation by *E. coli* TG1/pBS(Kan)Tmo.BTAi1.

1-naphthol and 2-naphthol formations were analyzed at 324 nm. Besides, 1-naphthol and 2-naphthol have maximum wavelengths at 292 nm and 274 nm, respectively (Figure 3.31 and Table 3.10).

Table 3.10 Initial formation rates of 1-naphthol and 2-naphthol synthesis from naphthalene oxidation by pBS(Kan)Tmo.BTAi1.

Products: 1-naphthol and 2-naphthol	Initial formation rate (nmol/min/mg of protein)		Initial formation rate (nmol/hr/mg of protein)	
	non-induced	IPTG-induced	non-induced	IPTG-induced
	0.0011	0.0045	0.065	0.269
	0.0014	0.0059	0.087	0.355

As a summary, *E. coli* TG1 expressing pBS(Kan)Tmo.BTAi1 cells are able to oxidize toluene into 100% *p*-cresol, benzene to 100% phenol, phenol to 88% catechol and 12% hydroquinone, nitrobenzene to 10% *m*-nitrophenol and 90% *p*-nitrophenol, *p*-nitrophenol to 100% 4-nitrocatechol and naphthalene to 42% 1-naphthol and 58% 2-naphtol (Table 3.11 and Figure 3.32-34).

Table 3.11 Retention times (RT) and maximum wavelengths (λmax) of compounds obtained from HPLC analysis by the oxidation of substrates. Formation rates were calculated with 0.122 mg/ml.OD protein of *E. coli* TG1/pBS(Kan)Tmo.BTAi1. Regiospecificity of products were measured via formation rate proportions.

Substrate	Product	RT (min)	λmax (nm)	Formation rate (nmol/min/mg of protein)		Formation rate (nmol/hr/mg of protein)		Regiospecificity (%)
				Non-induced	IPTG induced	Non-induced	IPTG induced	
Benzene	Phenol	8.5	270	0.0346	ND[a]	2.078	ND[a]	100
Naphthalene	1-Naphthol	27	324	0.0001±0.0002	0.0045	0.065	0.269	42
	2-Naphthol	23	324	0.0013±0.0002	0.0059	0.087	0.355	58
NB[b]	*p*NP	11.1	317	0.0048±0.0007	0.0092	0.287±0.040	0.554	90
	*m*NP	12	274	0.0006±0.0002	0.0013	0.033±0.009	0.080	10
Phenol	Catechol	5.9	275	0.0357	0.0868	2.141	5.208	88
	HQ	4.75	290	0.0050	0.0090	0.300	0.544	12
*p*NP	4NC	4.9	348	0.0011±0.0001	ND[a]	0.045±0.015	ND[a]	100
Toluene	*p*-Cresol	9.0	277	0.0208±0.0036	0.0356	1.254±0.219	2.135	100

[a]Not determined

[b]NB; nitrobenzene, *p*NP; *p*-nitrophenol, *m*NP; *m*-nitrophenol, HQ; hydroquinone, 4NC; 4-nitrocatehcol

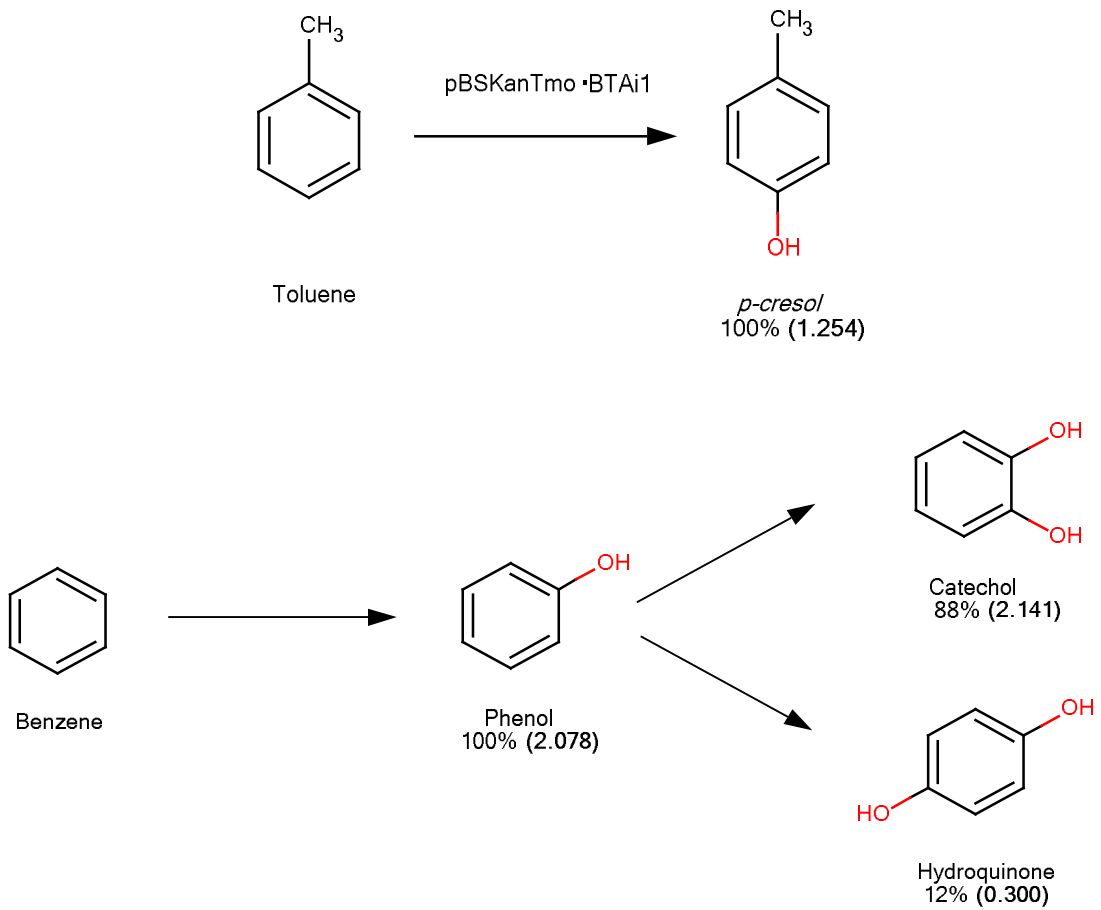


Figure 3.32 *E. coli* TG1/pBSKanT4MO.BTAi1 oxidation pathways for toluene (0.25 mM) and benzene (0.8 mM). Molar product percentages are shown followed by bold numbers in parenthesis, indicate the product formation rates in nmol/hr·mg protein.

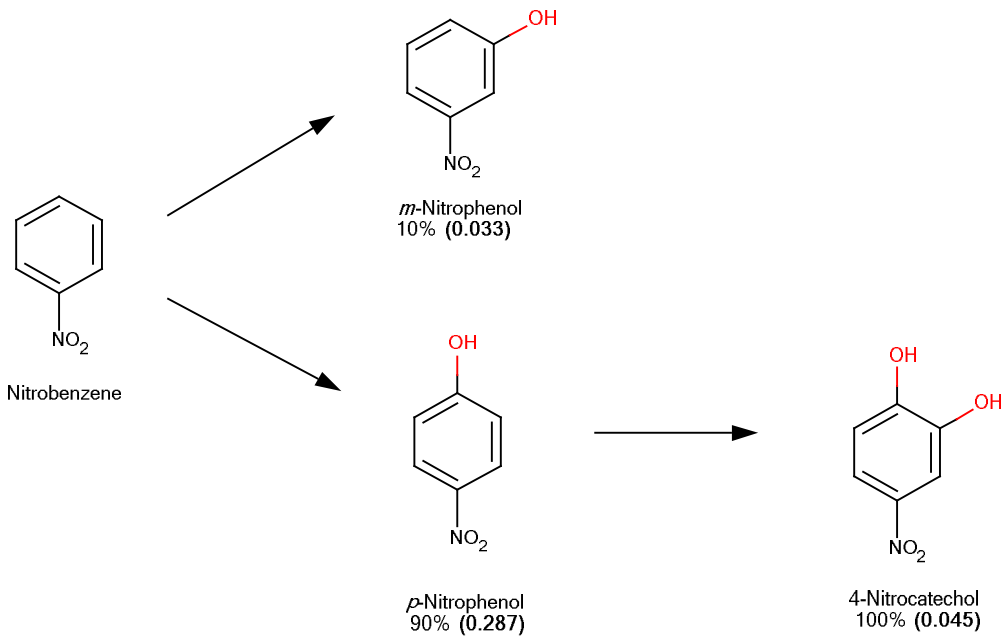


Figure 3.33 *E. coli* TG1/pBSKanT4MO.BTAi1 oxidation pathways for nitrobenzene (0.2 mM) to *m*-nitrophenol and *p*-nitrophenol (0.5 mM) to 4-nitrocatechol. Molar product percentages are shown followed by bold numbers in parenthesis, indicate the product formation rates in nmol/hr·mg protein.

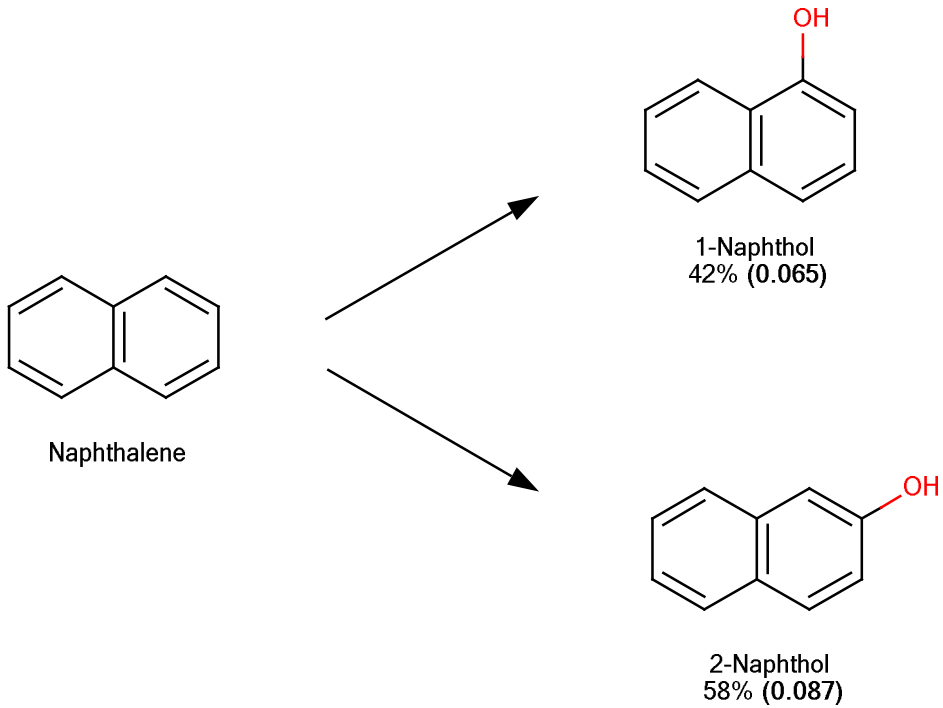


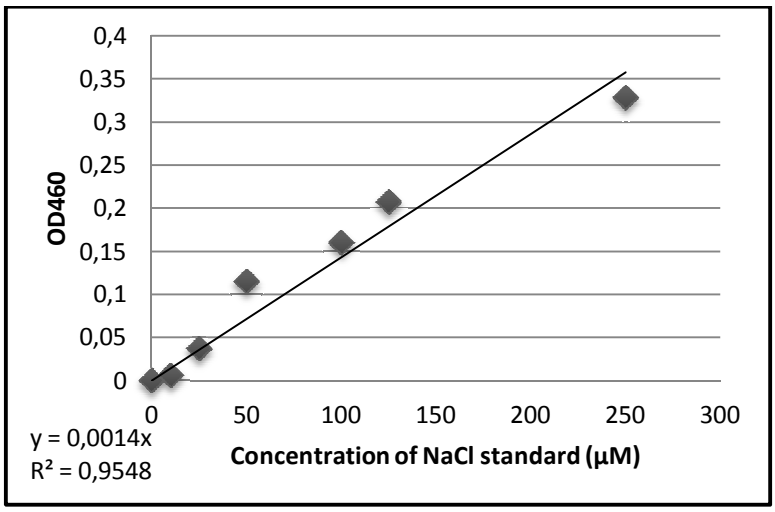
Figure 3.34 *E. coli* TG1/pBSKanT4MO.BTAi1 oxidation pathways for naphthalene (5 mM) to 1-naphthol and 2-naphthol. Molar product percentages are shown followed by bold numbers in parenthesis, indicate the product formation rates in nmol/hr·mg protein.

3.2.5 Chloride Assay

For trichloroethylene (TCE) degradation, *E. coli* TG1/pBS(Kan)ToMO from *Pseudomonas* sp. OX1 was used as a positive control.

TCE degradation assay was applied and chloride concentrations were measured for both non-induced ToMO, IPTG induced ToMO and IPTG induced BTAi1 after the calibration curve was obtained from NaCl standards (Figure 3.35).

The assay was repeated in six days with total fourteen different measurements (Table 3.12).



Concentration of NaCl (µM)	OD_{460}
0	0
10	0.006
25	0.037
50	0.115
100	0.160
125	0.207
250	0.328

Figure 3.35 Calibration curve of chloride concentration. Data was measured at 460 nm via spectrophotometer.

Table 3.12 Chloride concentrations of ToMO non-induced and IPTG-induced cultures and IPTG induced pBS(Kan)Tmo.BTAi1 culture.

	$[Cl^-]^1$	$[Cl^-]^2$
ToMO	16.19±1.67	21.19
ToMO+	nm[a]	44.00±25.46
BTAi1+	41.31±17.59	21.39±10.24

$[Cl^-]^1$: Concentration result of 30 minute incubation of cells with TCE.
$[Cl^-]^2$: Concentration result of overnight incubation of cells with TCE.
[a]nm; Not measured

CHAPTER 4

DISCUSSION

The nucleotide sequences of *Bradyrhizobium* sp. BTAi1 chromosome revealed the presence of toluene monooxygenase coding region, including six genes; *tmoABCDEF*. The gene clusters showed relevant similarities to the subunits of other toluene monooxygenases; ToMO, T4MO, T*p*MO and TOM.

In this thesis, the putative region of toluene monooxygenase, containing six gene clusters in *Bradyrhizobium* sp. BTAi1 was successfully cloned and expressed in *E. coli* TG1 strain. pBS(Kan)Tmo.BTAi1 expressed in *E. coli* TG1 host was characterized and analyzed by HPLC and spectrophotometer for its ability to oxidize certain aromatic hydrocarbons such as, toluene, benzene, phenol, nitrobenzene, *p*-nitrophenol and naphthalene and degrade a chlorinated ethene; trichloroethylene (TCE). It is clearly demonstrated that, the new toluene monooxygenase from BTAi produces 100% *p*-cresol (1.254±0.219 nmol/hr·mg protein) from toluene, 100% phenol (2.078 nmol/hr·mg protein) from benzene, 90% *p*-nitrophenol (0.287±0.040 nmol/hr·mg protein) and 10% *m*-nitrophenol (0.033±0.009 nmol/hr·mg protein) from nitrobenzene, 100% 4-nitrocatechol (0.045±0.015 nmol/hr·mg protein) from *p*-nitrophenol and finally 42% 1-naphthol (0.065 nmol/hr·mg protein) and 58% 2-naphthol (0.087 nmol/hr·mg protein) from naphthalene (Figure 3.32-34).

Table 4.1 summarizes the regiospecific oxidation of toluene, nitrobenzene, naphthalene and phenol by all the other isolated toluene monooxygenases and the new toluene monooxygenase from BTAi1 expressed in *E. coli* TG1 strain.

Table 4.1 Regiospecific oxidation of toluene, nitrobenzene (NB), naphthalene and phenol by TG1 cells expressing TMO.BTAi1, wild-type (WT) ToMO, WT T4MO, WT T*p*MO and WT TOM (Fishman et al., 2006; Tao et al., 2004; Vardar and Wood, 2005a).

	Toluene oxidation			NB oxidation			Naphthalene oxidation		Phenol oxidation		
Enzyme	*o*-cresol	*m*-cresol	*p*-cresol	*o*-NP	*m*-NP	*p*-NP	1-naphthol	2-naphthol	Catechol	Hydroquinone	Resorcinol
TMO.BTAi1	0	0	100	0	10	90	42	58	88	12	0
ToMO	32	21	47	0	72	28	88	12	100	0	0
T4MO	0	3	97	0	10	90	52	48	100	0	0
T*p*MO	0	10	90	0	34	66	62	38	100	0	0
TOM	100	0	0	0	0	0	100	0	100	0	0

pBS(Kan)Tmo.BTAi1 expressed in *E. coli* TG1 is the only one in response to the other toluene monooxygenases, that is capable of producing a novel product; hydroquinone (HQ) from phenol. ToMO, T4MO, T*p*MO and TOM can only form catechol from phenol oxidation. None of the wild type toluene monooxygenases have been reported to produce HQ. Only some mutants have been found to oxidize phenol into HQ (Vardar and Wood, 2005a). HQ has a wide range of usage in many areas which brings the need of consumption with it. It is used as an agent in photographic developers in films and papers, as an antioxidant and a supplier for X-ray films in medical and as a skin lightening in cosmetics (Vardar, 2005).

A regiospecific difference is also seen in naphthol' formations. pBS(Kan)Tmo.BTAi1 expressed in *E. coli* TG1 is able to produce 42% 1-naphthol and 58% 2-naphthol; that is the opposite of other subfamilies. Toluene monooxygenases mostly produces 1-naphthol, so this distinction increases the importance of pBS(Kan)Tmo.BTAi1 due to the various usage area of 2-naphthol. This naphthalene derived compound is used in pesticides, rubbers, and used as antioxidant and antiseptic (Canada et al, 2002).

Among toluene monooxygenases, TMO.BTAi1 is the only reported enzyme that is specific to *p*-cresol formation from toluene. Moreover, T4MO and T*p*MO are capable of hydroxylating toluene mainly at *para* position with regiospecificity of 97% and 90%, respectively. In addition to this, oxidation of nitrobenzene by pBS(Kan)Tmo.BTAi1 is resulted with the production of 90% *p*NP and 10% *m*NP which is also achieved by T4MO. These similarities demonstrate that, TMO.BTAi1 is more likely to be a *para*-hydroxylating enzyme. For this reason, it is more convenient to name the plasmid as pBS(Kan)T4MO.BTAi1.

Chlorinated ethenes, such as TCE, are one of the most hazardous chemicals, and their irregular release to environment causes contamination, even groundwater, which then may interfuse with tap water (Vardar and Wood, 2005b). Shields et al. demonstrated that chlorinated ethenes degradation is achieved by toluene monooxygenase pathway (Shields et al., 1991). This promising fact broadens toluene monooxygenases applications; so they can be involved in bioremediation of TCE. Rhizoremediation is an efficient way for degradation of such compounds due to using the advantage of plant-bacteria correlations (Yee et al, 1998).

E. coli TG1/pBS(Kan)T4MO.BTAi1 shows a tendency for the degradation of TCE. However, the activity of *E. coli* TG1/pBS(Kan)T4MO.BTAi1 towards aromatic hydrocarbons and TCE is found to be quite lower than the other four toluene monooxygenases. Nevertheless, the difference in regiospecificity of products and formation of novel products rise the importance of *E. coli* TG1/pBS(Kan)T4MO.BTAi1 for green chemistry and bioremediation applications.

Before this work, none of a native Rhizobium with toluene monooxygenase activity has been reported. Previous studies were included engineering of a gene coding for toluene monooxygenase cloning into a root-colonizing bacteria; however they were not naturally found in the environment (Yee et al, 1998). This work is novel in that the catalytic potential of a new toluene monooxygenase was investigated for biotechnology and bioremediation applications. In addition, a toluene monooxygenase gene cluster was characterized from a rhizobacterium for the first time.

CHAPTER 5

CONCLUSION

In this study, a new toluene monooxygenase gene cluster was cloned from a Rhizobium strain, *Bradyrhizobium* sp. BTAi1 that lives in the nodules of root of *A.indica,* and its potential activity towards certain aromatic hydrocarbons such as toluene, benzene, phenol, nitrobenzene, *p*-nitrophenol and naphthalene and a chlorinated ethene; TCE, was investigated. Differences in regiospecificity of products compared to other subfamilies; ToMO, T4MO, T*p*MO and TOM, and the formation of a novel compound, increases the significance of this new enzyme; that is named as T4MO.BTAi1 due to its similarity with T4MO.

This was the first study for characterizing a toluene monooxygenase from a rhizobacterium strain for bioremediation and green chemistry applications.

Following the investigations described in this thesis, to enhance the activity of *E. coli* TG1/pBS(Kan)T4MO.BTAi1 towards chlorinated aliphatics and aromatic compounds, protein engineering methods will be my future concern. Recent studies proved that, random mutagenesis techniques are extremely powerful tools for enzymes to enhance their activity, stabilize the structure and broaden the regiospecificity of products (Wood, 2008). One of these mutagenesis methods is error-prone polymerase chain reaction (ePCR) that is used to engineer proteins to increase their activity. An error prone library was established for pBS(Kan)T4MO.BTAi1, and 1080 number of different colonies were obtained and the library screening has been started. Moreover, the size of the library has to be expanded for obtaining several different characteristics of the enzyme to improve its activity for the oxidation of aromatic aliphatics and degradation of chlorinated ethenes.

Rational design, a genetic engineering method can be chosen to make better enzymes, which is widely used in bioremediation field (Wood, 2008). However, this methos depends on the information related to enzyme structure. For this reason, modelling of α-subunit of T4MO.BTAi1 was performed using Swiss-Model (Figure 5.1 and 5.2) and the results were analyzed on PyMOL Molecular Graphics System, Version 1.3, Schrodinger LLC. This will be helpful to find out the important residues in the enzyme which will be considered as my future work.

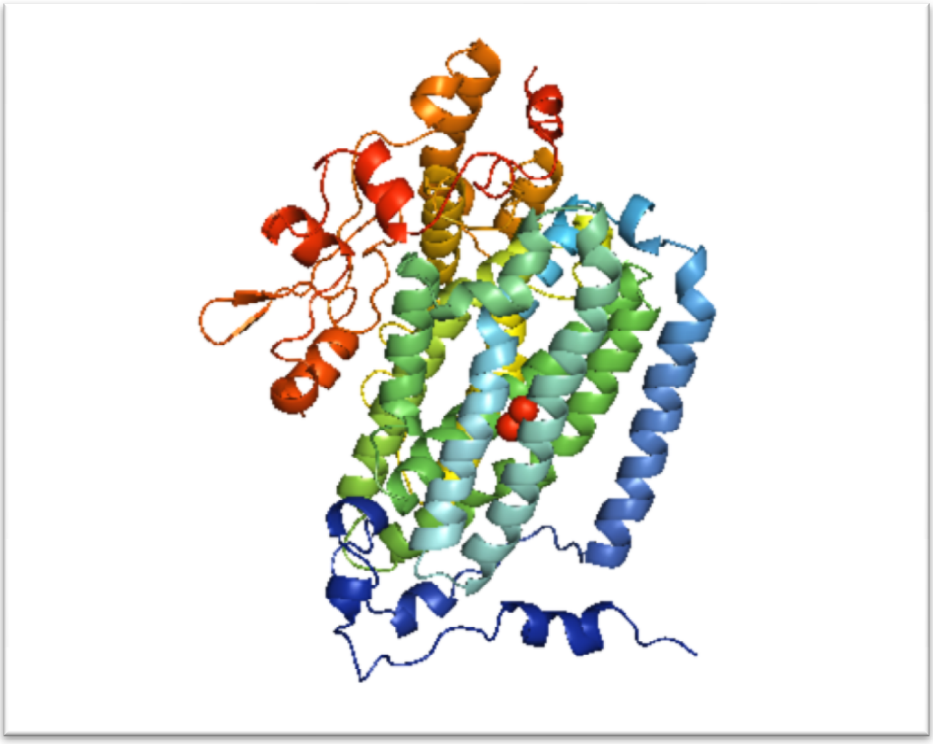


Figure 5.1 Modelling results of α-subunit of T4MO.BTAi1 using Swiss-Model and PyMOL, Schrodinger LCC programs. The subunit includes the active site which contains a diiron center shown as red spheres.

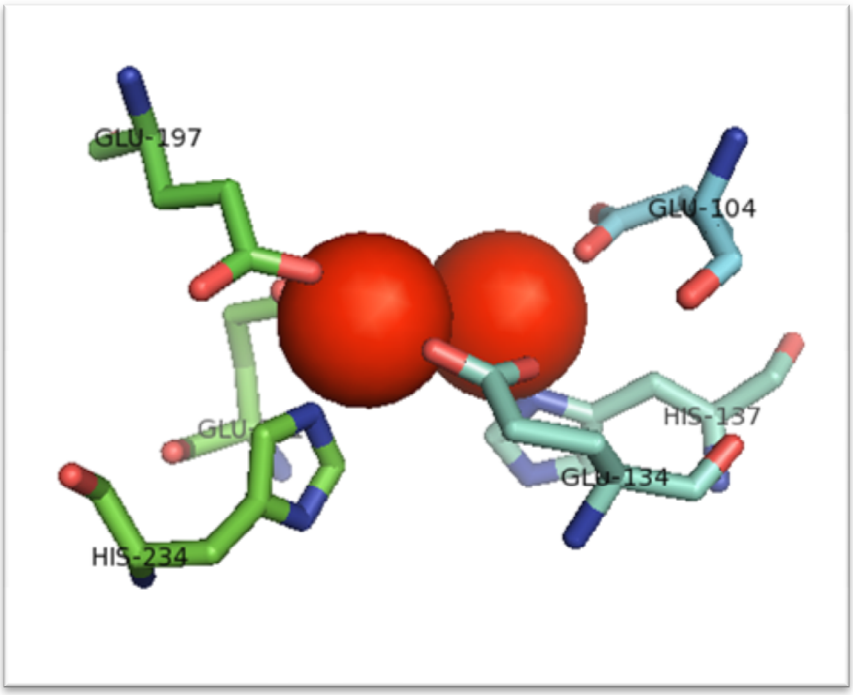


Figure 5.2 Modelling of α-subunit diiron center T4MO.BTAi1. Diiron binding residues; E104, E134, H137, E197, E231 and H234 are presented.

Protein engineering gives an opportunity for rapid and developed bioremediation and offers even degrading recalcitrant and persistent pollutants. Consequently, enhancing the activity of T4MO.BTAi1 with these methods will gain improvements in both bioremediation and rhizoremediation applications.

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APPENDIX A

List of Chemicals

1,2-Dihydroxybenzene (Catechol)	Sigma-ALDRICH	Germany
1-Naphthol	MERCK	Germany
2-Naphthol	MERCK	Germany
2-Nitrophenol	Sigma-ALDRICH	Germany
3-Nitrophenol	Sigma-ALDRICH	Germany
4-Nitrocatechol	Sigma-ALDRICH	Germany
4-Nitrophenol >=99%	Sigma-ALDRICH	Germany
Acetic acid (glacial) 100%	MERCK	Germany
Acetonitrile	MERCK	Germany
Acrylamide/Bis solution (30%)	Bio-Rad	USA
Agarose Low Melt	AppliChem	Germany
Ammonium iron (III) sulfate dodecahydrate	MERCK	Germany
Ammonium per sulfate	Sigma-ALDRICH	Germany
Benzene	MERCK	Germany
Coomassie Brilliant Blue R-250	Bio-Rad	USA
Ethanol 96%	MERCK	Germany
Ethanol absolute	MERCK	Germany
Ethidium Bromide	Sigma-ALDRICH	Germany
Ethylenediaminetetraacetic acid disodium salt	Carlo ERBA	Italy
Formic acid (98-100%)	MERCK	Germany
Gene Ruler 1 kb DNA Ladder	Fermentas	USA
Glycerol anhydrous	AppliChem	Germany
Glycine	MERCK	Germany

Hydrochloric acid (HCl)	MERCK	Germany
Hydroquinone	Sigma-ALDRICH	Germany
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Fisher	USA
Kanamycin sulfate	AppliChem	Germany
LB-Agar (MILLER)	MERCK	Germany
LB-Broth (MILLER)	MERCK	Germany
m-Cresol	Sigma-ALDRICH	Germany
Mercury (II) thiocyanate	MERCK	Germany
Methanol for liquid chromatography	MERCK	Germany
N,N-Dimethyl-formamide (DMF)	Carlo ERBA	Italy
Naphthalene	MERCK	Germany
Nitric acid fuming 100% extra pure	MERCK	Germany
Nitrobenzene >=99%	Sigma-ALDRICH	Germany
o-Cresol	Sigma-ALDRICH	Germany
p-Cresol	Sigma-ALDRICH	Germany
Phenol kristallin	AppliChem	Germany
Phenol:Chloroform:Isoamyl alcohol 25:24:1	AppliChem	Germany
Phenol:Chloroform:Isoamyl alcohol 25:24:1 + separate Tris - Solution	AppliChem	Germany
p-Nitrophenol	Carlo ERBA	Italy
Protein Ladder, PageRuler +Prestained, 26619	Pierce	USA
Resorcinol (1,3-Benzenediol)	Sigma-ALDRICH	Germany
SDS	MERCK	Germany
SDS loading buffer pack	Fermentas	USA
Sodium acetate anhydrous	AppliChem	Germany
Sodium Chloride	MERCK	Germany
Sodium Hydroxide pellets	MERCK	Germany
Sodium nitrate FCC, E 251	MERCK	Germany
TEMED	BIOMATIK	USA
Toluene (extra pure)	Riedel de Haen	Germany

Trichloroethylene (TCE)	Sigma-ALDRICH	Germany
Tris (hydroxymethyl)-aminomethane	MERCK	Germany

List of Enzymes

Restriction Endonucleases		
BamHI	Buffer 3	NEB, UK
KpnI	Buffer 1	NEB, UK
SacI	Buffer 1	NEB, UK
XhoI	Buffer 3	NEB, UK

DNA Polymerases		
Taq DNA polymerase	10x TaqPol Reaction Buffer (2mM Mg+2)	NEB, UK
Pfu Ultra II Fusion HS DNA polymerase	10x PfuII Reaction Buffer (2 mM Mg+2)	Stratagene, USA

DNA Ligase		
T4 DNA Ligase	10X Ligation Buffer	Fermentas, USA

APPENDIX B

List of Equipments

Analitical Balance	Explorer E12140, OHAUS, Switzerland
Autoclave	3870ELC, Tuttnauer, Netherlands
Centrifuge	Microfuge 16, Beckman Coulter, Germany
Centrifuge	Allegra X-22R, Beckman Coulter, Germany
Dry Bath	MK-10, BioJinn, China
Drying Oven	Ecocell, MMM Group, Germany
Electrophoresis Equipment	Bio-Rad, USA
Electrophoresis Power Supply	Power Source 300V, VWR, Singapore
Fume Hood	Labortex, Turkiye
High Performance Liquid Chromatography (HPLC)	LC-20A Prominence, Shimadzu, Japan
HPLC Column: Astec Cyclobond I2000	SupelCo analytical, USA
HPLC Column: Inertsil C8-3	GL Sciences Inc., Japan
HPLC Column: Nucleosil 100-5 C18	Agilent, USA
Ice Maker	AF80 Frimont, Scotsman, Italy
Incubator	EN500, Nüve, Turkey
Incubator Shaker	Innova 40R, New Brunswick Scientific, USA
Magnetic Stirrer	Labworld, Germany
Micro Syringe Pipette	Hamilton, Switzerland
Micropipettes	Research Plus, Eppendorf, USA
Microwave Oven	Beko, Turkey
NanoDrop	Thermo Scientific, USA
Orbital Shaker, Wisemix	RK-1D, Wisd Laboratory Instruments, Korea
pH meter	Hanna Instruments, Clarkson Laboratory, USA

Refrigerated Vapor Trap	Speed Vac Plus, USA
Refrigerator (-20°C/ +4°C)	Arcelik, Turkey
Spectrophotometer	6300, Jenway, UK
Thermal Cycler	VWR, Gene Technologies, UK
ThermoSavant	Speed Vac Plus, USA
Transilluminator	Bio-Rad GelDoc 2000, USA
Ultra Low Temperature Freezer (-80°C)	Elcold, Denmark
Ultrasonic Homogenizer	Sonopuls HD 2070, Bandelin, Germany
UV-Vis Spectrophotometer	Schimadzu, Japan
Vertical Electrophoresis System	MiniVE, PS300-B Hoefer, USA
Vertical Laminar Air Flow	SafeFast Classic, Italy
Vortex	ReaxTop, Heildolph, Germany
Water Purification System	Millipore, Milli-Q Direct-16, France

APPENDIX C

List of Molecular Biology Kits

TransformAid Bacterial Transformation Kit	Fermentas, USA
Coomassie (Bradford) Protein Assay Kit	Thermo Scientific, USA
PCR Clean-Up, Gel Extraction Kit	Nucleospin Extract II, MACHEREY-NAGEL, Germany
Plasmid DNA Purification Kit	Nucleospin Plasmid, MACHEREY-NAGEL, Germany
Protein Quantification Assay	MACHEREY-NAGEL, Germany

APPENDIX-D

List of Buffers and Solutions

10X Bovine Serum Albumin (BSA)	1. Add 10 µl BSA stock into 90 µl dH2O (autoclaved).
10X Running Buffer	1. Add 288 g glycine, 60.4 g Tris base, 20 g SDS in a glass bottle. 2. Complete the solution to 2 L by adding ddH_2O
30% Acrylamid/Bis (pH 7.0)	1. Add 29% acrylamide and 1% N,N'-methylene bisacrylamide in a dark bottle. 2. Store the solution at 25°C
50X TAE buffer (pH 8.5)	1. Add 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA in a glass bottle. 2. Complete the solution to 1 L by adding dH_2O.
Coomassie Blue Staining Solution	1. Add 53% ddH_2O, 40% methanol, 7% acetic acid and 0.25% Coomassie Brilliant Blue R-250 in a glass bottle.
Destaining Solution	1. Add 40% methanol, 7% acetic acid in a glass bottle. 2. Complete the solution to 1 L by adding ddH_2O.
EDTA (0.5 M, pH 8, final volume 200 ml)	1. Add 37.2 g disodium EDTA.$2H_2O$ into 150 ml dH_2O. 2. Adjust pH to 8.0 by adding NaOH pellets.

	3. Complete solution to 200 ml with dH_2O. 4. Autoclave the solution
Ferric Ammonium Sulfate Reagent (0.25 M, final volume 20 ml)	1. Add 0.25 M ferric ammonium sulfate in 10 ml, 9M HNO_3 solution. 2. Heat the solution at 50°C-100°C, for approximately 1 hour on magnetic stirrer, till the solution become clear.
Glycerol Stock Solution 50%	1. Add 5 ml ddH_2O into 5 ml glycerol 2. Autoclave the solution.
IPTG Stock Solution (0.8 M)	1. Add 0.2 g IPTG into 10 ml ddH_2O. 2. Filter the solution with 0.2 mm filter and syringe, load to microcentrifuge tubes. 3. Use 1 mM IPTG for induction of culture.
Kanamycin Stock Solution (25 mg/ml)	1. Into 0.25 g kanamycin, add 10 ml of ddH_2O. 2. Load solution into microcentrifuge tube via 0.2 mm filter and syringe. The concentration is 0.25 mg/ml. 3. For all experiments, adjust final concentration to 100 µg/ml.
Mercury (II) Thiocynate Reagent (saturated, final volume 20 ml)	1. Add 0.2 g mercury (II) thiocyanate into 20 ml 96% ethanol. 2. Shake the solution and then let to be sit for at least one hour. 3. Use supernatant for the sample measurement. The solution must be fresh in everyday usage.
Sodium Acetate (NaOAc) (3 M, pH 5.2)	1. Add 6.15 g NaoAc into 15 ml dH_2O. 2. Add 5 ml acetic acid till pH reachs to 5.2. 3. Complete solution to 25 ml by adding dH_2O. 4. Autoclave the solution.

TCE Stock Solution in DMF (100 mM, final volume 11 ml)	1. Inject 99 µl pure TCE into pure 11 ml dimethylformamide (DMF) in a serum bottle. 2. Cover the bottle with aluminum foil. TCE is light sensitive. 3. Shake the bottle at 37°C shaker, upside down. TCE must be fresh in everyday usage.
Tris (1 M, final volume 100 ml)	1. Add 12.1 g Tris (hydroxymethyl) aminomethane into 80 ml dH_2O. 2. Adjust pH to 7.8 by adding HCl (6 M). 3. Complete solution to 100 ml by adding dH_2O. 4. Autoclave the solution
Tris-EDTA (final volume 100 ml)	1. Add 10 mM Tris.Cl and 1 mM EDTA into 98.8 ml dH_2O (autoclaved).
Tris-HNO_3 Buffer (0.05 M, pH 7.0, final volume 1 L)	1. Add 6.06 g Tris (hydroxymethyl) aminomethane into 500 ml ddH_2O. 2. Prepare HNO_3 solution by adding 2.22 ml of 24 M stock in 500 ml ddH_2O. 3. Add 450 ml of HNO_3 into 500 ml Tris solution, and adjust pH to 7.0 by increasing HNO_3 amount. Complete the solution to 1 L with ddH_2O. 4. Autoclave Tris-HNO_3 solution before usage.