ERROR PRONE PCR MUTAGENESIS OF TOLUENE DIOXYGENASE OF *PSEUDOMONAS PUTIDA* T57 TO ENHANCE DEGRADATION OF 4-CHLOROANILINE

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การเปลี่ยนแปลงโทลูอีนไดออกซิจีเนสจาก *Pseudomonas putida* T57 โดยเทคนิค Error prone PCR เพื่อเพิ่มประสิทธิภาพการย่อยสลายของ 4-คลอโรอะนีลิน

นางสาวชนิกานต์ เลาหะจินดา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	ERROR PRONE PCR MUTAGENESIS OF TOLUENE DIOXYGENASEOF *PSEUDOMONAS PUTIDA* T57 TO ENHANCEDEGRADATION OF 4-CHLOROANILINE
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ชนิกานต์ เลาหะจินดา : การเปลี่ยนแปลงโทลูอีนไดออกซิจีเนสจาก *Pseudomonas putida* T57 โดยเทคนิค Error prone PCR เพื่อเพิ่มประสิทธิภาพการย่อยสลายของ 4-คลอโรอะนีลิน (ERROR PRONE PCR MUTAGENESIS OF TOLUENE DIOXYGENASEOF *PSEUDOMONAS PUTIDA* T57 TO ENHANCEDEGRADATION OF 4-CHLOROANILINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.อลิสา วังใน, 154 หน้า.

คลอโรอะนาลีนเป็นสารกลุ่มเอมีนอะโรมาติกที่เป็นส่วนประกอบสำคัญในการผลิตโพลียูรีเทน , สารเติมแต่งที่ใช้สำหรับผลิตยาง, สารกำจัดศัตรูพืช และ สีย้อม 4-คลอโรอะนิลีน (4-CA) เป็นสารหนึ่งในกลุ่มสารคลอโรอะนิลีน ซึ่งพบการสะสมจากการใช้และการผลิตสารในเกษตรกรรมและอุตสาหกรรม 4-คลอโรอะนิลีนมีความเป็นพิษต่อมนุษย์และสิ่งมีชีวิตจึงต้องมีการบำบัดด้วยกระบวนการที่เหมาะสม การบำบัดสารมลพิษทางชีวภาพเป็นหนึ่งในกระบวนการที่มีประสิทธิภาพโดยการใช้เชื้อจุลินทรีย์ทั้งเซลล์หรือเอนไซม์ จากรายงานเมื่อไม่นานมานี้แสดงให้เห็นว่า *Escherichia coli* (*E. coli*) รีคอมบิแนนท์ที่ได้รับยีน *todC1C2BA* ซึ่งเข้ารหัสให้เอนไซม์โทลูอีนไดออกซีจีเนสที่ได้มาจาก *Pseudomonas putida* T57 มีความสามารถในการย่อยสลายสาร 4-คลอโรอะนิลีนอย่างไรก็ตามการเร่งปฏิกิริยาดังกล่าวมีข้อจำกัดด้านปะสิทธิภาพ ดังนั้น ในการศึกษานี้จึงมีวัตถุประสงค์เพื่อที่จะเพิ่มประสิทธิภาพการย่อยสลายสาร 4-คลอโรอะนิลีนโดยการใช้เทคนิค Error prone PCR เพื่อเปลี่ยนแปลงหรือกลายยีน *todC1C2* แบบสุ่ม สร้างเป็นรีคอมบิแนนท์พลาสมิดที่มียีนโทลูอีนไดออกซีจีเนสกลายและทรานฟอร์มเข้า *E. coli* เพื่อ ศึกษาอัตราการย่อยสลายของสารโดยใช้วิธี Gibbs ที่มีการดัดแปลง การวิเคราะห์ผลโดยการเปรียบเทียบกับอัตราการย่อยสลายสารของสายพันธุ์ต้นแบบ จากนั้นระบุตำแหน่งของบริเวณที่มีการเปลี่ยนแปลงของกรดอะมิโนในเอนไซม์กลายโดยการวิเคราะห์ผลของลำดับนิวคลีโอไทด์ หลังจากผ่านกระบวนการเปลี่ยนแปลงแบบสุ่มหนึ่งครั้ง *E. coli* ที่มีการเปลี่ยนแปลงยีนโทลูอีนไดออกซีจีเนสจะได้รับการคัดเลือกด้วยการย่อยสลายของสาร 4-คลอโรอะนิลีน หรือ อะนิลีนที่สูงโดยวิธีการของ Gibbs ที่มีการดัดแปลง จากนั้นยืนยันผลโดยใช้เทคนิคไฮเปอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟฟี (HPLC) เมื่อเปรียบเทียบค่าจลนศาสตร์ของเอนไซม์ที่ได้รับการเปลี่ยนแปลงแสดงให้เห็นว่าประสิทธิภาพของตัวเร่งปฏิกิริยาดีขึ้นจากเอนไซม์ต้นแบบเดิม และตำแหน่ง E339 และ V345 ของ *todC1* ที่เกิดการเปลี่ยนแปลงนี้มีความเกี่ยวข้องกับบริเวณการจับกับสับสเตรท

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: 4-CHLOROANILINE / BIODEGRADATION / ERROR PRONE PCR / TOLUENE DIOXYGENASE / PSEUDOMONAS PUTIDA T57

CHANIKAN LAOHAJINDA: ERROR PRONE PCR MUTAGENESIS OF TOLUENE DIOXYGENASEOF *PSEUDOMONAS PUTIDA* T57 TO ENHANCEDEGRADATION OF 4-CHLOROANILINE. ADVISOR: ASSOC. PROF.ALISA VANGNAI, Ph.D., 154 pp.

Chloroanilines are important intermediates for the production of polyurethanes, rubber additives, pesticides and dye. 4-Chloroaniline (4CA) is one of chloroaniline group, which is an aromatic amine ubiquitously detected to accumulate during the growing of agricultural and industrial activities. 4CA is toxic toward human and living organism, so it requires appropriate technique for treatment. Bioremediation is an effective technique using whole-cell microorganisms or enzymes. The recent report demonstrated that *Escherichia coli* recombinant strain harboring *todC1C2BA*, the gene encoding toluene dioxygenase from *Pseudomonas putida* T57, was able to oxidize 4CA, but with limited efficiency. Consequently, this study aimed to enhance the enzyme efficiency toward 4CA using error prone PCR technique to random mutagenesis of *todC1C2* gene. The degradation rate of the obtained *E. coli* recombinant strain (pET21a*todC1C2'BA*) was determined by the modified Gibbs method. The degradation rate of the mutants was then compared with that of the wild-type. The changing in amino acid residues of the mutated enzyme was identified by nucleotide sequence analysis. After one round of random mutagenesis, the mutants were selected with the higher degradation rate of 4CA or aniline using the modified Gibbs method. The selected mutants were confirmed the degradation rate by HPLC analysis. The kinetics parameter of mutants showed the high catalytic efficiency compared to the wild-type enzyme. The mutated position (E339 and V345) of *todC1* revealed to be involved in substrate binding site.

Field of Study: Environmental Management Student's Signature .....

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CHAPTER 1

INTRODUCTION

1.1 State of problem

The growing agricultural and industrial activities increase the consumption of the chemicals in Thailand. The extensive use of these chemicals causes the accumulation and leads to a concern on their environmental impact. Among these, 4-chloroaniline (4CA), an intermediate of pesticides and herbicides, becomes one contaminant of interest. In addition to the release from agricultural activities, 4CA is one of the most important amines widely used in the production of polyurethanes, rubber, dyes (e.g., Acid Red 119:1, Pigment Red 184, and Pigment Orange 44), pharmaceutics and photographic chemicals (Jiang et al., 1996; Yu et al., 2001) The releases of industrial wastewater also contribute to the contamination of 4CA in the environment. 4CA is considered as an environmental pollutants by the Priority Pollutant List of U.S. Environmental Protection Agency (Eisenthal et al., 2007), which can be found in soil, water and sludge. The reported contamination range of 4CA is 1.5 - 50.4 µg/litre in soil (Freitag et al., 1985; Fuchsbichler, 1977; Rippen et al., 1982). 0.1 - 1.0 µg/litre in surface water (Abfall and Landesumweltamt, 1988), whereas 4CA was not detected in groundwater (detection limit 0.2 µg/litre) (Mutanen et al., 1988b).

The accumulation of 4CA can adversely affect to humans health *via* inhalation and simultaneous dermal absorption. This resulted in cyanosis, increased methaemoglobin and sulfhaemoglobin levels, the development of anaemia, and acute intoxication (Pacseri et al., 1958).

Due to its high toxicity, the remediation of 4CA is required. The contamination of 4CA can be treated via chemical, physical and biological processes (Arora et al., 2012b). Among these, the biological treatment gains a lot of interests since it is more environmental-friendly alternative (Tongarun et al., 2008). Chloroanilines (CAs) are proposed to be degraded *via* aniline dioxygenase, chloroaniline dioxygenase (Hongsawat and Vangnai, 2011b; Liu et al., 2002b).

Pseudomonas putida T57 is the bacterial strain, which was isolated from an activated sludge and was tolerant to organic solvents. They modified the toluene dioxygenase pathway in this strain for increasing the production of cresol from toluene in the two-phase system. (Koshland Jr, 2002). Normally, toluene could be a main substrate for toluene dioxygenase (Dennis and Zylstra, 2004; Morimoto et al., 2014; Parales, 2003); nevertheless, it also degraded aromatic compounds such as benzene, ethylbenzene, pyridine, phenol, biphenyl etc. (Brillas et al., 1995; Hurtubise et al., 1995; Kim and Lei, 2008; Wen-Chen and Gibson, 1994). Recently, Nitisakulkan and collaborators (2014) investigated that toluene dioxygenase from *P. putida* T57 is able to transform 4CA up to approximately 40%. The ability of toluene dioxygenase from *P. putida* strain T57 in 4CA degradation and the proposed pathway has been reported (Nitisakulkan et al., 2014). The information of this enzyme in 4CA degradation is still limited. Thus, this study aimed to improve ability of toluene dioxygenase to transform 4CA.

In conclusion, this study was divided in to 3 parts: i) overexpression of toluene dioxygenase in *E. coli* recombinant and random mutagenesis, ii) characterization and comparison of the enzyme catalytic activity (wild type and mutated ones) toward

4CA. iii) identification and analysis of the point mutation within toluene dioxygenase. The expected outcome of this study is the genetically engineered toluene dioxygenase with improved catalytic activity towards 4CA.

1.2 Objective

The main objectives of the research were to investigate of 4CA degradation by toluene dioxygenase (*todC1C2BA* gene) in recombinant strain and to improve the activity of toluene dioxygenase activity toward 4CA. This research focused on the kinetic of enzyme related to 4CA degradation and enhancing enzyme efficiency.

1. To improve degradation efficiency (activity or specificity) using error prone PCR technique.
2. To develop the method for high-throughput screening of mutated toluene dioxygenase activity.
3. To identify amino acid residue(s) involved in 4CA degradation enhancement.

1.3 Hypothesis

The modification of toluene dioxygenase increases catalytic efficiency of enzyme towards 4CA.

1.4 Scope of study

1. Overexpression of toluene dioxygenase in *E. coli* recombinant and random mutagenesis

1.1 Reconstruction of plasmid and expression of *todC1C2BA* in *E. coli* BL21(DE3) recombinant strain

1.2 Determination of whole cell biodegradation activity (HPLC analysis)

1.3 Random mutagenesis of *todC1C2* by error prone PCR technique and Screening the mutant clone by modified Gibbs method.

2. Characterization and comparison of the enzyme catalytic activity (wild type and mutated ones) toward 4CA

2.1 Determination of 4CA biotransformation rate

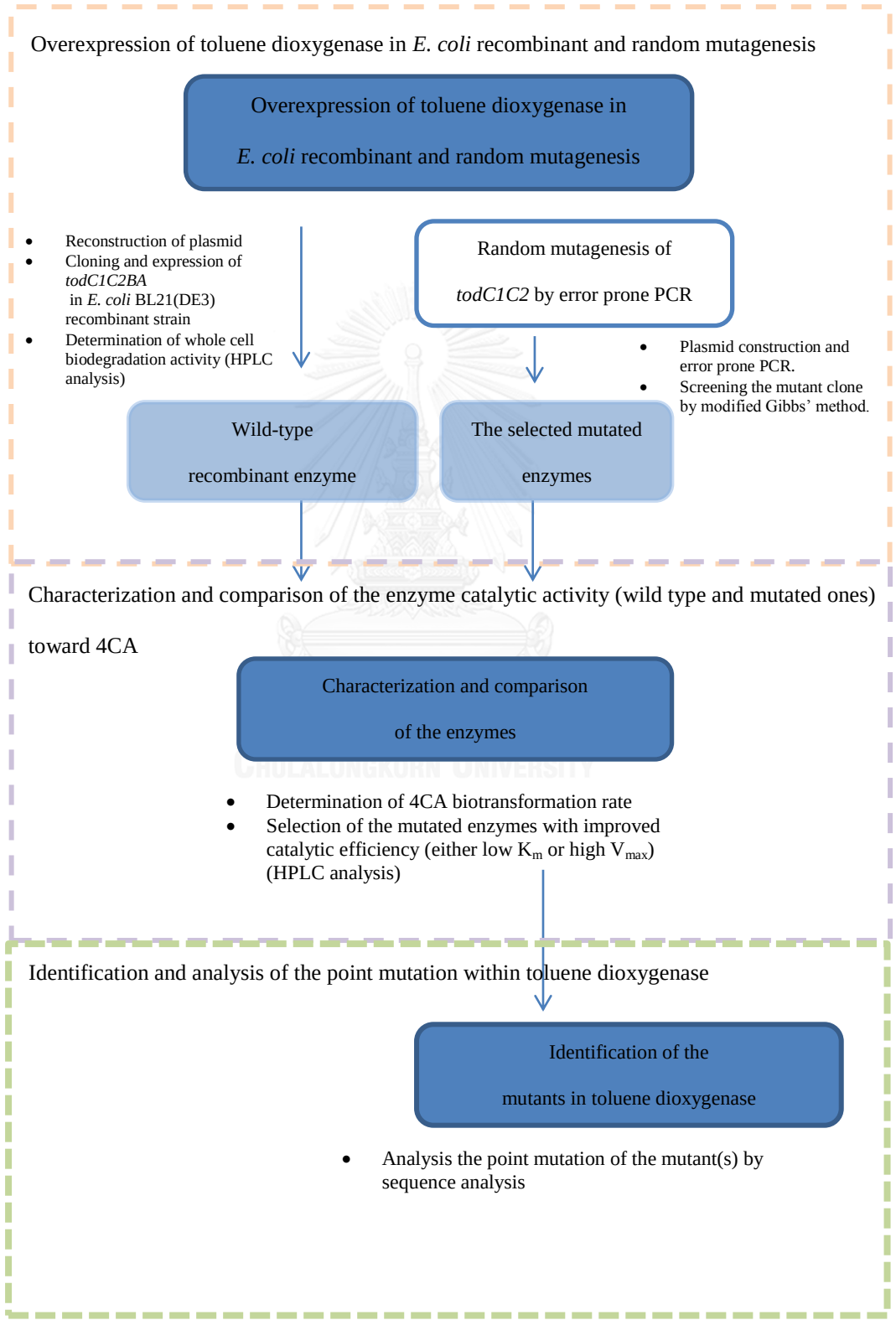
2.2 Selection of the mutated enzymes with improved catalytic efficiency

3. Identification and analysis of the point mutation within toluene dioxygenase.

Analysis the point mutation of the mutant(s) by sequence analysis

1.5 Experimental framework

Figure1.1 Frame work of the study



CHAPTER 2

LITERATURE REVIEW

This chapter was divided in to two topics, which are chloroanilines and toluene dioxygenase.

The first topic is Chloroanilines, This topic consists of following subtopics.

1.1 Properties of 4-chloroaniline

1.2 Chloroaniline production and usage

1.3 Contamination of chloroanilines in environment

1.4 Fate of 4-chloroanilie in the environment

1.5 The treatment methods of chloroanilines

1.6 Biodegradation of chloroanilines

Another is Toluene dioxygenase, this topic consists of following subtopics.

2.1 The properties of toluene dioxygenase

2.2 The toluene dioxygenase pathway involved in 4-chloroaniline degradation

2.3 Substrate specificity of toluene dioxygenase

2.4 Protein engineering and directed evolution of toluene dioxygenase

2.1 4-Chloroanilines

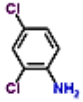
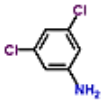
2.1.1 Properties of 4-Chloroanilines

Types of chloroaniline depend on the position of chloroanilines. 4-Chloroaniline (4CA) is colorless crystalline solid aniline which is para-position.

The chemical formula is C_6H_6ClN, and its relative molecular mass is 127.57. Its molecular structure shows in Figure 2. Its IUPAC name is 1-amino-4 chlorobenzene; other names include PCA, *p*-chloroaniline, 1-chloro-4-aminobenzene, 4-chloro-1-aminobenzene, 4-chlorobenzenamine, 4-chloroaminobenzene, and 4-chlorophenylamine. Depending on the purity of the product, the substance melts between 69 and 73 °C. Its boiling point is given as 232 °C (BUA, 1995b). The water solubility of 4CA is 2.6 g/L at 20 °C (Scheunert, 1981). The chloroanilines properties showed in Table 2.1.

Figure2.1 Structure of 4-chloroaniline

Table 2.1 Chloroaniline properties (Blank et al., 2008)

Chloroanilines	Abbreviation	formation	Chloro-position	Melting point (°C)	Water solubility (25°C)	Vapor pressure (25°C)	Octanol-water partition coefficient ($LogK_{ow}$)
2-chloroaniline	2CA		ortho- chloroaniline	-2	2241 mg/L	27.2 Pa (0.204 mm Hg)	1.90
3-chloroaniline	3CA		meta- chloroaniline	-10	2331 mg/L	8.8 Pa (0.066 mm Hg)	1.88
4-chloroaniline	4CA		para- chloroaniline	67-72 °C	2572 mg/L	10.6 Pa (0.0796 mm Hg)	1.83
2,4-Dichloroaniline	24DCA		ortho,para-dichloroaniline	59-62 °C	283.1 mg/L	4.59 Pa (0.0344 mm Hg)	2.78
2,5-Dichloroaniline	25DCA		para-Dichloroaniline	47-50 °C	300.3 mg/l	3.37 Pa (0.0253 mm Hg)	2.75
3,5-Dichloroaniline	35DCA		meta-dichloroaniline	49-53 °C	223.6 mg/L	2.01 Pa (0.0151 mm Hg)	2.90

2.1.2 4-chloroaniline productions and usage

4-chloroaniline (4CA) is normally used as an ingredient in the manufacture of products including herbicides and insecticides (e.g., monuron, diflubenzuron, monolinuron), azo dyes, pigments, pharmaceutical and cosmetic products. Consequently, releasing of 4CA into the environment may occur from industrial such as production, processing, dyeing /printing industry. 4CA is listed as an antimicrobial agent used in cosmetic product. During their degradation, 4CA may emerge and contaminate in environment (Yashrasayan, 2003).

Monuron is one of pesticides, which commonly used in Thailand. During the degradation of monuron, 4CA will accumulate in the environment. Bobu and colleague reported the monuron and its degradation products (Bobu et al., 2006). They showed that 4CA is monuron degradation product.

4CA is rapidly absorbed and metabolized. The reactive metabolites of 4CA bind covalently to haemoglobin and to proteins of liver and kidney. In humans, haemoglobin adducts are detectable as early as 30 min after accidental exposure, with a maximum level at 3 h. Oral LD_{50} values of 300.42 mg/kg body weight for rats, 228.5 mg/kg body weight for mice, and 350 mg/kg body weights for guinea-pigs are reported. Similar values have been found for intraperitoneal and dermal application of 4CA to rats, rabbits, and cats. An LC_{50} value for rats was given as 2,340 mg/m^3. The prominent toxic effect is methaemoglobin formation. 4CA is reported a more potent and faster methaemoglobin inducer than aniline. 4CA also exhibits a nephrotoxic and hepatotoxic potential (Boehncke et al., 2003). The International Agency for Research on Cancer (IARC, 1993) has classified 4-chloroaniline in Group 2B (possibly

carcinogenic to humans) based on inadequate evidence in humans and sufficient evidence in experimental animals for the carcinogenicity of 4-chloroaniline.

The LOAELs (lowest tested dosages, NOELs not derivable) for significant increases of methaemoglobin have been reported for a 13-week oral exposure by gavage of 5 mg/kg body weight in rats and 7.5 mg/kg body weight in mice and 2 mg/kg body weight per day for 26-103 weeks' oral (gavage) application to rats. WHO reported estimation of maximal consumer exposure at the maximum of 300 (ng/kg body weight per day) and the further effects was reported to concern, which are carcinogenicity and possibly skin sensitization (A. Boehncke et al., 2003)

2.1.3 Contamination of chloroanilines in environment

Chloroanilines have been one of the most important chemicals in many industrial pesticides, and polymers such as polyurethane and rubber additive (service, 1998; Yen et al., 2008). Owing to their widespread use and detected in many industrial waste water, it has the effect to aquatic organisms, which is impeded in the oxygen carrying capacity of the blood supply of aquatic organisms (Lacorte et al., 1999; Liu et al., 2002a; Rai et al., 2005; Sarasa et al., 1998). The effect resulting of chloroaniline acute exposure by inhalation or oral route, is methemoglobinemia, usually accompanied with anoxia, erythrocyte damage and spleen effects. (Amund et al., 2013; Bomhard and Herbold, 2005; Edgell and Wilbers, 1989; Site).

The EPA Great Lakes Water Quality Initiative were reported about the food chain values for water by screening benchmarks for freshwater in Region 3 (Mid-Atlantic) found that 4CA had screening value 232 µg/L in the fresh water.(Pluta, 2006)

The releases from the production of 4CA at the German manufacturers in 1990 were as follows: <20 g/tonne produced released into air at each site (derived from the registry limit of the emission declaration of 25 kg/year), and 13 g/tonne produced released into surface water. The annual wastes are estimated to be a maximum of 400 g 4CA/tonne produced. These wastes are disposed of in special company incinerators (BUA, 1995a)

4CA was reported to release into surface waters from the use of dyed textiles and printed papers. BUA (1995) reported that the residual 4CA content of <100 mg/kg is reported for German dye products (BUA, 1995a). In 1985, the 6.1 tonnes of monochloroanilines (sum of 2CA, 3CA,and 4CA) was detected from industrial processes, were estimated to be released to the river Rhine (IAWR, 1998).

In the 1980s and 1990s, 4CA was also found in German drinking-water samples at concentrations between 0.007 and 0.013 µg/L (BUA, 1995). In Finland (1984), 4CA was not detected (detection limit 0.2 µg/L) in groundwater after the application of the insecticide diflubenzuron (Mutanen et al., 1988a) In the groundwater below a Danish landfill site containing domestic wastes and wastes from pharmaceutical production, PCA was detected at concentrations between <10 µg/L (depth 5.5 m) and 50 µg/L (depth 8.5 m). For the pharmaceutical production (e.g., sulfonamides), 4CA was form from the waste water during the processing. (Holm and Sander, 1995). In 1995–96, 4CA was found in groundwater from three sites in an industrialized area near Milan, Italy. Fattore and colleague reported that 4CA concentrations was detected between 0.01 and 0.06 µg/L (positive results in four of seven wells) (Fattore and Jommi, 1998)

USEPA reported the production and usage of pesticide that several classes of nitrogenous pesticides have been produced since 1945. Herbicidal activity was discovered in 1946 for substituted urea and a series of “uron” were introduced subsequently, for example, monuron, diuron, fenuron, and noruron. During the degradation of these pesticides, 4CA is accumulated in the environment (Parry, 1998).

In Thailand, the pesticides including monuron and diuron have been reported to import for usage in agricultural fields (OIE). The Central Lab Thai, Department of Agriculture (DOA), Thailand reported the regulation of pesticide screening on aubergines and ginger. Chloroaniline is also concerned by Central Lab Thai (CLT, 2010).

2.1.4 Fate of 4-chloraniline in the environment

Chloroanilines are commonly found in wastewater (Lacorte et al., 1999; Sarasa et al., 1998) during the production of polyurethanes, rubber, azo dyes, drugs, photographic chemicals, varnishes, and pesticides at concentrations ranging from 12 (Lacorte et al., 1999) to 230 mg/L (Jen et al., 2001b). Thus, it is targeted to remove from environmental water. 4CA has been reported to contaminate about 0.1 μg/L of the Rhine River during 1976-78. For the bank and dune filtration of water, the levels were 0.03-0.1 μg/L and less than 0.01 μg/L of 4CA, respectively. The half-life of 4-chloroaniline in water was 0.3-3 day in river water and 30-300 days in ground water (Zoeteman et al., 1980). The detection threshold level in Canada was 20-30 pg. In soil, 4CA binds to humid materials and it is slowly degraded by aerobic and anaerobic processes (Hamby, 1996). Releasing of 4CA in environment from the application of pesticides was also reported (Jen et al., 2001a; Lacorte et al., 1999; Zoeteman et al.,

1980). Nevertheless, 4CA concentration in wild mushrooms, blueberries, and cranberries were below the detection limit of 10–20 µg/kg after the treatment of diflubenzuron in a forest area in Finland in 1984 (Mutanen et al., 1988).

In Finland (2008), Ulla Karlström and coworker reported the residues of 256 different pesticides which detected in fruit, vegetables, cereals and processed foods. They reported the chloroaniline also found in fruit and vegetable (Ulla Karlström, 2008).

The Central Lab Thai, Department of Agriculture (DOA), Thailand reported the regulation of pesticide screening on aubergines and ginger. Chloroaniline is also concerned by Central Lab Thai. They reported the Maximum Residue Limits (MRL) of 3-chloroaniline (3CA) is 0.05 and 0.1mg/kg, respectively (CLT, 2010).

2.1.5 The treatment methods of chloroanilines

The treatment methods can be divided in to 3 treatments which are physical, chemical and biological treatments.

The physical treatments are the removal of the hazard through physical methods including capping, cementitious waste forms, electro kinetic remediation, incineration techniques, etc. (Hamby, 1996). HSU and BARTHA (1973) proposed that chloroaniline residues can be immobilized by physical absorption (HSU and BARTHA, 1973). Garcia et al.,(2006) reported that Multi-walled carbon nanotubes (MWCNT) can be efficiently used to remove aniline and its derivatives. .The 100% aniline removal was achieved after 45 min of reaction with an initial aniline removal rate of 245 µmol min^{-1} g^{-1}_{cat} (Garcia et al., 2006).

Air stripping is one technique ,which applied in industrial wastewater treatment (A. Srinivasan, 2008). But, it also has the limitation that air stripping is the simplify method to transfer the contaminant from one medium to another. Thus, it has to provide the necessary protection to public health (Michael D. LaGrega et al., 2001). The air stripping was applied to use in the fermentation by microorganism to degrade phenolic substrate. This air stripping parameter was found to have a value of 0.0033 h^{-1} for phenol molecules in a typical fermentation situation. (Singh and Hill, 1987)

The chemical treatment

The chemical treatments involve the application of agents to promote extraction of the hazardous substance for instance, electrochemical technologies, chemical immobilization, critical fluid extraction, oxidation, photodegradation, etc. (Hamby, 1996)

Electrochemical technologies have many methods such as electrodeposition, electrocoagulation (EC), electroflotation (EF) and electrooxidation. Electrodeposition is used in heavy metals recovery effectively from wastewater streams. Electrocoagulation (EC) is used for water production or water treatment. For the treatment water of the flocculated sludge is achieved by using electroflotation (EF) which is efficient in removing colloidal particles, oil and grease, as well as organic pollutions (Chen, 2004). Kadar and coworker also used the electrochemical oxidation technique to isolate and identify the product of 4-chloroaniline, 2,4-dichloroaniline and 2,4,6-trichloroaniline in acetonitrile(Kádár et al., 2001). Zettersten and coworker (2009) was studied the oxidation of 4CA by electrochemistry (EC) coupled on-line with electrospray ionization mass spectrometry (ESI-MS) using two electrochemical

flow cells of different design. They reported that the conversion efficiency of 4CA was about 50% under mass transport controlled conditions (Zettersten et al., 2009)

Sarasa and colleague reported that ozone and chemical coagulation is used to degrade the chlorinated wastewater such as the production of azoic dye. The aniline derivatives and azo compounds are the majority of compounds, which found in the waste water. Chloroanilines, chloronitrobenzenes and chlorophenols are subject to be treated. The concentration of compounds was decreased after ozonation Nevertheless, chloroanilines and its derivatives are formed after chemical coagulation due to the strong basic medium at which the sample in this process is set. After ozonation, C.O.D. was reduced 25%, while it decreased 50% after the complete treatment. Coagulation treatment with $Ca(OH)_2$ was effective in removing the remaining compounds of ozonation (Sarasa et al., 1998).

Photodegradation was also the technique to treat 4CA. This method can be complete mineralization of toxic substrates. The final products are CO_2, H_2O and mineral acids. Augugliaro and colleague reported that the heterogeneous photocatalysis is an appropriate method for the complete photodegradation of aniline, 4-ethylaniline and 4-chloroaniline in aqueous medium. They investigated the complete mineralization of substrates lasted 14 h (Augugliaro et al., 2000). Gosetti and colleague reported that the natural degradation behavior of 4CA in the surface water when the sun light irradiation was exposed and the calculated $t_{1/2}$ value is around 29 min, but the photodegradation product was reported more toxic than precursor (Gosetti et al., 2010).

The reported of Sanchez and coworkers (2002) showed that 4CA could be degraded by gamma rays in aqueous solution. The results from radiation-induced decomposition of 4CA under various conditions showed that 4CA was degraded around 40-80% in various conditions (Sánchez et al., 2002). The degradation of 4CA by persulfate activated with zero-valent iron (ZVI) was investigated by Hussain and colleague. The results showed that the complete degradation of 4CA was obtained by ZVI-activated persulfate at pH 4.0 at 12 min. Thus, 4CA and its intermediates could be mineralized into CO_2 and H_2O (Hussain et al., 2012)

The physical-chemical treatment can be treated 4CA, but they have some limitation, for example one step of treatment can't be complete degradation (Sarasa et al., 1998) and after the photodegradation, Gosetti and coworkers (2010) reported that the ecotoxicological biotests results indicated that the photodegradation products are more toxic than 4CA itself (Gosetti et al., 2010). The methods cannot be complete mineralization the pollutions in one step compare with biological treatment. Although the physical-chemical treatments are more effective, but they are expensive and require high energy demand and consumption of many chemical reagents (Mrozik and Piotrowska-Seget, 2010). And the other reason is some method required to excavate contaminated soil for treatment (Michael D. LaGrega et al., 2001)

The biological treatments

The several methods, which had effective to remove the pollutant from soil and water, were reported. They involved in physical, chemical and biological approaches. The physical and chemical approaches are effective methods, but they are

expensive and require the energy and many chemicals reagent for operation. Thus, microorganism capable to degrade toxic compound known as bioremediation has be attractive to apply in treatment method.

The biological treatment is biodegradation of organic compounds by microorganisms. This technique is generally used because of their advantages including low land requirement, low capital and operating costs. It is also a cost-effective technique for degradation of CAs in the soil and water. Moreover, its capability for in-situ treatment is not having to excavate contaminated soil (Arora et al., 2012a; Michael D. LaGrega et al., 2001). The biological treatment processes have been conducted with the optimum temperature range, moisture, acidity or alkalinity. However, biological treatment can be mineralized toxic pollutants to be non-toxic that is the most advantage of this treatment (Michael D. LaGrega et al., 2001). The biological treatment consists of many technologies, for example, the established technologies, which are biofilm processes, nitrogen removal, membrane processes (membrance bioreactor; MBR), anaerobic processes, such as Anaerobic Sequencing Batch Reactor (ASBR®), Upflow Anaerobic Sludge Blanket (UASB), ANFLOW (ANaerobic FLuidized Bed Reactor) (water.epa.gov, 2013). The innovative technologies, which are bioaugmentation (Parker, 2007), solids minimization, solids settleability., etc (water.epa.gov, 2013)

Biostimulation is the method that adds the nutrients to a polluted site in order to encourage the growth of naturally occurring chemical-degrading microorganisms. While, bioattenuation is the method to relies on natural processes to dissipate contaminants through biological transformation. The bioaugmentation approach

should be applied when the biostimulation and bioattenuation have failed. Mrozik and Piotrowska-Seget (2010) reported that the percentages of 4CA removal from soil reached the values of 43% and 45% for bioaugmentation and biostimulation, respectively. whereas only 4% of added 4CA was degraded by bioattenuation (Mrozik and Piotrowska-Seget, 2010).

Zhu and colleague (2012) reported the degradation of chloroanilines and aniline by aerobic granule. The aerobic granule is self-immobilised and mixed-culture microbial aggregates. It has ability to degrade toxic organic compounds. They showed the enhancing the degradability of toxic organic compounds because of the formation of aerobic granule favours. The results showed that aniline was completely degraded after 2 h. While, mono-chloroanilines (2CA, 3CA and 4CA) were completely degraded at 4 h, and 3,4-DCA degradation occurred 4 h after the disappearance of mono-chloroanilines (Zhu et al., 2012).

The biodegradation have been developed to degrade 4CA, based on different microorganisms and techniques. (Hongsawat and Vangnai, 2011a; Nitisakulkan et al., 2014; Vangnai and Petchkroh, 2007; Zhang et al., 2010)

2.1.6 Biodegradation of chloroanilines

Biodegradation is a process to remove or convert toxic substance into nontoxic ones by microorganisms. This process is widely used because it is environmental friendly and cost effectively. Chlorinated nitroaromatic compounds such as aniline, chloroanilines has been studied and several metabolic pathways have been proposed. Liu et al. 2002 proposed the pathway of aniline degradation in *Delftia* sp.AN3 (Figure

2.2). They indicated that cleavage of the benzene ring via catechol 2, 3-dioxygenase. Chloroanilines can be degraded by microorganisms. According to various studies, bacteria species of *Pseudomonas* strain G (Figure 2.4) (Zeyer et al., 1985), *Acinetobacter baumannii* CA2, *Pseudomonas putida* CA16, *Klebsiella* sp. CA17(Vangnai and Petchkroh, 2007), *Delftia tsuruhatensis* H1(Zhang et al., 2010), *Acinetobacter baylyi* GFJ2 (Figure 2.3) (Hongsawat and Vangnai, 2011b) are able to degrade CAs via aniline dioxygenase or chloroaniline dioxygenase. The pathway of monochloroaniline degradation in some of these strains was found to lead directly to a modified *ortho*- or *meta*-cleavage pathway after oxidation of the monochloroaniline to the corresponding chlorocatechol. The previous reported showed the chloroanilines and biodegradation by whole cell. Generally, chloroanilines group was degraded *via* chloroaniline dioxygenase or aniline dioxygenase. Interestingly, 4CA could also degraded by toluene dioxygenase of *Pseudomonas putida* T57 (Table 2.2).

Recently, Nitisakulkan and collaborators (2014) reported the ability of toluene dioxygenase from *P. putida* T57 in 4CA degradation. The 4CA degradation rate by *P. putida* T57 is 5.7 mM/h. The metabolic pathways of 4CA by toluene dioxygenase have also been reported.

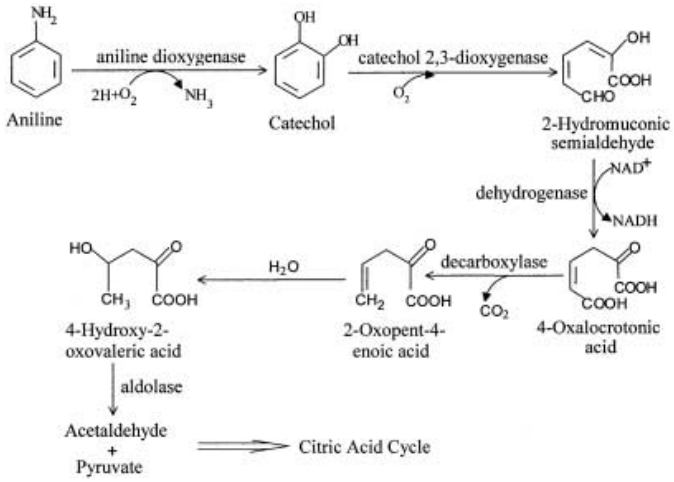


Figure2.2 Proposed pathway of aniline degradation in *Delftia* sp. AN3 (Ang et al., 2009)

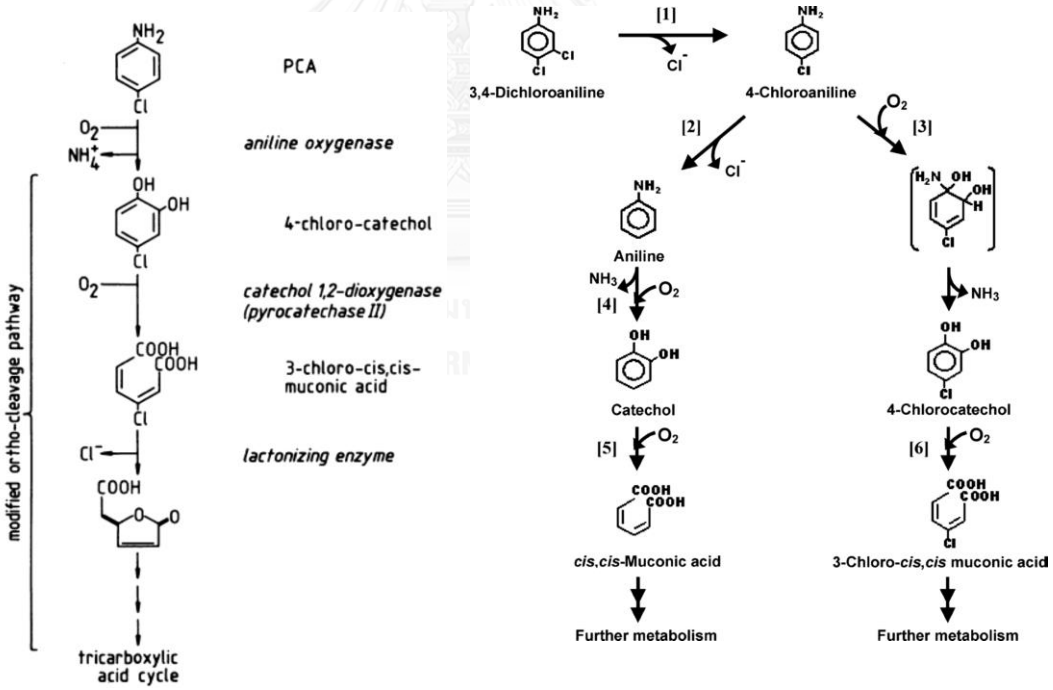


Figure2.3 Biodegradation pathway of 3,4-dichloroaniline and 4-chloroaniline in *Acinetobacter baylyi* GFJ2. (Hongsawat and Vangnai, 2011b)

Figure2.4 Pathway of 4CA degradation by *Pseudomonas* strain G (Zeyer et al., 1985)

Table 2.2 Chloroanilines degradation by microorganism

Strains	Key enzyme	Substrate	Performance	References
P. putida UWC3(pWDL7::*rfp*)	*dcaA1A2B*	3-chloroaniline (3CA)	The *dcaA1A2B* gene products expressed from a high-copy-number vector were shown to convert 3-CA to 4-chlorocatechol in *Escherichia coli*.	(Dejonghe et al., 2002; Krol et al., 2012)
Chryseobacterium indologenes SB1	Catechol 1,2-dioxygenase (C12DO)	Aniline+4CA	The enzyme had a much higher conversion rate for catechol [156 U (g protein)$^{-1}$] than for 4-chlorocatechol [17.2 U (g protein)$^{-1}$]	(Radianingtyas et al., 2003)
Comamonas testosteroni SB2				
Pseudomonas corrugata SB4				
Stenotrophomonas maltophilia SB5				
Acinetobacter baumannii CA2	Chlorocatechol 1,2-dioxygenase (CC12DO) (Modified ortho-cleavage pathway	4-chloroaniline (4CA)	61.5±3.44% the degradation efficiency within 12 days	(Vangnai and Petchkroh, 2007)
Pseudomonas putida CA16			63.7±3.67% the degradation efficiency within 12 days	
Klebsiella sp. CA17			73.1±3.72%. the degradation efficiency within 12 days	
Acinetobacter sp. YAA	*AtdB* :Catechol 2,3-dioxygenase (C23O)	Aniline	V_{max} 53.7±0.9 Umg-1 Km 1.47 ±0.01 µM	(Takeo et al., 2007)
Delftia tsuruhatensis H1	Catechol 1,2-dioxygenase (C12DO) and Chlorocatechol 1,2-dioxygenase (CC12DO)	2CA	degradation rate 0.30 ± 0.06 mg/hr	(Zhang et al., 2010)
		3CA	13.0 ± 0.08 mg/hr	
		4CA	12.6 ± 0.08 mg/hr	
Variovorax sp. WDL1	Aniline dioxygenase	3,4-dichloroaniline (3,4 DCA)	ND	(Breugelmans et al., 2010)

Table 2.2 Chloroanilines degradation by microorganisms (Continued)

Acinetobacter baylyi GFJ2	Catechol 1,2-dioxygenase (C12DO) and Chlorocatechol 1,2-dioxygenase (CC12DO)	Aniline	81±7% degradation in 72 hr 0.062±0.002 µmole h^{-1} mgcell $protein^{-1}$	(Hongsawat and Vangnai, 2011)
		2CA	68±5% degradation in 72 hr 0.022±0.003 µmole h^{-1} mgcell $protein^{-1}$	
		3CA	94±1% degradation in 72 hr 0.082±0.014 µmole h^{-1} mgcell $protein^{-1}$	
		4CA	97±3% degradation in 72 hr 0.087±0.016 µmole h^{-1} mgcell $protein^{-1}$	
Pseudomonas putida T57	Toluene dioxygenase ; ortho-cleavage pathway and meta-cleavage pathway	2CA	50 % degradation in 6 hr	(Nitisakulkan et al., 2014)
		3CA	80% degradation in 6 hr	
		4CA	Complete degradation in 2 hr	
		34DCA	< 30% degradation in 6 hr	
		35DCA	< 30% degradation in 6 hr	
E. coli DH5α (pHK-C1C2BA)		4CA	40% degradation in 12 hr	

2.2 Toluene dioxygenase

Aniline was reported to be complete degradation *via* aniline dioxygenase to eventually TCA cycle intermediate and it also reported about improving of their efficiency (Ang et al., 2009). Nevertheless, chloroanilines degradation is still limited the specific gene and enzyme for the transformation of chloroanilines. Latorre and colleague (1984) reported that during 2CA and 3CA metabolism generated 3-chlorocatechol (3CC), while 4-chlorocatecchol (4CC) was generated during 3CA and 4CA metabolism (Latorre et al., 1984). Dejonghe and coworker (2002) investigated that 3CA and 3,4 DCA can degrade by several microorganism (*Comamonas*, *Delftia*, *Acidovorax*, *Achromobacter*, and *Pseudomonas*), which contained an IncP-1L plasmid that was identified as an important role for degradation (Dejonghe et al., 2002) and the gene was identified as *dcaA1A2B*. Meanwhile, the gene was involved in 4CA metabolism was limited reported. Recently, *Pseudomonas putida* T57 was reported to completely degrade 4CA. Toluene dioxygenase from *P. putida* T57 was reported to involve in 4CA degradation.

2.2.1 The properties of toluene dioxygenase

Toluene dioxygenase is multiple component enzymes which encoded by *todC1C2BA* gene that is a heterohexamer containing a catalytic and a structural subunit. The catalytic subunit contains a Rieske [2Fe–2S] cluster and mononuclear iron at the active site. The toluene dioxygenase system is consisted of three main proteins that is a reductase (TDO-R), a Rieske [2Fe–2S] ferredoxin (TDO-F) and two

subunits of terminal dioxygenase (TDO-O), which is *todA todB* and *todC1C2* respectively. The tod*B* shuttles electrons from NADH *via* a flavin to *todA* to *todC1C2* (Figure 2.5) (Friemann et al., 2009).

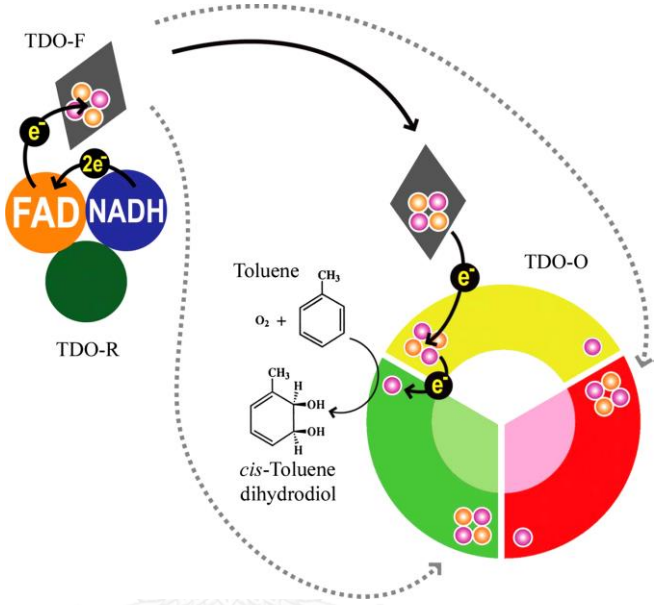


Figure2.5 Schematic representation of electron transfer in the TDO enzyme system.

Toluene dioxygenase pathway (*todC1C2BADE*) is an important in bioremediation of aromatic compounds due to their wide-substrate range. The previous study reported that *P. putida* F1 can utilize aromatic compounds such as benzene, toluene and ethylbenzene as carbon and energy sources (Gibson et al., 1973; Gibson et al., 1970; Gibson et al., 1968). The aromatic compounds are sequentially transformed to TCA cycle intermediate by toluene degradation (*tod*) pathway (Finette and Gibson, 1988; Zylstra and Gibson, 1989). The pathway consists of seven enzymatic reactions that encoded by tod*FC1C2BADEGIH*, arranged in the order

(Eaton, 1996; Zylstra and Gibson, 1989; Zylstra et al., 1988). Toluene dioxygenase (TDO; *todC1C2BA* gene) is the first enzymatic step of this pathway (Figure 2.6).

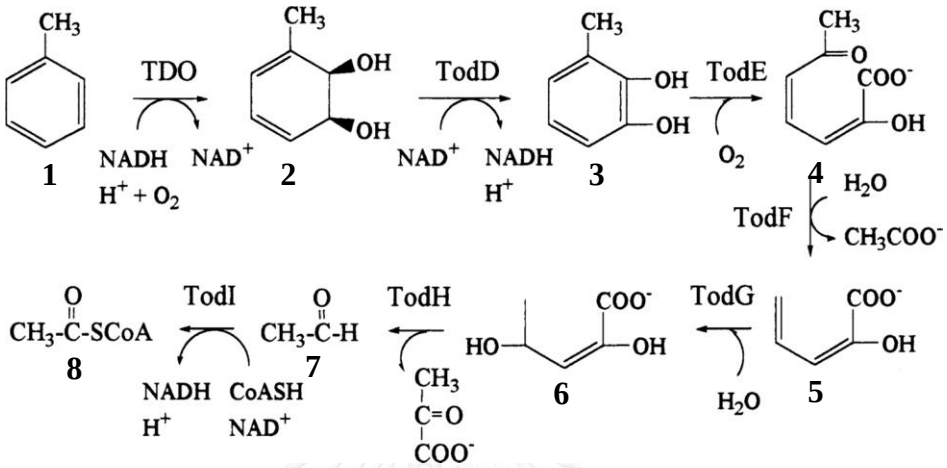


Figure2.6 Toluene degradation (tod) pathway from *P. Putida* F1 (Cho et al., 2000). The abbreviations for enzymes are described: TDO; toluene dioxygenase encoded by *todC1C2BA* genes, TodD; *cis*-toluene dihydrodiol dehydrogenase, TodE; 3-methycatechol-2,3-dioxygenase, TodF; 6-methyl-HOHD hydrolase, TodG; 2-hydroxypenta-2,4-dienoate hydratase, TodH; 4-hydroxy-2-oxovalerate aldolase, TodI; cyclating aldehyde dehydrogenase. The abbreviations for intermediates are described: 1; toluene (substrate), 2; *cis*-toluene dihydrodiol, 3; 3-methylcatechol, 4; 2-hydroxy-6-oxo-6-methylhexa-2,4-dienoate (6-methyl-HOHD), 5; 2-Hydroxypenta-2,4-dienoate, 6; 4-Hydroxy-2-oxo-valerate , 7; acetaldehyde, 8;acetyl-CoA

2.2.2 The toluene dioxygenase pathway involved in 4-chloroaniline degradation

The toluene dioxygenase (TDO) pathway, which is a meta cleavage enzyme (Smith and Ratledge, 1989)..

Recently, Nitisakulkan and colleagues studied the toluene dioxygenase from *P. putida* T57 could degraded 4CA. They proposed metabolic pathways of 4CA in *E. coli* DH5α and *P. putida* T57 recombinant strain (Figure 2.7). The oxidation of 4CA by toluene dioxygenase from *P. putida* T57 was expressed in *E. coli* showed two oxidation routes. The first route is same as primary toluene dioxygenase pathway, which is a meta cleavage pathway. Toluene dioxygenase generated 2-amino-5-chlorophenol shows that toluene dioxygenase also catalyzes 2,3-dioxygenation of 4CA, which generated 4-chloroaniline-2,3-cis-dihydrodiol and it then converted to 2-amino-5-chlorophenol by spontaneous dehydration and another is toluene dioxygenase catalyzes 1,2-dioxygenation of 4CA (ortho cleavage pathway) which, generates 4-chloroaniline-1,2-cis-dihydrodiol and it converts to 4-chlorocaetchol by spontaneous deamination (Figure 2.7). The oxidation of 4CA in *E. coli* recombinant strain (pHK*todC1C2BA*-DH5α) showed the accumulation of 5-chloropyrogallol in the reaction (Figure 2.7A) while, the metabolic pathway of 4CA of *Pseudomonas putida* T57 could be complete degradation (Figure 2.7B) (Nitisakulkan et al., 2014).

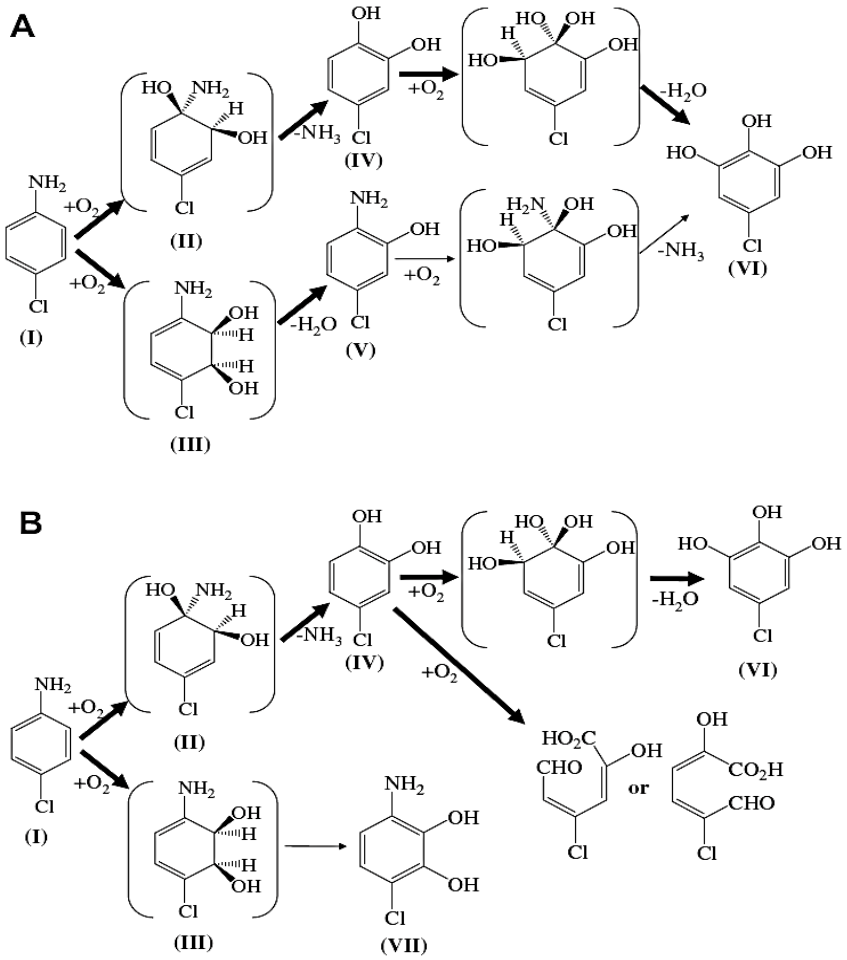


Figure2.7 The metabolic pathways of 4CA by *tod* gene in *E. coli* DH5α (Fig. 2.7A) and *P. putida* T57 recombinant strain (Fig. 2.7B) I, 4-chloroaniline; II, 4-chloroaniline-1,2-cis-dihydrodiol; III, 4-chloroaniline-2,3-cis-dihydrodiol; IV, 4-chlorocatechol; V, 2-amino-5-chlorophenol; VI, 5-chloropyrogallol; VII, 3-amino-6-chlorocatehol.

The parent strain of *P. putida* T57 showed only weak 4CA degradation activity. They enhanced 4CA degradation ability of *P. putida* T57 by introducing

recombinant plasmid harboring *tod* operon into *P. putida* T57. The results showed that the degradation rate of 4CA by *P. putida* T57 recombinant strain is approximately 250-fold (1.5 mM/h) higher than parental strain (5.7 μM/h). Although T57's toluene dioxygenase has catalytic activity toward 4CA, there is no report on enzyme kinetics.

2.2.3 Substrate specificity of toluene dioxygenase

Toluene dioxygenase has ability to degrade aromatic compounds. Table 2.3 shows the specific activity of toluene dioxygenase from *Pseudomonas putida* sp.

Table 2.3 The specific activity of toluene dioxygenase.

Specific activity [μmol/min/mg]	**Organisms**	**Substrate**	**Literature reference**
0.0075	*Pseudomonas putida*	-	(Subramanian et al., 1979)
0.045	*Pseudomonas putida*	benzene	(Bagneris et al., 2005)
0.054	*Pseudomonas putida*	ethylbenzene	(Bagneris et al., 2005)
0.056	*Pseudomonas putida*	toluene	(Bagneris et al., 2005)

Hori and collaborators investigated the degradation rate of toluene and trichloroethylene (TCE) by four strains including *P. putida* F1 and toluene degrading bacteria (A1071, IB5 and B6122) (Hori et al., 2005). The kinetic constants (K_m) and maximum substrate degradation (V_{max}) are shown in Table 2.4.

Table 2.4 Kinetic constants of toluene degradation

Strains	**Toluene**		**References**
	K_m (µm)	**V_{max} (nmol min^{-1} mg^{-1} cell)**	
P. putida F1	5.2	$2.9x10^2$	(Hori et al., 2005)
P. putida A1071	8.6	$2.8x10^2$	
P. putida IB5	10	$4.9x10^2$	
P. putida B6122	14	$1.6x10^2$	

Toluene dioxygenase has a wide-substrate specific range including toluene, benzene, biphenyl, phenol, chloroanilines etc. Table 2.5 showed the efficiency of toluene dioxygenase with various substrates.

Table 2.5 Substrate specificity and enzyme activity of toluene dioxygenase

Whole cell/ Enzyme	Strain	Substrate	End Product	Degradation rate	Enzyme activity (Cell free extract) (nmol min^{-1} mg protein^{-1})	References
Toluene dioxygenase (Whole cell)	*E. coli* DH5α (pHK-C1C2BA)	4CA	5-chloropyrogallol	-		(Nitisakulkan et al., 2014)
	P. putida T57 (pHK-C1C2BADE)	2CA		0.15 mM/h		
		3CA		0.5 mM/h		
		4CA		1.5 mM/h		
		34DCA		0.1 mM/h		
Toluene dioxygenase	*P. putida* NCIMB 11767	Toluene	**Ring cleavage:** Catechol 2,3-dioxygenase		365	(Heald and Jenkins, 1996)
			Catechol 1,2-dioxygenase		13	
		Phenol	**Ring cleavage:** Catechol 2,3-dioxygenase		235	
			Catechol 1,2-dioxygenase		9	
		Benzoate	**Ring cleavage:** Catechol 2,3-dioxygenase		7	
			Catechol 1,2-dioxygenase		688	
Toluene dioxygenase	*E. coli* JM109 recombinant strain harboring *todC1C2BADE*	biphenyl	2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HPDA)		< 0.1	(Furukawa et al., 1993)

Table 2.5 Substrate specificity and enzyme activity of toluene dioxygenase (continued)

Whole cell/ Enzyme	Strain	Substrate	End Product	Degradation rate	Enzyme activity (Cell free extract) (nmol min^{-1} $mg\ protein^{-1}$)	References
Dioxygenase	*Pseudomonas* sp. NCIB 10643	Ethylbenzene	Catechol		235	(Smith and Ratledge, 1989)
		n-Propylbenzene	Catechol		233	
		Isopropylbenzene	Catechol		235	
		n-Butylbenzene	Catechol		210	
		tert-Butylbenzene	Catechol		194	

2.2.4 The protein engineering and direct evolution of toluene dioxygenase

The protein engineering is a common technique, which has been applied in various fields including research, medicine, agriculture and industrial biotechnologies. Direct evolution is a method use in protein engineering, for example site directed mutagenesis and error prone PCR, that mutates the gene to evolve protein or nucleic acid toward enhanced activity. Normally, protein engineering is famous to use for modification of enzymes such as substrate specificity (Ang et al., 2009; Hirose et al., 1994; Hupert-Kocurek et al., 2014; Lee et al., 2005; Ni and Chen, 2005; Sakamoto et al., 2001; Suenaga et al., 2006), selectivity (Chockalingam et al., 2005; Zhang et al., 2000), enzyme efficiency(Gursky et al., 2010; Jiang et al., 1996), thermostability, and thermotolerant (Kim and Lei, 2008; Morimoto et al., 2014; Niu et al., 2006). Table 2.6 showed the genetic engineering of various enzymes to improve its activity. For the summary, protein engineering has been most successful in three areas; first is to improve protein stability for biotechnology, which use at high temperature or solvent tolerant. Second is to improve binding affinity of enzyme, which high efficiency. Another is to improve substrate specificity of enzyme, which is often used in industry.

Error prone PCR is a common technique, which is used for protein engineering. This technique is a mostly used for improving enzyme efficiency. Basak and colleagues (Parales et al., 2000) used error prone PCR technique to enhance organic solvent tolerance. The mutants showed better growth than wild type when cultured in organic solvent. The thermostability of phytase (*App*A2) was improved by error prone PCR (Kim and Lei, 2008). They reported that the catalytic efficiency of mutants, K46E and K65E/K97M/S209G were enhanced 56% and 152%

respectively, comparing to wild type. Error prone PCR technique is also used in improving of thermotolerant of malic enzyme (Chen, 2004). Compared to wild type, mutants showed higher malate production and less amount of lactate, which is by product. This technique is used to enhance the degradation of aromatic compounds i.e. picoline and indene by toluene dioxygenase (tod*C1C2BA*) (Wen-Chen and Gibson, 1994; Zhang et al., 2000). In the study by Zhang et al., (2000), error prone PCR is used to mutate the target gene. After that, the high throughput assay was used to screen the tentative mutants capable to use the aromatic compounds as a substrate. They reported that the achievement of using aromatic compounds as substrate can be obtained by changing of amino acid in the active site to fit with substrates (Zhang et al., 2000).

Table 2.6 The genetic engineering of enzyme to improve the activity

Enzyme	Strains	Directed evolution	Improvement/ Modification	Potentiality	References
Toluene dioxygenase	*Pseudomonas putida* F1	Site direct mutagenesiss (*todC1*)	Enzyme efficiency	The residues, Glu-214, Asp-219, His-222, and His-228, are essential for catalytic activity	(Jiang et al., 1996)
Toluene dioxygenase	*Pseudomonas putida* F1	Error prone PCR (*todC1C2BA*)	Selectivity	Increasing 40% of product (cis-indandiol) yield that is expected product	(Zhang et al., 2000)
Toluene dioxygenase	*Pseudomonas putida* sp.	Error prone PCR (*todC1*)	Substrate specificity	The results indicated a key role for amino acid 451 in determining activity towards 4-picoline	(Sakamoto et al., 2001)
Toluene dioxygenase	*Pseudomonas putida* F1	Insertion gene (*Cmt*E and *tod*s)	Substrate specificity	The mutant proteins could be obtained from F1 strains adapted to assimilate n-propylbenzene, n-butylbenzene, cumene and biphenyl	(Choi et al., 2003)
Toluene dioxygenase	*Pseudomonas putida* sp.	Insertion gene Tn*10*	Substrate specificity	The modifications on membrane structure with lipoprotein mutation	(Ni and Chen, 2005)
Toluene dioxygenase and Biphenyl dioxygenase	*Pseudomonas putida* sp.	Hybrid gene cluster (*bphA1A2A3A4* and *todC1C2BA*)	Substrate specificity	hybrid dioxygenase (composed of TodC1::BphA2A3A4 and TodC1C2::BphA3A4) showed a wide substrate specificity rather similar to that of the wild-type toluene dioxygenase (TodC1C2BA)	(Hirose et al., 1994)
Aniline dioxygenase	*Acinetobacter* sp. strain YAA	Error prone PCR (Atd*A*)	Substrate specificity	The mutated aniline dioxygenase with enhanced activity and capable of hydroxylating a wider range of aromatic amines than the wild type	(Ang et al., 2009)
Catechol 2,3-dioxygenase	*Planococcus sp.* strain S5	Error prone PCR (*C23O*)	Substrate specificity	The mutants (C23OB58 and C23OB65) showed that amino acid residues located outside the active site pocket can affect substrate specificity and activity of catechol 2,3-dioxygenasetoward substrates (3-methylcatechol, 4-methylcatechol and 4-chlorocatechol).	(Hupert-Kocurek et al., 2014)

Table 2.6 The genetic engineering of enzyme to improve the activity (continued)

Enzyme	Strains	Directed evolution	Improvement/ Modification	Potentiality	References
Biphenyl dioxygenase	*Pseudomonas pseudoalcaligenes* KF707	Site-directed mutagenesis (*bphA1*)	Substrate specificity	The mutated at the active site of Biphenyl dioxygenase (Thr376Phe) exhibited enhanced and expanded degradation activities toward all of the PCBs.	(Suenaga et al., 2006)
2-nitrotoluene dioxygenase	*Acidovorax sp. strain* JS42	Site-directed mutagenesis (*ntdAc*)	Substrate specificity	The residues JS765 and Asn258 of active site appears to contribute to substrate specificity through hydrogen bonding to the nitro group of nitrotoluenes.	(Lee et al., 2005)
Styrene monooxygenase	*Pseudomonas putida* CA-3	Error prone PCR (*styAB*)	Enzyme efficiency	The mutated enzyme exhibited eight- and 12-fold improvements in styrene and indene oxidation rates compared to the wild-type enzyme	(Gursky et al., 2010)
Phytase	*Aspergillus fumigatus*	Error prone PCR (*App*A2)	Thermostability	The mutants (K46E and K65E/K97M/S209G) showed over 20% improvement in thermostability (80°C for 10 min), and 6–7°C increases in melting temperatures (T_m) than that wild-type.	(Kim and Lei, 2008)
Lipases	*Rhizopus arrhizus*	Error prone PCR (*RAL*)	Thermostability	. The optimum temperature of the mutant lipase was higher by 10 °C than that of the wild-type RAL (WT-RAL).	(Niu et al., 2006)
Malic enzyme	*Thermococcus kodakarensis*	Site direct mutagenesis (*Tk*ME)	Thermotolerant	The mutants showed higher malate production and less amount of lactate compared with wild-type.	(Morimoto et al., 2014)
Nuclear hormone receptors	The yeast two-hybrid *(Y2H)* strain YRG2	Error-prone PCR (hERα ligand binding domain)	Selectivity	The resulting ligand–receptor pair is highly sensitive to the synthetic ligand in human endometrial cancer cells and is essentially fully orthogonal to the wild-type receptor–natural ligand pair.	(Chockalingam et al., 2005)

In order to improve catalytic of enzyme including specificity and stability, site-directed or random mutagenesis is generally conducted (Ang et al., 2009; Nolan and O'Connor, 2008; Peng et al., 2010; Ramos-González et al., 2003; Zhang et al., 2000)

Toluene dioxygenase (TDO) that is the terminal oxygenase contains TDO active site, which consist of a large α subunit and a small β subunit. It is important to specific with the substrate (Parales et al., 1998; Sakamoto et al., 2001; Tan and Cheong, 1994). The α subunit assists the Rieske [2Fe-2S] center and the mononuclear iron which is necessary for activity and plays a major role in substrate specificity, whereas the β subunit is proposed to have structure role (Jiang et al., 1999) and it is also interact with the substrate. Consequently, TDO active sites are purposed to study the performance of toluene dioxygenase activity.

Tod*C1C2* (the terminal oxygenase) is critical for the initial oxidation of toluene (Furukawa et al., 1993), thus it is the main point to improve enzyme activity. Friemann and colleagues reported the crystal structure of toluene dioxygenase of *P. putida* F1 (Friemann et al., 2009). Since the amino acid sequences of *P. putida* F1 tod*C1C2BA* products are completely identical to *P. putida* T57, which is the strain in previous research (Nitisakulkan et al., 2014). The further protein model structure will be conducted using toluene dioxygenase crystal structure (PDB; 3EN1).

Table 2.7 shows the previous studies of the genetically engineered toluene dioxygenase to improve its efficiency and substitution of amino acid residues.

Table 2.7 The genetically engineered toluene dioxygenase to improve its activity and substitution of amino acid residues

Microorganism	Gene	Direct evolution	Mutation position	Substrates	Commentary	References
E. coli recombinant strain	*todC1*	Site direct mutagenesiss	Alpha subunit (*todC1*) E214A, D219A, H222A,H228A, Y221A, T266A		o All of mutant of *todC1* loss or reduction of TDO activity. o The results investigate that Glu-214, Asp-219, His-222, and His-228 are essential for catalytic activity and may act as mononuclear iron ligands.	(Jiang et al., 1996)
E. coli recombinant strain	*todC1C2BA*	error prone PCR	*todC1* : L306P, L306P, A212T, A234T, L306P *todC2*: I53V *todB* : G22S, E61V *todA* : V5M	Indene (indene Bioconversion)	o To reduce the undesirable byproducts 1-indenol and 1-indanone formed during indene bioconversion o Mutant 1C4-3G was identified to have a threefold reduction in 1-indenol formation over the wild type (20% vs 60% of total products) and a 40% increase of product (cis-indandiol) yield.	(Zhang et al., 2000)
E. coli recombinant strain	*todC1*	error-prone PCR	*todC1* Stop codon TGA→451R (CGA), R459L (G1376T) Stop codon → 451P, 451T R450K	4-picoline (4-methylpyridin)	o The results indicate a key role for amino acid 451 in determining activity towards 4-picoline. o Mutation of the stop codon leads to extension of *todC1* and a C-terminal extension of the protein which improve activity toward 4-picoline by increasing expression and altering the enzyme's substrate specificity.	(Sakamoto et al., 2001)

Table 2.7 The genetically engineered toluene dioxygenase to improve its efficiency and substitution of amino acid residues (continued)

Microorganism	Gene/Enzyme	Direct evolution	Mutation position	Substrates	Commentary	References
Pseudomonas putida F1	CmtE and Tods	Construct recombinant plasmid and conjugate to F1 Insertion gene	Tod S mutation the nucleotide substitutions A1410G, C1956G, C2318A, G1976C and C1736T	n-Propylbenzene n-Butylbenzene Cumene Biphenyl Toluene	o These modified proteins could be obtained from F1 strains adapted to assimilate n-propylbenzene, n-butylbenzene, cumene and biphenyl.	(Choi et al., 2003)
Recombinant strain	Toluene dioxygenase *todC1C2BA*	Tn*10* insertion	E609L (Lipoprotein)	Toluene, *o*-cresol, ethylbenzene, 2-ethylphenol, 2-indanone, ethyl acetate, and butyl acetate	o The enhancement of the reaction rate, an increase of up to 6-fold	(Ni and Chen, 2005)

CHAPTER 3

METHODOLOGY

The Methodology of this study is divided into 4 parts as follows:

3.1 Laboratory equipment and chemicals

3.2 Culture medium and cultivation conditions

3.2.1 Chemicals and bacterial media

3.2.2 Cultivation condition and resting cell preparation

3.3 Experimental procedures

3.4 Analytical method

3.1 Laboratory equipment and chemicals

3.1.1 Laboratory equipment

Laboratory equipment	**Company**	**Country**
Autocalve NLS-3020	Sanyo Electric Co., Ltd	Japan
Autoclave HV-110	Hirayama	Japan
Centrifuge (Prism R)	Labnet	USA
Cooling & heating block	Bioer Technology	USA
C 18 column (Hyperclone 5u BDS C18 130A, a250X4.6mm)	Phenomenex	USA
Digital dry bath	Labnet	USA
Finemixer (SH 2000, Finemould)	Precision Ind. Co.	USA
Gel Doc™XR + Image Lab™ Software	Bio-Rad	USA

Laboratory equipment	**Company**	**Country**
High Performance Liquid Chromatography (HPLC) LC-20	Shimazu	Japan
Hotplate stirrer	Lab Tech	Korea
Incubator shaker, innova 4000	New Brunswick scientific	USA
Incubator shaker, innova 4340	New Brunswick scientific	USA
Micropipette 20, 100, 200, 1000 µl	Gilson	France
MiniRun Gel Electrophoresis	Bioer Technology	USA
pH meter	Mettler Toledo	USA
Quick spin (Micro one)	TOMY	Japan
Refrigerated Centrifuge, Avanti ™ J301	Beckman Coulter	USA
Refrigerated Centrifuge, 5804R	Eppendrof	USA
Spectrophotometer DU 650	Beckman	USA
UV transilluminater, BioDoc-It™ System	UVP	USA
Vacuum pumps	GAST	USA
Vortex (Touch mixer model 232)	Fisher Scientific	USA

3.1.2 Laboratory chemicals

The chemicals used in this study are shown as follow

Chemicals	**Company**	**Country**
Absolute ethanol (99.9% purity)	Merck	Germany
Acrylamide	Bio basic Inc	USA
Acetonitrile (99.9% purity)	B&J	Korea
Agar	Scharlau Chemic Microbiology	Spain

Chemicals	**Company**	**Country**
Agarose	BMA	USA
Ammonium persulfate	Bio-Rad	USA
Ampicillin	Sigma	USA
Aniline (≥ 99% purity)	Merck	Germany
Bovine serum albumin (BSA)	Sigma	USA
Calcium chloride	Ajax	Australia
Chloroform (≥ 99.5% purity)	Sigma	USA
Coomassie brilliant blue R-250	Sigma	USA
DNA marker: 1kb DNA Ladder	Fermentas	USA
2,6-Dichloroquinone-4-chloroimide (Gibbs' reagent)	Merck	Germany
Dimetyl sulfoxide (DMSO)	Sigma	USA
Ethidium bromide (95% purity)	Sigma	USA
Glacial acetic acid	BDH	England
Glycerol	Ajax	Australia
Hydrochloric acid	Merck	Germany
Kanamycin	Sigma	USA
Ligation high ver.2	Toyobo	Japan
6X loading dye	Toyobo	Japan
Nitrocellulose membrane	Pall corporation	Germany
N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)	BDH	England
Phenol (≥ 99% purity)	BDH	England

Chemicals	Company	Country
Restriction enzyme	Fermentas	Thermo Scientific
Sodium chloride	Ajax	Australia
Sodium dodecyl sulfate	Sigma	USA
Tris (hydroxymethyl) aminomethane	USB	USA
Tryptone	Scharlau Microbiology	Spain
Yeast extract	Scharlau Microbiology	Spain
4-chloroaniline (≥ 99% purity)	Merck	Germany

3.2 Culture medium and cultivation conditions

3.2.1 Chemicals and bacterial media

4-chloroaniline (EC Number: 203-401-0) was obtained from Merck. The chemical formula is $ClC_6H_4NH_2$. *E. coli* DH5α was used for transformation and maintenance of recombinant plasmids. *E. coli* BL21 (DE3) was used as a host strain for expression of recombinant strain. pET21a was used as an expression vector that has Ampicillin as a selective antibiotic. The bacterial strains and plasmids used in this study are listed in Table 3.1.

Table 3.1 Bacterial strains and plasmids were used in this study

Strain or plasmid	Characteristics	Reference
Strain		
Pseudomonas putida T57	The strain containing *todC1C2BA* gene	(Faizal et al., 2005)
Recombinant strains		
E. coli (pHK*todC1C2BA*)	*E.coli* JM109 harboring pHK*todC1C2BA*	(Nitisakulkan et al., 2014)
E. coli (pET21a*todBA*)	*E. coli* DH5α harboring pET*todBA*	This study
E. coli (pET21a*todC1C2BA*)	*E. coli* Bl21(DE3) harboring pET*todC1C2BA*	This study
Plasmid		
pHK*todC1C2BA*	pHK1 with a 3.8-kb containing kan promoter-todC1C2BA; Km^r	(Nitisakulkan et al., 2014)
pET21a*todC1C2BA*	pET21a+ containing T7 promoter-*todC1C2BA*; Amp^r	This study
pET21a*todBA*	pET21a+ containing *todBA*; Amp^r	This study

Amp^r, Ampicilin

3.2.2 Cultivation condition and resting cell preparation

Recombinant stain was grown in 5 ml LB medium as a starter, with a 200 rpm shaking condition, overnight (18 h.), at 37 ºC and used as an inoculant (2 %, v/v) into MSBY medium (Nitisakulkan et al., 2014) (100 ml). After 4 h, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) added for an induction. Bacterial culture grew overnight at 30 °C, with a 200 rpm shaking condition reach to optical density (OD_{600}) of 1. Bacterial cell culture was harvested by centrifugation at 5,000 rpm, 4 °C for 15 min. Cell pellet washed once with 25 mM sodium phosphate buffer pH 7.0 (Appendix

A), and resuspended with the same buffer in 5 ml buffer. The biodegradation test started by adding various concentration of 4CA and incubated at 30 °C.

3.3 Experimental procedures

3.3.1 DNA manipulation

The standard protocol for DNA cloning technique use to prepare plasmid, digest with restriction enzyme, ligate, transform and run agarose gel electrophoresis (Sambrook and Russell, 2001). Polymerase chain reaction (PCR) was performed by KOD Plus NeO DNA polymerase. *E.coli* was used as genetic host (Appendix A).

3.3.2 Construction of the *E.coli* recombinant strain (wild-type)

pET21a was used as an expression vector of *tod* genes. DNA fragment containing *todC1 todC2 todB todA* gene was amplified from pHK*todC1C2BA* reccombinant plasmid. DNA fragment of *todBA* was amplified by using 5'ATCGGAATTC-ATGACTTGGACATACATATTGCGG 3' and 5' ATCGGAGCTCTCACGTTAG-GTCTCCTTCATT 3'. PCR product was ligated to pET21a between *Eco*RI and *Sac*I site as a vector. Then, *todC1C2* was amplified by using 5'ATCGGGATC-CATGAATCAGACCGACACATC3' and 5'ATCGGAATTCTAGAAGAAGAAA-CGAGGTTAT3'. The PCR products was ligated to pET21a*todBA* between *Bam*HI and *Eco*RI site. The thermal cycling reacion (KOD Plus NeO; Takara) using the following method: 95ºC for 3 min; 35 cycles of 98ºC for 10 s, 55ºC (*tod*BA); 58ºC (*tod*C1C2) for 30 s, and 68ºC for 1.5 min; and a final extension at 72ºC for 10 min. Recombinant plasmid (wild-type) was over-expressed in *E.coli* BL21(DE3) strain. The *todC1C2BA* genes was controlled under T7 promoter.

3.3.3 Whole-cell biodegradation activity test

The concentrated cell preparation is described above. Five milliliter of the concentrated cells was aliquoted into a 30-ml glass vial. The biodegradation test started by adding 4CA concentration and then incubated at 30°C. At time interval, the sample was taken to analyze the remaining 4CA using high performance liquid chromatography (HPLC) as described below.

3.3.4 The improvement of *todC1C2BA* genes by error prone PCR technique

1. Plasmid construction for error prone PCR

DNA fragment of *todBA* was amplified by using 5'ATCGGAATTC-ATGACTTGGACATACATATTGCGG 3' and 5' ATCGGAGCTCTCAC-GTTAGGTCTCCTTCATT 3'. PCR product was ligated to pET28b between *Eco*RI and *Sac*I site as a vector. Two kb of *todC1C2* DNA fragment was a target for random mutagenesis technique. The *todC1* and *todC2* were amplified by using Diversify™ PCR Random Mutagenesis Kit (Clontech; USA) with 5'ATCGGGATCC-ATGAATCAGACCGACACATC3' and 5'ATCGGAATTCTAGAAGAAGA-AACGAGGTTAT3'. Thermal cycling reacion (Diversify™ PCR Random Mutagenesis Kit) using the following method: 98ºC for 30 s; 35 cycles of 98ºC for 10 s, 58ºC for 30 s, and 72ºC for 1 min; and a final extension at 72ºC for 10 min. The 2-kbp fragment generated by EPPCR wad digested with *BamH*I and *Eco*RI and ligated to pET21a*todBA* between *Bam*HI and *Eco*RI site to generate pET21a*todC1C2'BA*. The recombinant plasmid was transformed to *E. coli* BL21(DE3) for 4CA

biotransformation screening. The condition, which used in this study shows in Table 3.2

Table 3.2 The mutagenesis reactions of Diversify™ PCR Random Mutagenesis Kit

Condition	**Volume by buffer condition (µl)**		**Final concentration**
	5	**Standard**	
Mutagenesis per 1,000 bp	**4.6**	**-**	
PCR grade water	36	41	
10X titanium *Taq* buffer	5	5	1X
$MnSO_4$ (8 mM)	4	0	0.64 mM
dGTP (2 mM)	1	0	0.04 mM
50X Diversify dNTP mix	1	0	1X
50X dNTP Mix	0	1	
F Primer: *tod*C1F-*Bam*HI (10µM)	0.5	0.5	0.1 µM
R Primer: todC2R-EcoRI (10µM)	0.5	0.5	0.1 µM
Template: pHK*todC1C2BA*	1	1	
TITANIUM *Taq* polymerase	1	1	
Total volume	50	50	

2. The optimization of modified Gibbs method for screening the mutated clones

The Gibbs' reagent was used to detect the phenolic compound by substitute at *para*-position. For the modification of Gibbs method, the investigation of optimum condition for screening was tested that are the maximum absorbance of each chemical, the optimum reaction time with Gibbs' reagent, and the optimum of transformation time. Chloroanilines and its intermediates was used to test reaction with Gibbs' reagent, which were 2CA, 3CA, 4CA, aniline, catechol, 4CC and 3CC, by vary the chemical concentration (0.1 – 3 mM).

1) The maximum absorbance of each chemical

The chemical, which used to be substrate in this experiment, was 4CA and aniline. While, 4CC and catechol are the chemical was used to detect with Gibbs' reagent. Gibbs' reagent was used in the spectrophotometric determination of aniline, catechol, and 4CC. Due to the similarity of chloroanilines structure, the maximum absorbance of each chemical could be identified. Thus, the maximum absorbance (λ_{max}) was determined by varying concentration of the chemicals. The reaction was test in 96-wellplate. The 150 µl of substrate in MSBY was added in each well. Then, 10 µl of 0.32% (w/v) Gibbs' reagent in ethanol was added directly to each well. The wavelength of each chemical was scanned by using micro plate reader (Thermo scientific; multiskan go) after 10 min of incubation.

2) The optimum reaction time with Gibbs' reagent

The reaction was tested in 96-well plate. The optimum reaction time with Gibbs reaction was investigated by fixing wavelength at maximum absorbance of each chemical (aniline, catechol, and 4CC) and varying the concentration of chemical (0.1 – 3 mM) and the reaction time (0, 2, 3, 5, 8, 10, 12, 15, and 20 min). The 150 µl of substrate in MSBY was added in each well. Then, 10 µl of 0.32% (w/v) Gibbs' reagent in ethanol was added directly to each well. The absorbance of each chemical was detected by using micro plate reader (Thermo scientific; multiskan go). The optimum reaction time of chemical with Gibbs' reagent was determined by the relationship between the maximum absorbance and concentration of chemical.

3) The optimum of transformation time

The pHK*todC1C2BA*-JM109 was used as a model strain for investigation of the transformation time. The 1 mM 4CA was used as substrate for transformation. At each time interval, the sample was taken to measure 4CC transformation. The reaction was tested in 96-well plate. The recombinant strain (pHK*todC1C2BA*-JM109) was grown in 5 ml LB medium as a starter, with a 200 rpm shaking condition, overnight (18 h.), at 37 ºC and used as an inoculant (10 %, v/v) into MSBY medium containing 0.1 mM IPTG and 50 µg/ml of kanamycin. The plates were incubated at 30°C with 200 rpm shaking for 4 h. Then, 100 µL of MSBY media with 50 $mg.L^{-1}$ kanamycin, 0.1 mM IPTG, and 2 mM substrate was added into each well plate (final substrate concentration is 1 mM). The plates were incubated at 30°C with 200 rpm. At time interval, the plate was taken to analyze. Cell density was measured (OD_{600}) after incubation. Then, cells was centrifuged at 1,600 xg for 10 min The supernatant was transformed to a new microtiter plate where 10 µl of 0.32% (w/v) Gibbs' reagent in ethanol was added directly to each well. The reaction was measured at 590 nm after 5 min of incubation. The optimum transformation time was determined by the relationship between the A_{590}/OD_{600} and transformation time.

3. Screening the mutant clones by Gibbs method

The screening method was adapted from Sakamoto (2001), Ang (2009) and collaborators (Ang et al., 2009; Sakamoto et al., 2001) with modifications. Aniline was used as a substrate for screening. Each colony from library was picked into 500 µL of LB containing Ampicilin (100 $mg.L^{-1}$) in a rack containing microtubes, with 3 wild-type (WT) clones as positive controls in each rack. The racks were incubated

overnight at 37°C with shaking at 200 rpm. Twenty microliters of the overnight culture was inoculated into new 96-wells microplate containing 80 μL of MSBY media supplemented with 50 $mg.L^{-1}$ Ampicilin and 0.1 mM IPTG. The plates were incubated at 30°C with 200 rpm shaking for 4 h. Then, 100 μL of MSBY media with 50 $mg.L^{-1}$ Ampicilin, 0.1 mM IPTG, and 2 mM substrate was added into each well plate (final substrate concentration is 1 mM). The plates were incubated at 30°C with 200 rpm shaking for 4 h. Cell density was measured (OD_{600}) after incubation. Then, cells was centrifuged at 1,600 xg for 10 min The supernatant was transformed to a new microtiter plate where 10 μl of 0.32% (w/v) Gibbs' reagent was added directly to each well. The reaction was measured at 576 nm within 2 min of incubation. The activity of each mutant was indicated by the absorbance at 576 nm of catechol formation. Then, normalize to its cell density (OD_{600}). The positive mutants were subjected to secondary screening.

3.3.5 Determination of 4CA degradation based enzyme activity

For enzyme activity assay, the reaction mixture is described below (Table 3.3). The enzyme assay was modified from Jiang and coworkers (1996). The crude enzyme of pET21a*todC1C2BA* or its mutant derivatives were added to 25 mM sodium phosphate buffer, pH 7. The reactions were started by the addition of NADH and FAD (0.50 mM final concentration) and substrate (4CA/Aniline). The initial 4CA concentration was varied from 0.01 to 5 mM. The mixture was incubated at 30°C and analysed by HPLC. The initial reaction velocities was measured at the various concentration of

4CA and fitted into the Lineweaver-Burk equation of the Michaelis-Menten to kinetic. The Lineweaver-Burk plot is showed on Figure 3.1.

The Michaelis-Menten Equation : $$v = \frac{d[P]}{dt} = \frac{V_{\max}[S]}{K_m + [S]}$$

The Lineweaver-Burk equation : $$\frac{1}{V_0} = \frac{K_M + [S]}{V_{\max}[S]}$$

Where, V = Substrate degradation rate (mg.L^{-1}.day)

V_{max} = Maximum substrate degradation rate (mg.L^{-1}.day)

K_m = Michaelis constant (mg.L^{-1})

S = Substrate concentration (mg.L^{-1})

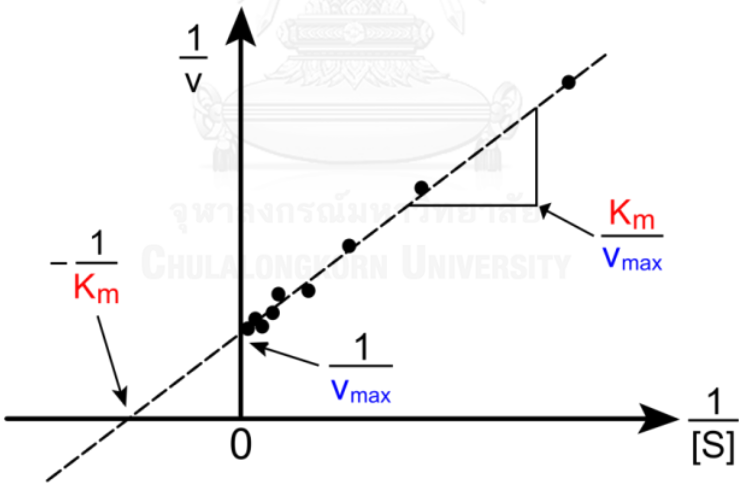


Figure 3.1 The Lineweaver-Burk plot

Table 3.3 The reaction mixture for catalytic study

Components	Final concentration
25mM Phosphate buffer (pH 7)	-
Substrate: 4-chloroaniline/ Aniline	0.01, 0.05, 0.1, 0.3, 0.5, 1, 3, 5 mM
10 mM NADH	50 µM
10 mM FAD	50 µM
Crude enzyme (pET21a*todC1C2BA)*	300-400 µg protein
Total volume 1 ml	

3.4 Analytical method

3.4.1 Protein quantification

Protein concentrations was determined by the modified method of Lowry (Lowry et al., 1951), with bovine serum albumin as a standard protein. SDS-PAGE was prepared according to Bollag (1996) (Daniel M. Bollag, 1996).

1.SDS-PAGE analysis preparation

The denaturing gel was prepared according to Bollag *et al.,* (1996). The gel was prepared with 0.1% (w/v) SDS in 12.5% (w/v) separating and 5% (w/v) stacking gels. The electrode buffer was prepared with Tris-glycine buffer pH 8.3 containing 0.1% SDS. The sample was prepared by maxing with sample buffer and boiled for 5 min. before analyze by gel (Appendix A). The electrophoresis was performed at constant current of 15 mA per gel in a Mini-Gel electrophoresis (Bio-rad, USA). The proteins were separated based on molecular weight.

3.4.2 HPLC analysis

4CA and aniline concentration was determined using a reverse phase HPLC (LC-10AD, Shimadzu, Japan). The separation was performed at 40°C on C18 column (5

µm, 250 x 4.6 mm; Hyperclone, Phenomenex, USA) using acetonitrile: water mixture (70:30, v/v) as a mobile phase at a flow rate of 1 ml min^{-1} and the detection wavelength of 240 nm. The retention time of 4CA and aniline under this condition was expected at 3.4 and 3.1 min, respectively.

3.4.3 Sequencing analysis

The recombinant wild type and mutants was identified the point mutation by using sequencing service of 1st BASE DNA Sequencing Division.

3.4.4 General techniques in genetic engineering

1. Genomic DNA extraction for Gram negative bacteria

The single colony of *Pseudomonas putida* T57 was cultured in 5 ml of LB in tube; incubate at 30°C, overnight (10-12 h.). The 1.5 ml of cell culture was collected by centrifugation at 13,000 rpm for 30 sec and wash with TE or UP water for once. The cell pellet was resuspended in 50 µl of UP water. Then 50 µl of 2 mg/ml, Lysozyme in TE pH 8.0 was added. The mix sample was incubated at 37°C for 15 min. Then, 450 µl of dH_2O, 6 µl protenase k (10 mg/L) and 60 µl of 10% SDS and mixed thoroughly. The DNA was incubated 37°C for 15 min, then 50°C for 15 min. The 600 µl of phenol: chloroform: isoamyl (25: 24: 1) was added and mixed with fine mixer for 2-3 min, then centrifuge at 13,000 rpm for 3 min and transfer 500 µl of upper layer to new tube. The 40 µl of 3 M sodium acetate (pH 7.0) was added in sample. DNA was precipitated by adding 1 ml of Absolute ethanol and stored at -20°C for 1-2 h. After that DNA pellet was collected by centrifugation at 13,000 rpm for 10 min. The supernatant was discarded. The pellet was wash with 70% ethanol. After drying, the

pellet was dissolved in 50 µl of distilled water containing Rnase (20µg/ml), then incubated at 37°C for 10 min. The DNA solution was then stored at -20°C until use.

2. Plasmid DNA extraction: Alkaline lysis with SDS (mini preparation)

Plasmid harboring cells were cultured in 5 ml of LB culture supplement with 100 µg/ ml of ampicillin or 50 µg/ ml of kanamycin at 37°C, 200 rpm for 16 h. and cells were collected by centrifugation at 13,000 rpm, 30 seconds, discard the supernatant. Then, the cells were resuspened by adding 100 µl of solution I (GTE buffer-ice cold) (Appendix A) and 200 µl of solution II (fresh prepared) (Appendix A) was added, invert tube 5 times rapidly, the microtubes were on ice 5 min. Next, 150 µl of solution III (ice-cold) (Appendix A) was added and the tube was inverted several times, the microtubes were on ice 5 min. And then centrifuge at 13,000 rpm, 30 seconds, collect the upper layer to new tube. The 450 µl of phenol: chloroform: isomyl (lower layer) was added in to samples. The samples were mixed thoroughly 2 min by fine mixer and then centrifuge at 13,000 rpm, 30 sec. The 380 µl of upper layer was transfer to the new tube. The plasmid DNA was precipitated by adding 1 ml of Absolute ethanol and stored at -20°C for at least 1 h. The pellet was collected by centrifugation at 13,000 rpm, 10 min. The pellet was wash with 70% ethanol. After drying by vacuum aspirator, the pellet was dissolved in 50 µl of distilled water containing Rnase (20 µg/ml), then incubated at 37°C for 10 min. The Plasmid solution was then stored at -20°C until use.

3. Agarose gel electrophoresis

Agarose gel electrophoresis was prepared to separate and purify fragment of DNA using 0.8% or 1% (w/v) agarose gel depending on DNA fragment size. DNA samples were mixed in 1X loading buffer and loaded into the wells of agarose gel covering with 0.5X TAE buffer. DNA was run in chamber at 100 volts until dye reached the bottom of the gel. After electrophoresis, the gel was stained in ethidium bromide for 15-30 min at room temperature. DNA fragment was analyzed under a long wavelength UV light. The concentration and molecular weight of DNA sample was analyzed with the standard DNA marker.

4. Preparation of *E. coli* competent cell for heat shock method

A single colony of *E. coli* was cultured into 5 ml of LB medium and incubated at 37°C, 200 rpm for 16-18 h. Then, 500 µl of inoculum was transferred to 50 ml of LB medium and incubated at 37°C, 200 rpm until OD_{600} reached 0.4-0.6. The culture was incubated on ice 10 min. The cells were collect by centrifugation at 4,000 rpm, 4°C for 10 min, and then the medium was completely removed. The pellet was resuspened gently in 35 ml of ice-cold Inove transformation buffer and collected by centrifuge at 3,800 rpm, 4°C for 10 min, then resuspened cell gently in 4 ml of ice-cold Inove transformation buffer (Appendix A). For long term storage of competent cell, 300 µl of DMSO was added, mix gently and stored on ice for 15 min. And aliquot 300 µl into chilled sterile 1.5 ml microcentrifuge tube, then the competent cell was dropped in to -80°C freezer immediately. The competent cells were stored at -80°C until use.

3.4.5 The programs were used in this research

1. Clone Manager Suite

Clone Manager Suite was used to design primer, plan cloning, do restriction enzyme analysis, draw graphic maps, and protein analysis functions. This program was used for planning molecular work and analysis the sequencing information.

2. Swiss-Pdb Viewer

Swiss-Pdb Viewer is program, which use to predict the mutated point of toluene dioxygenase. The model structure of *todC1C2* (α-subunit and β-subunit) is 3EN1 (pdb.files). The procedure of amino acid substitution by using Swiss-Pdb Viewer was shown in appendix D3.

CHAPTER 4

RESULTS AND DISCUSSION

Experimental overview

The research experiment is divided into 3 parts as follows:

4.1 Overexpression of toluene dioxygenase in *E. coli* recombinant and random mutagenesis

4.1.1 Construction of the *E. coli* recombinant strain (wild-type) for whole cell degradation of 4CA and toluene

4.1.2 Determine of whole-cell biodegradation activity test toward 4CA and toluene by HPLC analysis

4.1.3 The construction of recombinant plasmid containing *todC1C2BA* genes for error prone PCR technique

4.1.4 Optimization of the modified Gibbs method for screening of the mutant clones

1. Reaction of Gibbs' reagent with chloroanilines and its intermediates
2. The modified Gibbs method to screen the mutant clones; optimum maximum absorbance, reaction time with Gibbs' reagent, transformation time
3. Screening of the mutant clones by the modified Gibbs' method.

4.2 Characterization and comparison of the enzyme activity (wild type and mutated ones) toward 4CA

4.2.1 Selection of the mutated enzymes with improved catalytic efficiency

4.2.2 Determination of 4CA biotransformation rate

4.3 Identification and analysis of the point mutation within toluene dioxygenase.

4.3.1 Identification the mutated points by sequencing analysis

4.3.2 The prediction of mutated points by comparing with reference protein structure.

4.1 Overexpression of toluene dioxygenase in *E. coli* recombinant and random mutagenesis

4.1.1 Reconstruction of plasmid and expression of *todC1C2BA* in *E. coli* BL21(DE3) recombinant strain (wild-type)

1. PCR amplification of the *todC1C2BA* gene cluster

Toluene dioxygenase gene (*todC1C2BA*) with 3,591 bp was constructed in pET21a+, which was controlled under T7 promoter, generating the recombinant plasmid (pET21a*todC1C2BA*). The pET21a*todC1C2BA* was used to study in this research as the wild-type strain.

The construction of pET21a*todC1C2BA* (wild-type) was shown in Figure 4.1. The template for amplification is pHK*todC1C2BA*-SacI (Nitisakulkan et al., 2014). The expected PCR product size of *todBA* was 1,576 bp. The primers for amplification were shown in Appendix D. The *todBA* (Figure 4.1A) was cloned in to pET21a as a backbone using *Eco*RI and *Sac*I sites (Figure 4.1B). After that, the *todC1C2* gene was ligated to pET21a*todBA* at *Bam*HI and *Eco*RI sites (Figure 4.1C).

The PCR products of *todBA* and *todC1C2* were shown in Figure 4.2. The recombinant plasmid (pET21a*todBA*) was transformed to *E. coli* BL21(DE3). After that, the plasmids were extracted and digested at *Not*I site and double digested at *Eco*RI and *Sac*I sites. The expected sizes of pET21a*todBA* digested by *Not*I enzymes were 1,085 and 5,914 bp, whereas the digestion by *Eco*RI and *Sac*I showed the products with the expected size of 1,558 and 5,441bp. The digestion patterns of the recombinant plasmids (Figure 4.3) were then compared to the predicted pattern. The

obtained digestion patterns indicated that pET*todBA*-8 was the expected recombinant. Accordingly, the pET21a*todBA*-8 was used as a backbone for the construction of recombinant plasmid (pET21a-*todC1C2BA*). The fragment of *todC1C2* was cloned into the backbone pET21a*todBA*. Finally, the recombinant plasmid (pET21a*todC1C2B*A) was confirmed by *Bam*HI and *Eco*RI digestion. The recombinant plasmid (pET21a*todC1C2BA*) was controlled under T7 promoter.

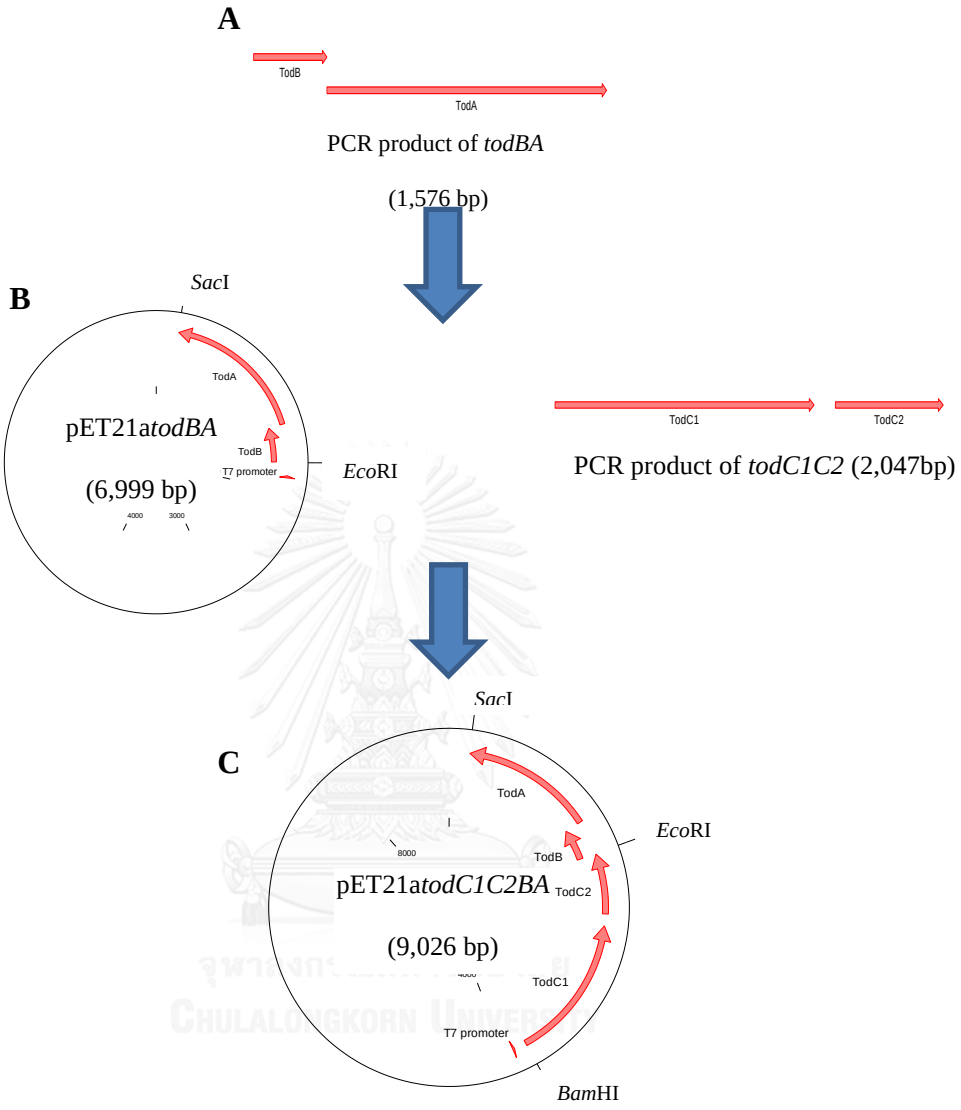


Figure 4.1 The procedure of pET21a*todC1C2BA* construction. A) The *todBA* PCR product. B) The *todC1C2* as an insert and pET21a*todBA* as backbone. C) The construction of pET21a*todC1C2BA* with the restriction site for insertion.

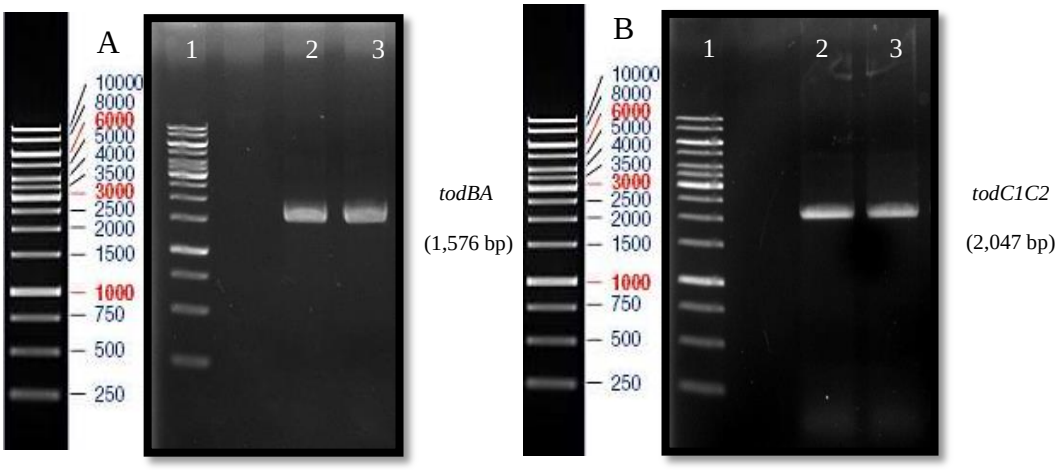


Figure 4.2 Agarose gel electrophoresis of PCR products. A) PCR product of *todBA* B) PCR product of *todC1C2*. Lane 1: GeneRuler 1 kb DNA ladder (Thermo scientific); Lane 2-3: PCR product amplified from pHK*todC1C2BA-Sac*I (as a template) with 10x dilution and 100x dilution, respectively.

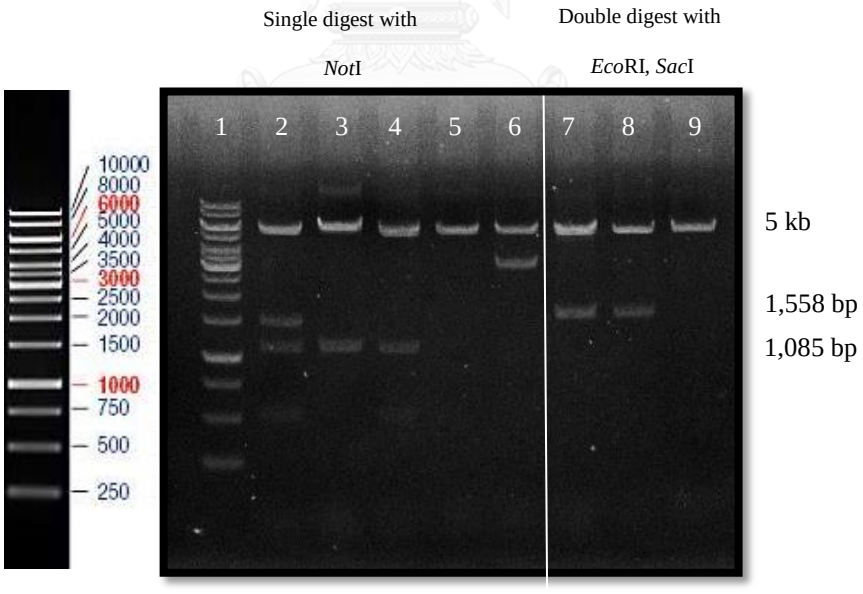


Figure 4.3 Agarose gel electrophoresis of the constructed plasmid cut check with *Not*I (lane2-5) and double digested by *Eco*RI and *Sac*I (lane 6-9). The expected sizes of pET21a*todBA* digested by *Not*I enzymes were 1,085 and 5,914 bp, whereas the

digestion by *Eco*RI and *Sac*I showed the products with the expected size of 1,558 and 5,441 bp. Lane 1: GeneRuler 1 kb DNA ladder (Thermo scientific); Lane 2,6: pET*todBA*-1; Lane3,7: pET*todBA*-6; Lane 4,8: pET*todBA*-8; Lane 5,9: pET21a (negative control).

2. The overexpression of pET21a*todC1C2BA* analyzed by SDS-PAGE analysis

After the construction of the recombinant plasmid (pET21a*todC1C2BA*-wild-type), the overexpression of pET21a*todC1C2BA* was checked by SDS-PAGE analysis

Toluene dioxygenase was encoded by *todC1C2BA* of which the sizes are 52.5, 20.8, 15.3, 46 kilodaltons (kDa), respectively. After the induction by various concentrations of IPTG (0.1, 0.2, 0.5, 0.8. and 1 mM), the recombinant strain showed the overexpression of the protein (*todC1*) at the expected size of 52.5 kDa as shown in Figure 4.4. The overexpression of *todC1C2BA* in pHKtodC1C2BA-JM109 (Nitisakulkan et al., 2014) was also presented. The pET21a*todC1C2BA* was controlled under T7 promoter of pET system, while pHK*todC1C2BA* was controlled kanamycin promoter. Both recombinant plasmids were controlled by strong promoter.

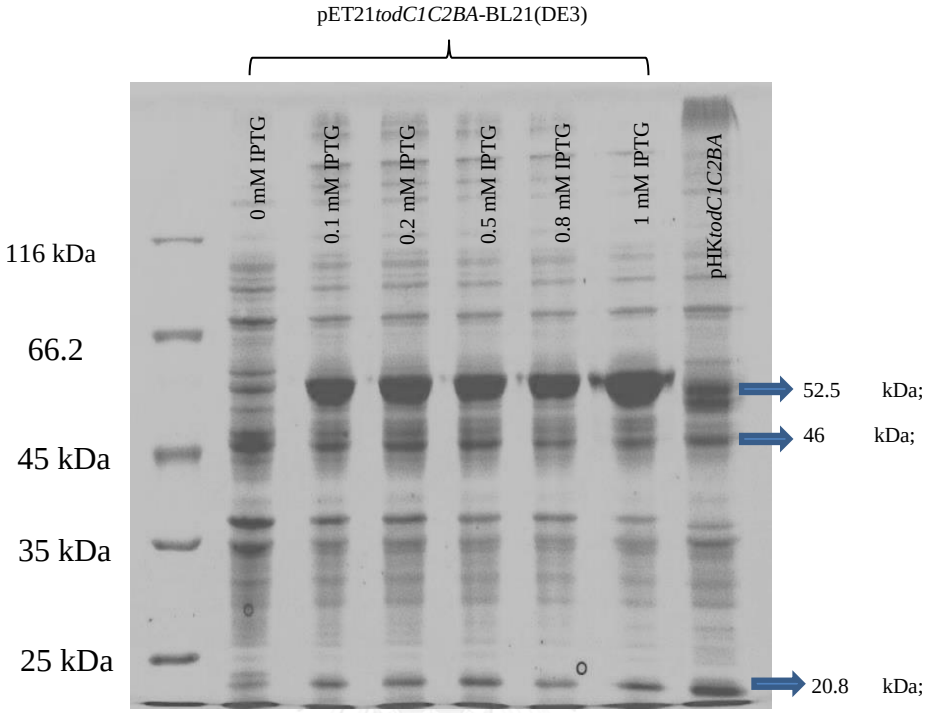


Figure 4.4 The overexpression of recombinant strain (pET21a*todC1C2BA* BL21(DE3), pBS*todC1C2BA*-DH5α and pHK*todC1C2BA*-JM109) by SDS-PAGE analysis (10 µg protein).

4.1.2 Determination of whole cell biodegradation activity toward 4CA and toluene (HPLC analysis)

In this study, the reconstructed *E. coli* recombinant strain (pET21a*todC1C2BA*-BL21(DE3)) was used to study the whole cell biodegradation activity, in comparison to that by *E. coli* (pHK*todC1C2BA*-JM109) (Nitisakulkan et al., 2014). The 4CA degradation (the initial concentration of 1 mM) by resting cell of *E. coli* (pHK*todC1C2BA*-JM109) and *E. coli* (pET21a*todC1C2BA*) were 97% ± 0.19 and 89% ± 4.27, respectively (Figure 4.5). The standard curve for HPLC analysis was shown in Appendix B.

Resting cell reaction test results indicated that *E. coli* recombinant strain (pET21a*todC1C2BA*) was able to degrade 4CA, and confirmed that *tod* gene is involved in 4CA degradation, as previously shown by Nitisakulkan and coworkers (2014).

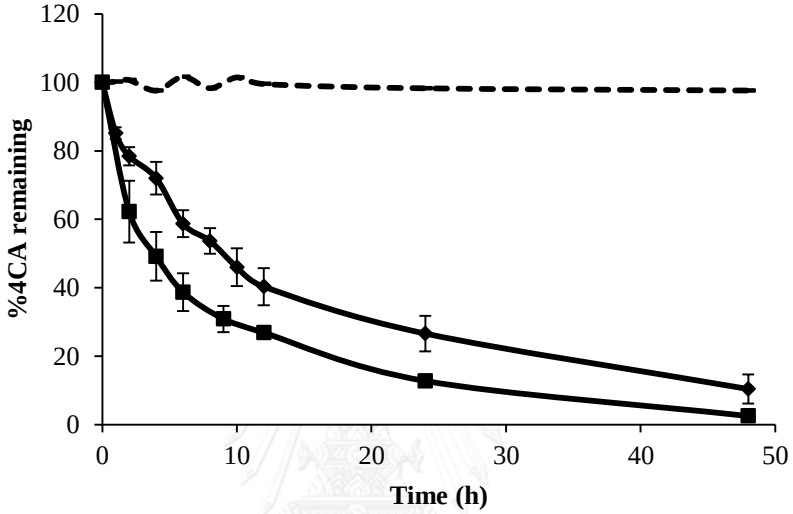


Figure 4.5 4CA degradation by *E. coli* recombinant strains. The test was conducted as a growth-independent resting cell test using *E. coli* (pHK*tod*) (■), *E. coli* (pET*tod*) (♦) and *E. coli* BL21(DE3) with an empty vector of pET21a (a control) (-).

4.1.3 The construction of the recombinant plasmid containing *todC1C2BA* genes for error prone PCR technique

Random mutagenesis is a technique used to mutate *todC1C2*, which is the target gene to improve the activity of toluene dioxygenase toward 4CA. The construction of the recombinant plasmid containing *todC1C2BA* genes for error prone PCR technique was shown in Figure 4.1. The pET21a*todBA*-8 was used as a backbone (described above) for recombinant plasmid construction. The *todC1C2*, the

targeted gene for random mutagenesis by error prone PCR technique **(**Diversify® PCR Random Mutagenesis Kit; Appendix A), showed the expected size of 2,047 bp (Figure 4.6). The mutagenesis reactions of Diversify™ PCR Random Mutagenesis Kit were shown in Table 3.2. The user manual of kit suggested that buffer condition 5 has minimal mutational bias and is the best choice for most directed evolution applications (Laboratories, 2008; Shafikhani et al., 1997). Consequently, buffer condition 5 was selected for random mutagenesis of the target gene (*todC1C2*) in this study. The primer for amplification was shown in Appendix D. The mutated *todC1C2* was designed as *todC1C2'*.

The fragments of *todC1C2'* was then ligated into pET21a*todBA* between *Bam*HI and *Eco*RI sites. After that the plasmid was transformed to *E. coli* BL21 (DE3) and plated on the selective medium. From the first round of error prone PCR, 833 clones was obtained and subjected for further screening by modified Gibbs method.

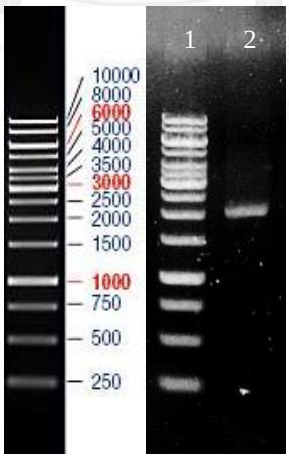


Figure 4.6 Agarose gel electrophoresis of *todC1C2'* PCR products. Lane 1: GeneRuler 1 kb DNA ladder (Thermo scientific); Lane 2: Random mutagenesis PCR product of *todC1C2'* by using error prone PCR technique.

4.1.4 The optimization of the modified Gibbs method for screening of the mutant clones

This subtopic describes reaction of Gibbs' reagent with chloroanilines, and the optimization of modified Gibbs method to screen the mutant clones. The optimization included reaction time with Gibbs' reagent, optimum maximum absorbance, optimum transformation time, and screening the mutant clones by modified Gibbs method.

1. Reaction of Gibbs' reagent with chloroanilines and its intermediates

2,6-dichloro-*p*-benzoquinone-4-chloroimide (Gibbs' reagent) is a chemical that can form a complex with a phenolic compound at *para*-position, generating a blue color (Joern et al., 2001). According to the colorimetric changes, this method can be applied to detect the aromatic oxidation reaction. The structure of Gibbs' reagent and schematic of Gibbs reaction were shown in Figure 4.7 and Figure 4.8, respectively.

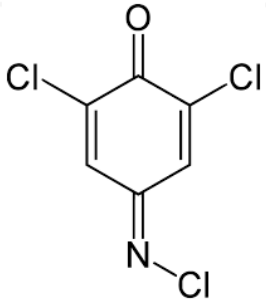


Figure 4.7 Structure of 2,6-dichloro-*p*-benzoquinone-4-chloroimide (Gibbs' reagent)

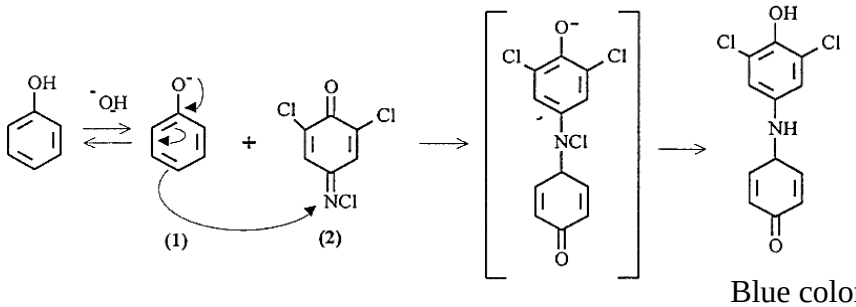


Figure 4.8 The schematic of the mechanism of the Gibbs' reagent reaction with phenol (Quintana et al., 1997).

In this study, chloroanilines and its intermediates were tested with Gibbs' reagent. The chemical structures of 2-chloroaniline (2CA), 3-chloroaniline (3CA), 4-chloroaniline (4CA), aniline, catechol, 4-chlorocatechol (4CC), and 3-chlorocatechol (3CC) were shown in Figure 4.9. When Gibbs' reagent was added, 3CA, catechol, 4CC, and 3CC, immediately generated the blue color of complex chemical (Figure 4.10A). As previously mentioned, Gibbs' reagent can form the complex at the para position of the phenolic compound, resulting in a blue color of indophenol (Josephy and Van Damme, 1984). In Figure 4.10, the reaction of Gibbs with 4CA did not show the blue color because 4CA has the chlorine at *para*-position. Nevertheless, the reaction with 4CC could generate the blue color. The 4CC structure (Figure 4.9) showed that the *para*-position of hydroxyl is free, do it may react with Gibbs' reagent to generate the blue color. Accordingly, this reagent can be used to determine the presence of *ortho*- and *meta*- substituted phenolic compounds where the substituent is a halide or alkoxy group. After 10 min, the reaction between Gibbs' reagent and aniline resulted in the light blue color because the *para*-position of aniline is free. It may take time to react at the *para*-position to give the complex compound (Figure

4.10B). In screening method aniline was selected to be a substrate for screening due to transformation of aniline to be catechol, the catechol could react with Gibbs' reagent.

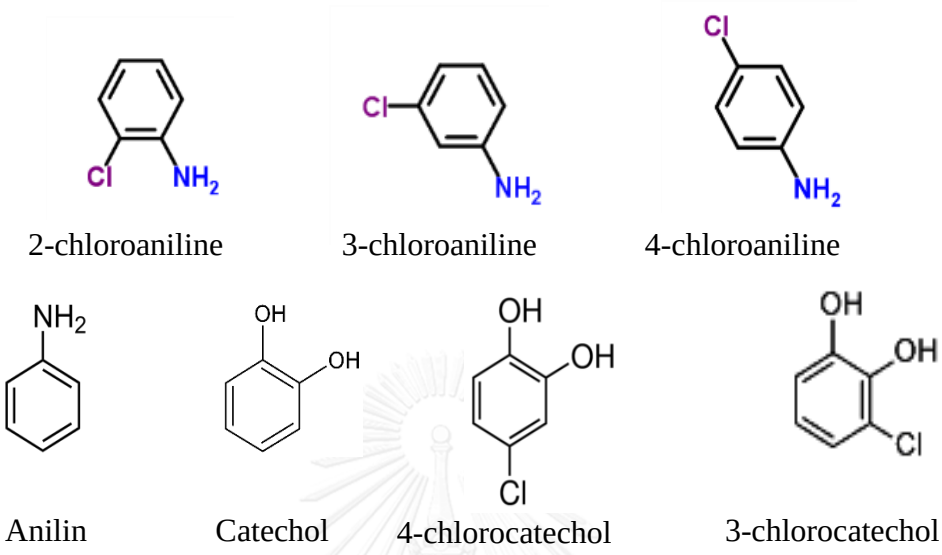


Figure 4.9 The structure of chloroanilines and its intermediates used in this study

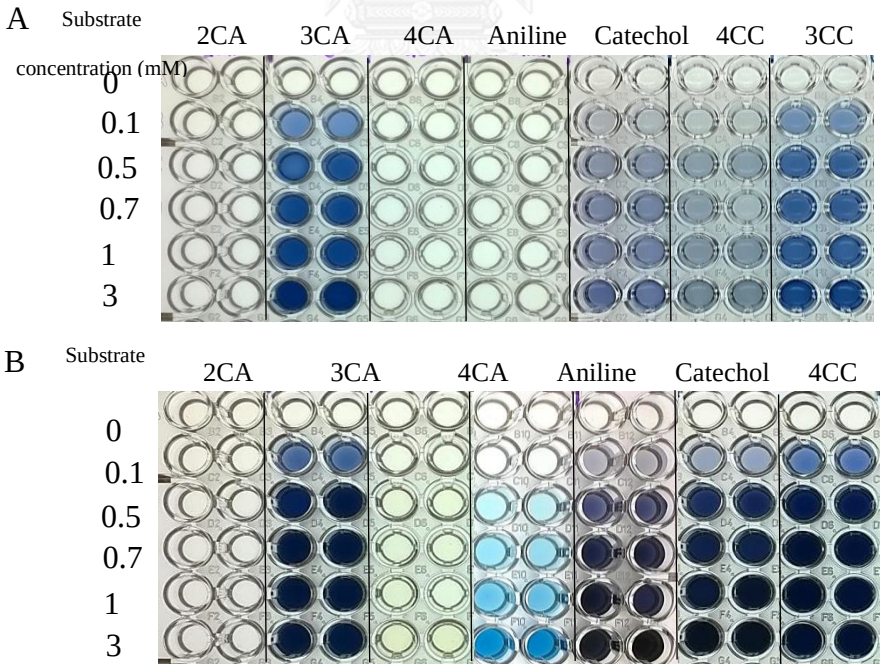


Figure 4.10 The reaction of Gibbs reagent with chloroanilines and its intermediates at A) 0 min and B) 10 min.

2. The optimization of the modified Gibbs method for screening the mutated clones

1) The maximum absorbance of each chemical complexed with Gibbs' reagent.

The previous study (Nitisakulkan et al., 2014) showed that toluene dioxygenase is able to transform 4CA. Gibbs' reagent was used in the spectrophotometric determination of catechol, and 4CC. Due to the similarity of chloroanilines structure, the maximum absorbance of each chemical should be distinguished and identified. The maximum absorbance (λ_{max}) was determined by varying concentration of each chemical. The complex formed between Gibbs'reagent and each chemical was scanned by using a micro plate reader (Thermo scientific; multiskan go). The maximum absorbance (λ_{max}) of the complex formed between Gibbs' reagent and each chemical are as follows: Gibbs-aniline, Gibbs-catechol, and Gibbs-4CC were 660, 576, and 590 nm, respectively (Figure 4.12). In this experiment, aniline or 4CA was used as a substrate for screening by determining the increasing A_{660}, A_{576}, and A_{590}. If 4CA is transformed to 4CC, the A_{590} of Gibbs-4CC would increase the reaction of 4CA or aniline with Gibbs' reagent was shown in Figure 4.11. If aniline is transformed to catechol, the absorbance of both (Gibbs-aniline at A_{660} and Gibbs-catechol at A_{576}) would increase due to the interference of aniline (Figure 4.11E). However, with a fixed incubation time, it can be differentiated between aniline oxidation and catechol formation using the maximum absorbance at 576 nm.

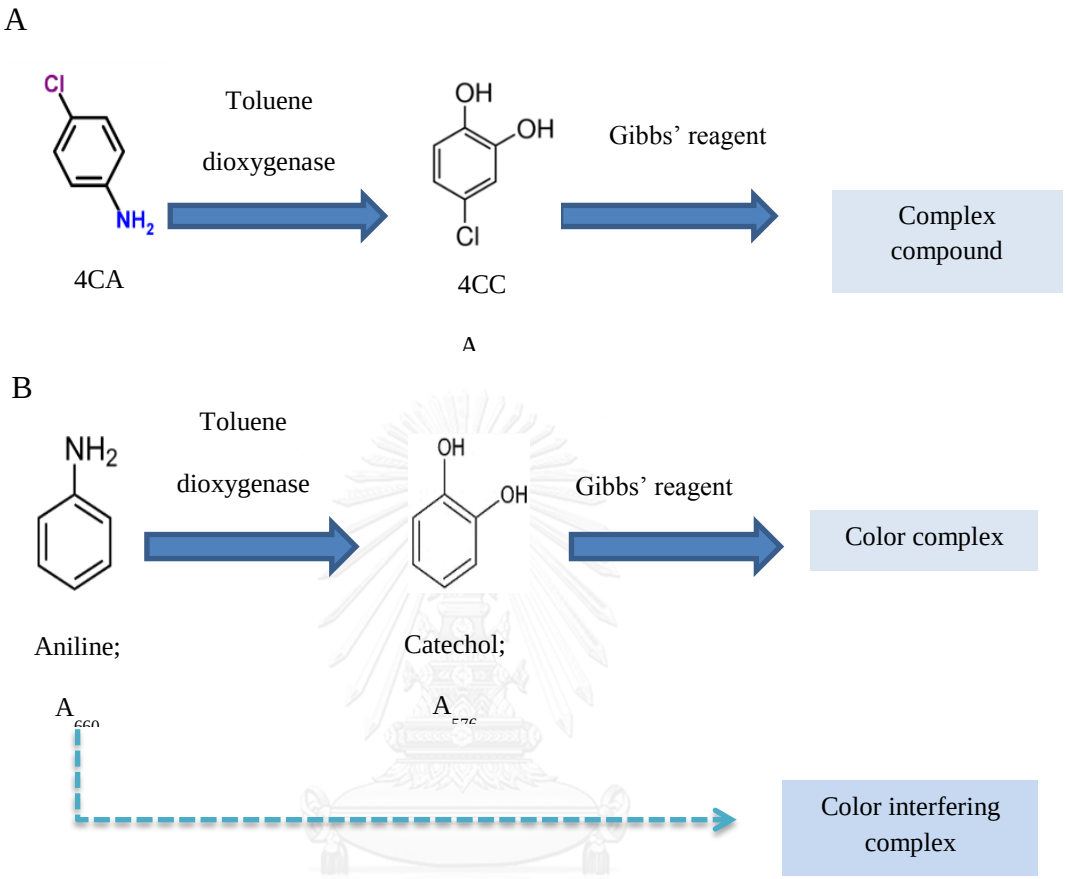


Figure 4 11 The equation of the reaction of 4CA or aniline with Gibbs' reagent.

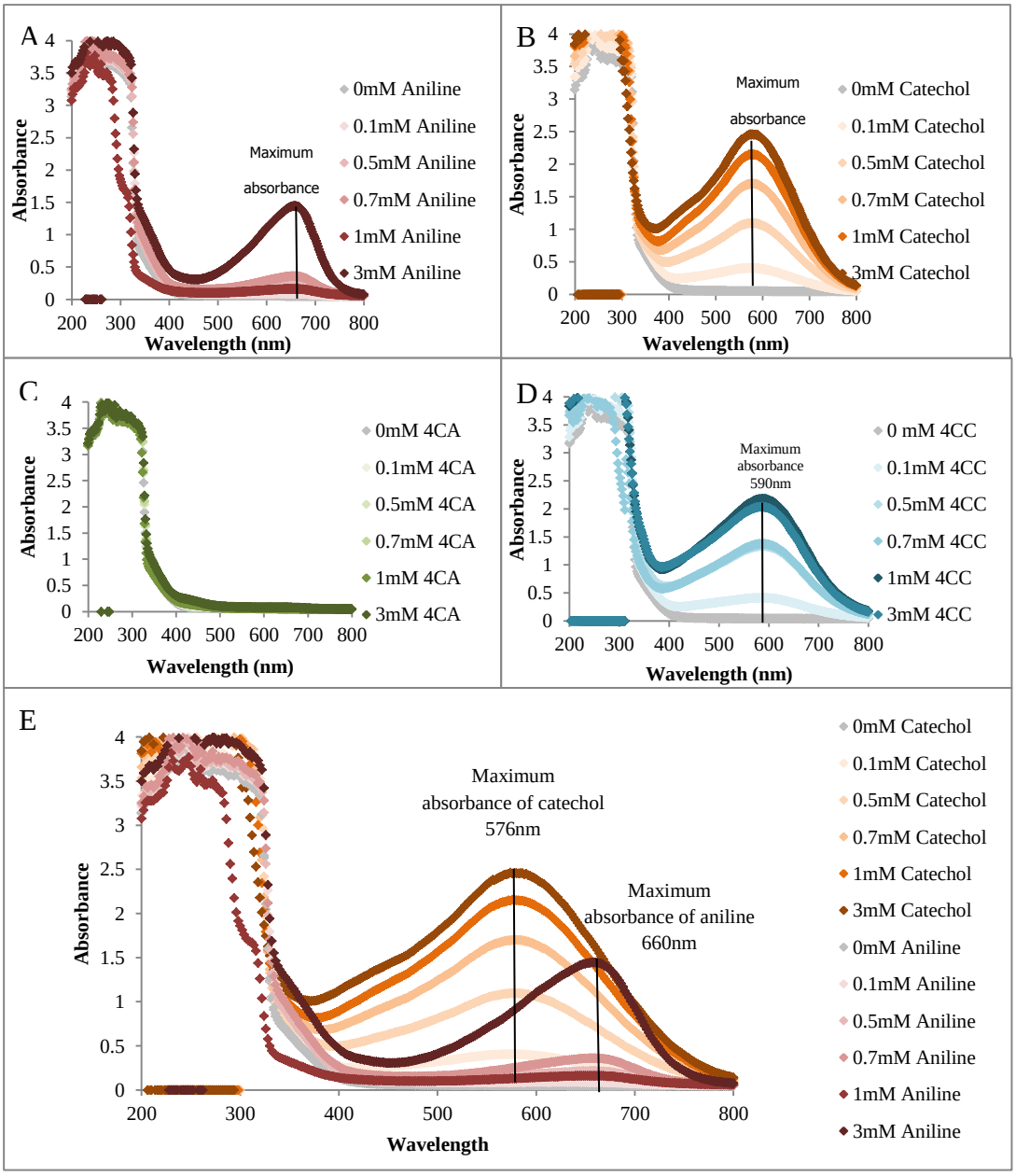


Figure 4.12 The maximum absorbance of aniline (A), catechol (B), 4CA (C), 4CC (D), aniline and catechol (E).

2) The optimum reaction time with Gibbs' reagent

The optimum reaction time with Gibbs reaction was investigated by fixing wavelength at the maximum absorbance of each Gibbs-chemical complex and varying the concentrations of chemical and the reaction time. Then, the graph was plotted between the maximum absorbance and the concentration of chemical (Figure 4.13).

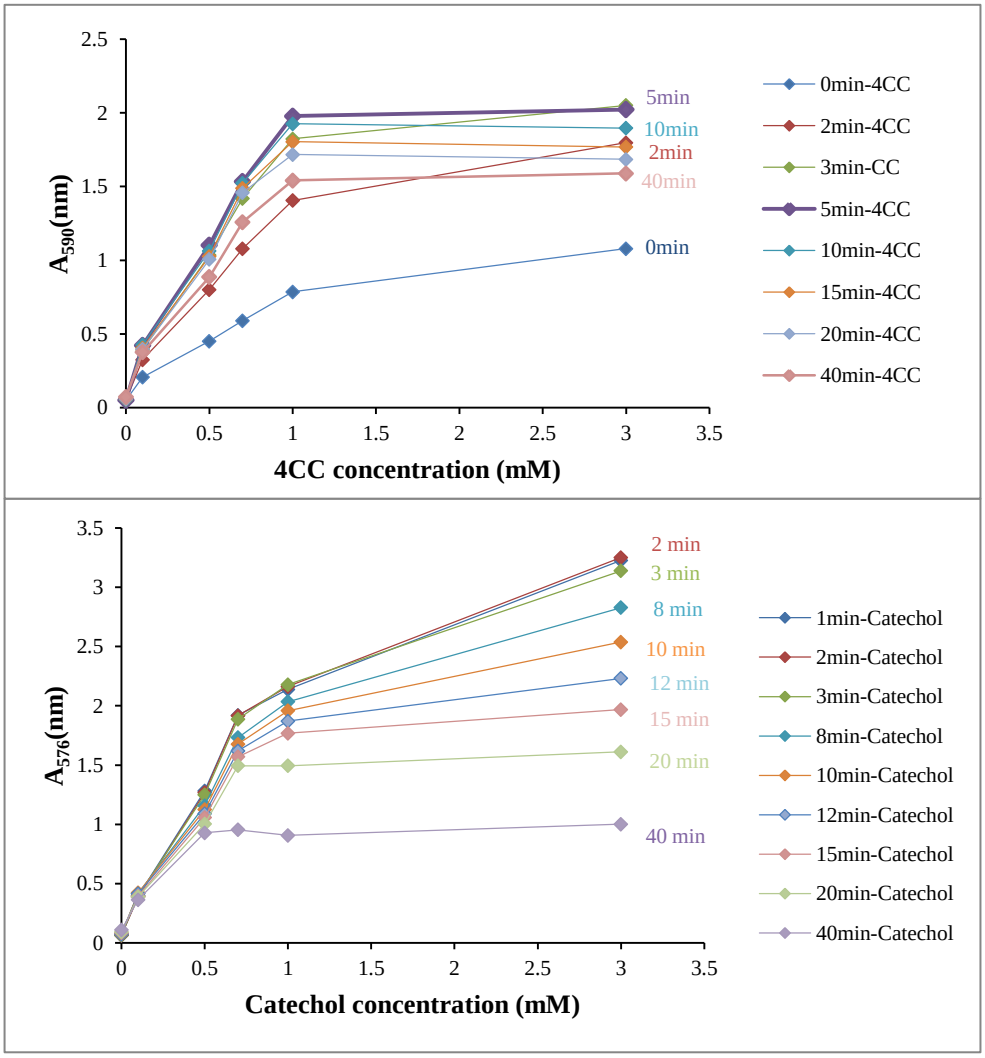


Figure 4.13 The relationship between maximum absorbance and concentration of chemical with Gibbs' reagent; 4CC (A), catechol (B).

4CC and catechol was selected to determine the optimum reaction time of chemical with Gibbs' reagent because they are the target intermediate from 4CA and aniline conversion. The optimum reaction time of chemical with Gibbs' reagent was determined by the relationship between the maximum absorbance and concentration of chemical. The color of Gibbs-4CC and Gibbs-catechol were decreased during the periods of time (0 – 40 min). Thus, the optimum reaction time between Gibbs' reagent and each chemical was selected at the time in which the color was stably formed reacted when the chemical react with Gibbs' reagent (Figure 4.13). The optimum reaction time of Gibbs-catechol, and Gibbs-4CC were, 2 min, and 5 min, respectively. The raw data was shown in Appendix C.

3) The optimum of substrate transformation time

The optimum transformation time of 4CA was determined by using *E. coli* (pHK*todC1C2BA*-JM109) as a model strain. 4CA with the initial concentration of was used as substrate for transformation. At each time interval, the sample was taken to measure 4CC formation. The supernatant was tested with Gibbs' reagent as described below. After 5 min of Gibbs reaction, the A_{590} was measured by a micro plate reader. To normalize the absorbance value obtained by each reaction, the A_{590} value was normalized with its cell density (OD_{600}). Four hours of transformation time showed the highest value of A_{590}/OD_{600}, while the declining 4CC formation value was observed after prolonged transformation time. Therefore, the optimum transformation time was 4 h. (Figure 4.13). The obtained data indicated that 4CA was transformed to be 4CC within 4 h. After prolonged exposure, 4CC is apparently further transformed to other compounds, which are unable to react with Gibbs' reagent. Thus, the

transformation period longer than 4 h, was not appropriate for detecting 4CC as the intermediate of 4CA conversion. The calculation of the interval formation of 4CC detected by the modified Gibbs method (Figure 4.14) was shown in Appendix C.

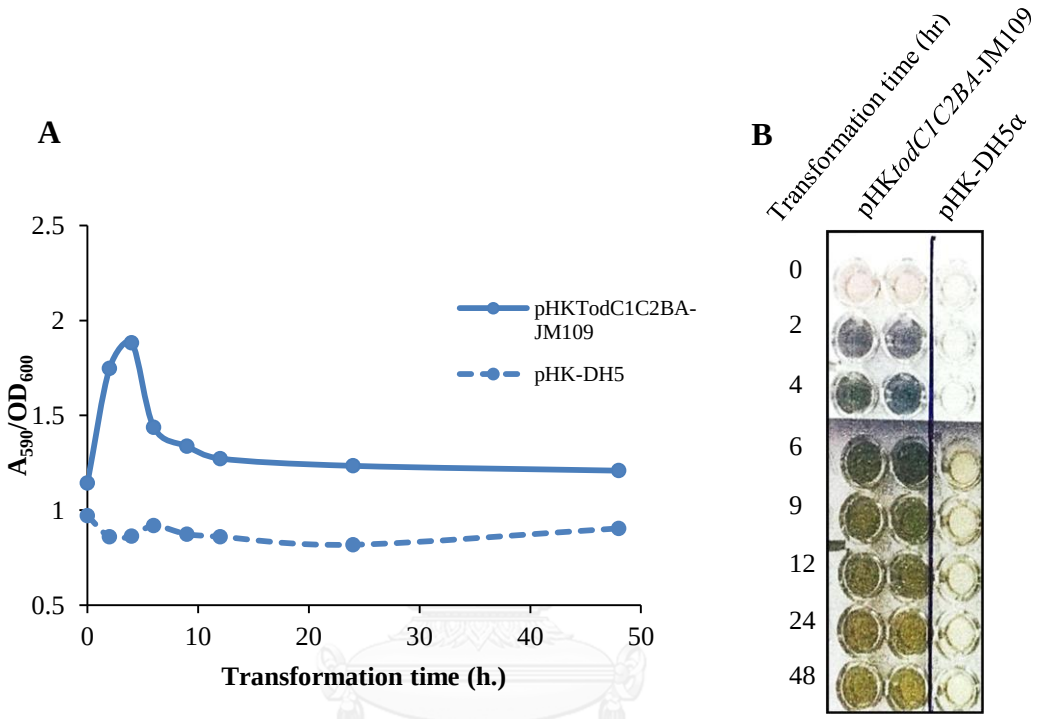


Figure 4.14 The interval formation of 4CC detected by the modified Gibbs method. The relation between A_{590}/OD_{600} and transformation time (A). The color of 4CC formation detected by Gibbs' reagent (B).

The optimum condition for using modified Gibbs method for screening the mutant was summarized in Table 4.1. Because of the interference of peak aniline and catechol as shown in Figure 4.12E, the A_{660} could not show the decreasing of aniline. Thus, for the interpretation of screening result, the higher value of product formation, which is catechol (A_{576}/OD_{600}) and 4CC (A_{590}/OD_{600}) was expected. In conclusion,

the optimized procedure to use the modified Gibbs method for this detection was shown in Figure 4.15.

Table 4.1 The optimum condition of the modified Gibbs method

	aniline	**catechol**	**4CC**
1.The maximum absorbance	A_{660}	A_{576}	A_{590}
2.Transformation time	4 h.		
3. Reaction time with Gibbs' reagent	10 min	2 min	5 min
Analysis value	-	A_{576}/OD_{600}	A_{590}/OD_{600}

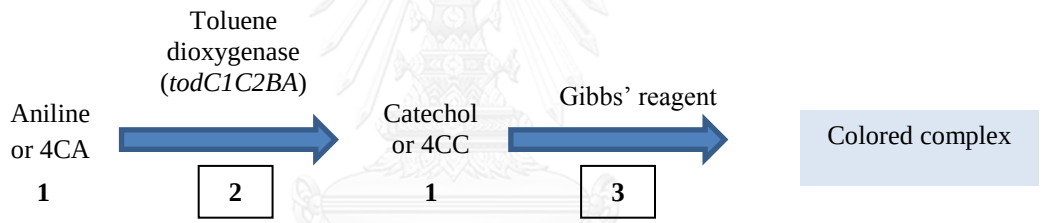


Figure 4 15 The procedure of optimized of the modified Gibbs method.

3. Screening the mutant clones by the modified Gibbs method.

Toluene dioxygenase (*todC1C2BA*) is encoded by three components: reductase (*todA*), ferredoxin (*todB*), and the two subunits of terminal dioxygenase (*todC1C2*) (Friemann et al., 2009). These components play different roles in toluene dioxygenase system. The terminal dioxygenase (*todC1C2*) is the subunit of interest because it is where the catalytic reaction takes place. The active site is also located involved in this subunit (Friemann et al., 2009). Consequently, *todC1C2* is the target for random mutagenesis by error prone PCR technique **(**Diversify® PCR Random

Mutagenesis Kit; Appendix A). The procedure for construction the mutated recombinant plasmid for screening is previously shown in Figure 4.1. The mutated recombinant plasmid was transformed to *E. coli* BL21 (DE3). Totally, 833 mutants were screened with Gibbs' reaction by using aniline or 4CA as a substrate. In this step, 41 mutants showed the higher A_{576}/OD_{600} than that of the wild-type. Thus, the second round of screening was used in replicate. Three clones of the mutants immediately generated blue color after added Gibbs' reagent (Figure 4.16). Consequently, the three mutants showed higher catechol formation than that the wild-type. When aniline was used as substrate, it can also be detected by Gibbs' reagent and gave the light blue color. The green color of catechol was detected as a product for this transformation. Aniline and catechol can react with Gibbs' reagent. They can be detected by different wavelength, which are A_{660} and A_{576} of Gibbs-aniline and Gibbs-catechol, respectively. Thus, the three positive mutants, R1P1-26, R1P2-39 and R1P13-2, were obtained. This method can be used as qualitative as well as quantitative screening method. They could be showed the colorimetric (quality) compare with the recombinant wild type (Figure 4.16) and they also showed the higher relative value (quantity) of mutants compared with wild-type. Table 4.2 shows the formation rate of catechol, which calculated by A_{576}/OD_{600} divided by transformation time (4 h). The mutants (the recombinant strain) showed the higher formation rate than that of the wild-type. Moreover, the mutants showed higher the fold of catechol formation rate 1.5-2.3 folds than that by the wild-type recombinant.

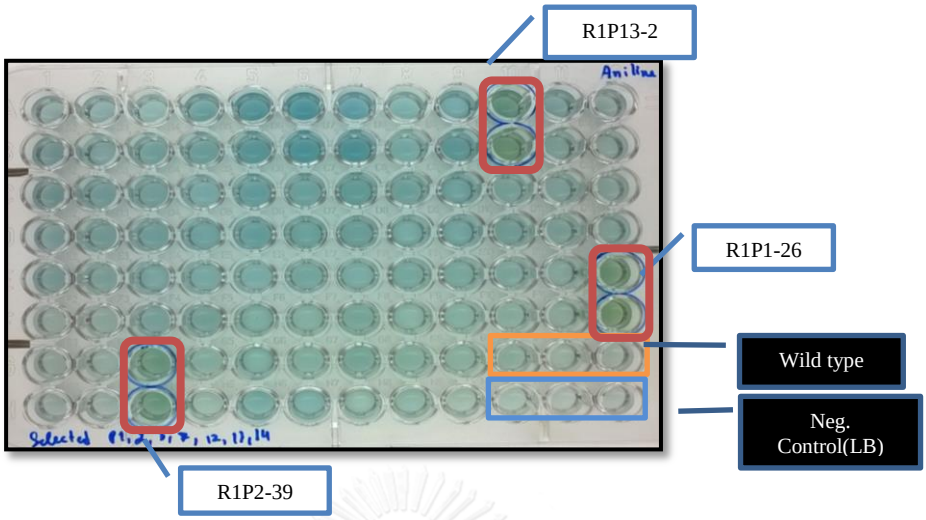


Figure 4.16 The second round of the screening of the positive mutants using the modified Gibbs method.

Table 4.2 The relative rate of aniline conversion detected by Gibbs' reagent of the tentative mutant compared with recombinant wild type

Strains	**Formation rate of catechol A_{576}/A_{600}/h.**	**Fold of Catechol formation rate**
R1P1-26	0.149	1.520
R1P2-39	0.227	2.316
R1P13-2	0.197	2.010
wild-type	0.098	1.000

In protein engineering, the mutagenesis of gene can be obtained *via* two general approaches: site-directed mutagenesis and random mutagenesis. Site direct mutagenesis is the technique to random only specific positions. The important

residues of gene should be identified first. While, the random mutagenesis including error prone PCR technique introduces changes randomly at positions throughout the gene sequence. In this study, random mutagenesis by error prone PCR technique was selected to mutate the targeted gene, although some of the important residues in the substrate binding site of toluene dioxygenase are reported. Due to the large size and complexity of toluene dioxygenase, it takes long time to predict the optimal condition of interaction of enzyme with substrate using protein modeling. In addition, the conserved region of the substrate binding site (at 226-384 residues) is in the wide range and residue(s) involved in specific interaction with other substrate such as 4CA is still unclear. Thus, the error prone PCR technique was selected to mutate the targeted gene and the screening method was developed to select the mutants, which had high catalytic efficiency toward 4CA.

Random mutagenesis is one approach of directed evolution, which can be applied to a protein to produce mutated genes with enhancing properties. The microorganism or cells may be disclosed to mutants by UV radiation or mutagenic chemicals. Error prone PCR is one approach for generating random mutations in the target proteins, which is a common technique to use for protein engineering. Joo et al., 1999 was enhanced the activity of cytochrome $P450_{cam}$ in *Pseudomonas putida* by error prone PCR and they also developed the method for screening the mutants library by colorimetric method, which is Horseradish peroxidase (HRP). It couples the phenolic products of hydroxylation of aromatic substrates (Joo et al., 1999). Toluene dioxygenase was used error prone PCR technique to extent the range the range of substrate. Sakamoto and coworkers (2001) reported the improvement of the toluene

dioxygenase activity by laboratory evolution to accept 4-Picoline as a Substrate. The *todC1* is the target gene, which they improved by error prone PCR technique (Sakamoto et al., 2001). They got the mutants, which had the activity between 1.4-5.6 folds higher than that of the wild-type. Ang and colleague (2009) used error prone PCR technique for enhanced bioremediation of aniline dioxygenase. They also developed the screening method by using Gibbs' reagent. The results showed that mutated enzyme had high activity between 2-98 folds compared with the wild-type.

In bioremediation field, Gibbs' reagent was also developed to use as a high throughput screening for dioxygenase. Ang et al., (2009) used Gibbs method to screen the mutants of *E. coli* harboring aniline dioxygenase gene (*AtdAB)* form *Acinetobacter* sp. stain YAA, which showed the enhanced activity between 2-98 folds of relative activity compared with the wild-type of aniline dioxygenase and capable of hydroxylating a wider range of aromatic amines than the wild-type. The activity of their mutants was indicated by A_{620} normalized by its cell density for investigation of positive mutants (Ang et al., 2009).

The expected result for using the direct evolution is the enhancement of the activity of interest. The error prone PCR technique generated the mutated gene, and then the screening method was used to select the positive mutants that have higher activity compared with its wild-type (Ang et al., 2009; Bartel et al., 2011; Joo et al., 1999; Kim and Lei, 2008; Morimoto et al., 2014; Sakamoto et al., 2001; Zhang et al., 2000). The relative activity of the mutated enzyme was shown higher than that wild-type, which showed in Table 4.3.

Table 4.3 Comparison of the enhancement activity by directed evolution

Enzyme	Strain	Substrate	Directed evolution approach	Screening method	Activity compared with wild-type (Fold of relative activity)	References
Toluene dioxygenase	*Pseudomonas putida* F1	4-Picoline	Error prone PCR	Gibbs' reagent	1.7-5.6	(Sakamoto et al., 2001)
		Toluene			1.4-1.9	
Toluene dioxygenase	*Pseudomonas putida* F1	Coumarin	Error prone PCR	High-throughput fluorometric and spectrophotometric assays	1.1-1.6	(Zhang et al., 2000)
		Napthalene			6-9	
		Indene			1.2	
Aniline dioxygenase	*Acinetobacter* sp. strain YAA	Aniline	Error prone PCR	Gibbs' reagent	8.9	(Ang et al., 2009)
		2,4-dimethylaniline			98	
		2-isopropylaniline			2	
Phytase	*Saccharomyces cerevisiae*	-	Error prone PCR	Screening for improved thermostability	110	(Kim and Lei, 2008)
Styrene monooxygenase	*Pseudomonas putida* CA-3	Indene	Error prone PCR	indigo production rates	8-12	(Gursky et al., 2010
cytochrome $P450_{cam}$	*Pseudomonas putida* strain.	3-phenylpropionate (3-PPA)	Error prone PCR	Fluorescence image analysis	2.4-3.2	(Joo et al., 1999)
malic enzyme	*Pseudomonas diminuta*	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	Error prone PCR	The MTT colorimetric assay	1.6-3.9	(Morimoto et al., 2014)
Toluene dioxygenase	*Pseudomonas putida* T57	Aniline	Error prone PCR	Gibbs' reagent	1.5-2	This study

Table 4.3 showed the comparison of the enhanced activity by directed evolution of previous studies. The fold of relative activity of mutants compared with wild-type of previous studies showed higher than this study because the fold of relative activity was shown from mutants, which used two or three round of mutagenesis by using mutant that had high activity from round one as a template. In this study, the mutants were generated from one round of random mutagenesis showed the 1.5 – 2.3 fold of increasing relative activity of mutants compared to its wild-type In order to increase the activity, the second round of mutagenesis is planned. However, the characterization of mutants and the substituted residues have to be completed first; since this information of the first round of mutagenesis is essential for the secondary mutagenesis.

4.2 Characterization and comparison of the enzyme catalytic activity (wild type and mutated ones) toward 4CA

4.2.1 Determination of 4CA biotransformation rate

After the positive mutants were selected, the determination of 4CA degradation was investigated. 4CA biotransformation rate was determined by using HPLC analysis. Cell- free extract was prepared as shown above, and further used for degradation test by using 4CA or aniline (1 mM) as a substrate. Table 4.4 shows the degradation rate by using 4CA or aniline. HPLC analysis results indicated that the catalytic rate of mutants was higher than that of the wild-type, but the extent is different between aniline and 4CA substrate. The results of aniline degradation rate and the fold of aniline degradation rate (Table 4.4) agreed with catechol formation rate detected from the Gibbs' method. The mutants showed 1.3-2.0 higher folds of aniline degradation rate than that of the wild-type.

Table 4.4 The degradation rate of 4CA and aniline by using crude enzyme

Recombinant Strain	**Degradation rate (μM/h)**			
	4CA	Fold of 4CA degradation rate	**Aniline**	Fold of aniline degradation rate
Wild-type	97.164	1.00	70.987	1.00
R1P1-26	72.046	0.74	NA	-
R1P2-39	99.444	1.02	141.748	2.00
R1P13-2	10.374	0.11	93.718	1.32

NA = not determined

4.2.2 Selection of the mutated enzymes with improved catalytic efficiency

The study of catalytic efficiency of toluene dioxygenase was conducted using the crude enzyme of the wild-type and the mutants and varying the 4CA or aniline concentration (0.01, 0.05, 0.1, 0.3, 0.5, 1, 3, 5 mM).

The biodegradation kinetic study by cell free extract was studied to determine the kinetic parameter of enzyme. The Michaelis-Menten model was used to find the relationship between substrate concentration and degradation rate. Furthermore, The Lineweaver-Burk plot was used to calculate the kinetic parameter. Michaelis-Menten model of 4CA and aniline degradation was shown in Figure 4.17 and 4.18 respectively. The raw data of catalytic study was shown in Appendix C.

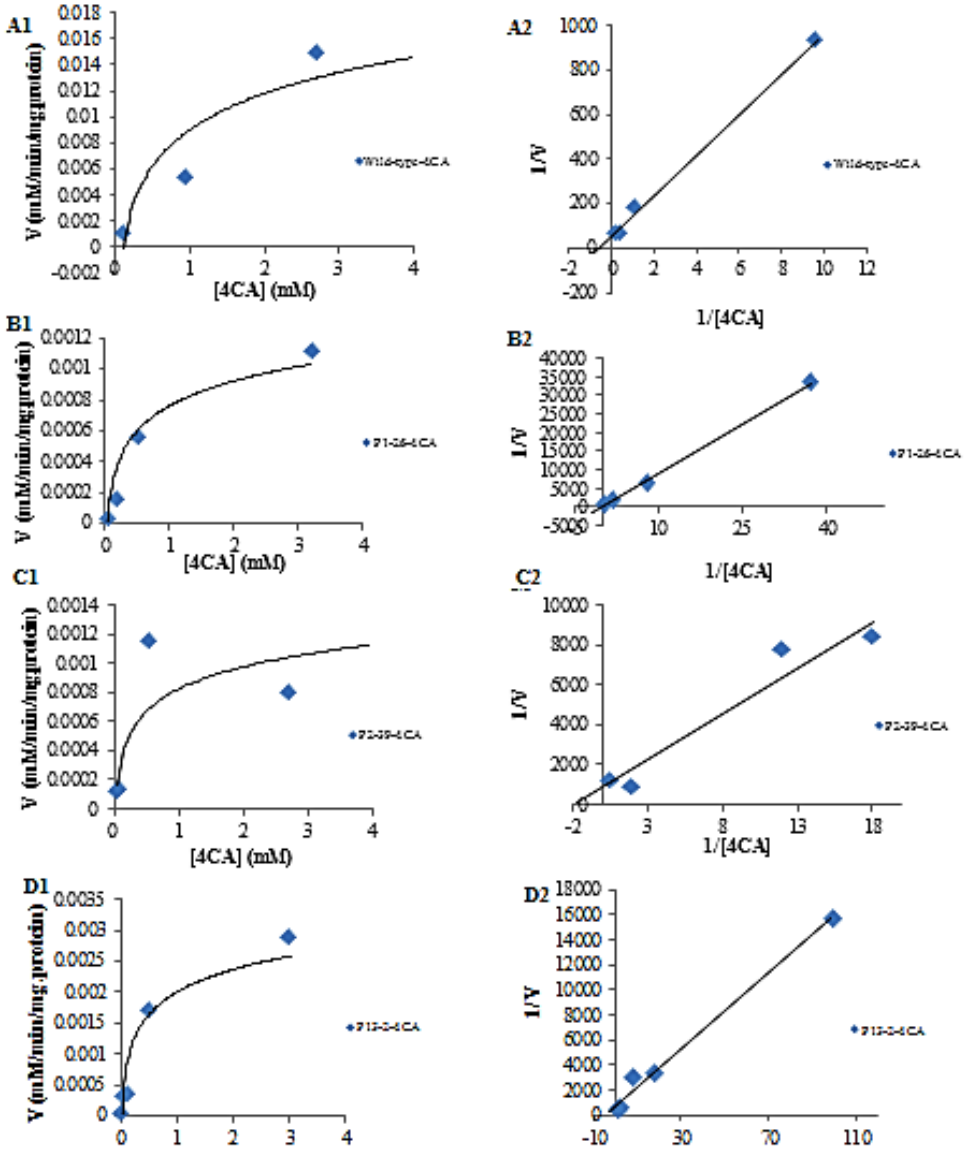


Figure 4.17 1) The relationship between reaction rate (V) and substrate concentration (S) of 4CA degradation by the wild-type recombinant (A), and the mutant recombinant strains R1P1-26 (B), R1P2-39 (C), and R1P13-2 (D). 2) The relationship between 1/V and 1/[4CA] of 4CA degradation by wild-type (A), and the recombinant strain R1P1-26 (B), R1P2-39 (C), and R1P13-2 (D).

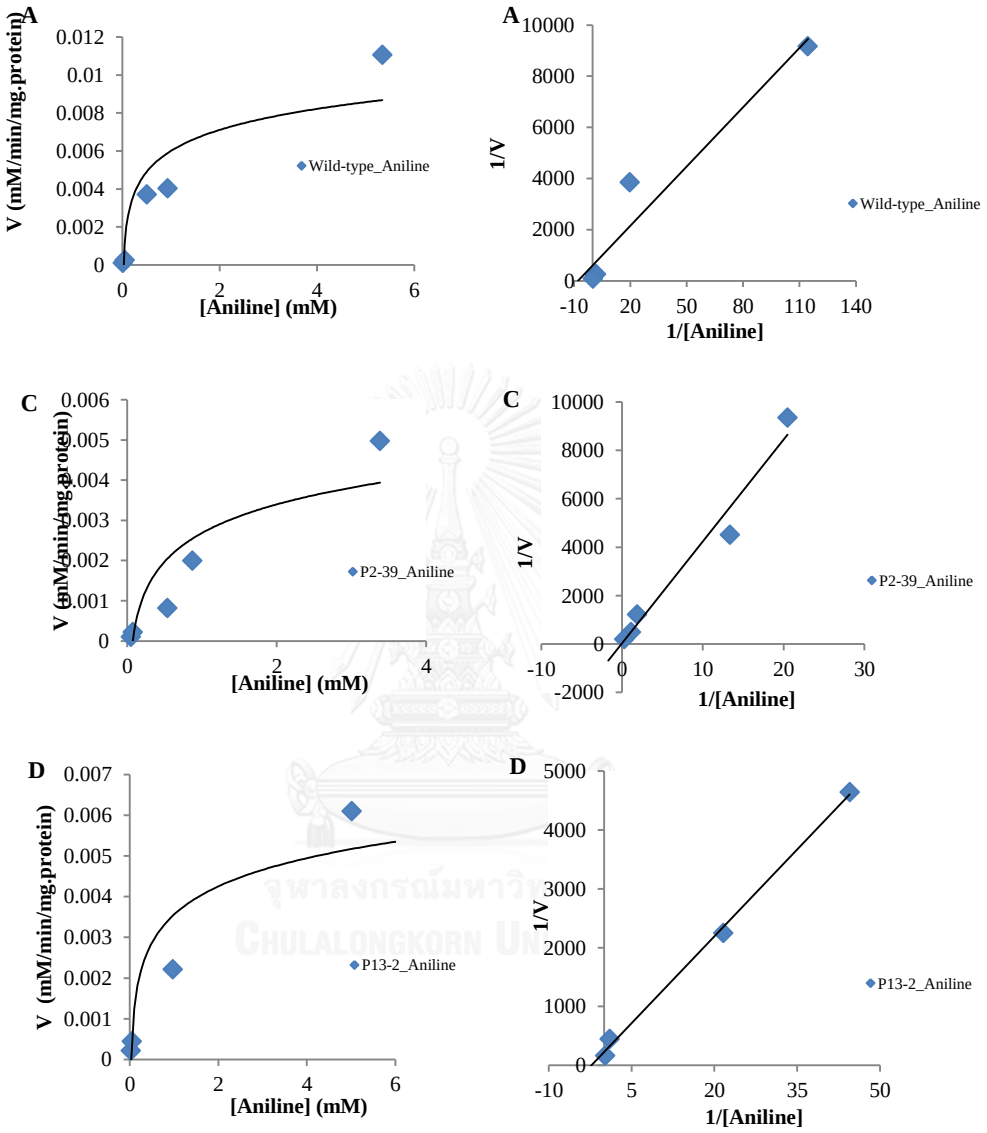


Figure 4.18 1) The relationship between reaction rate (V) and substrate concentration (S) of aniline degradation by the wild-type recombinant (A), and the mutant recombinant strain R1P2-39 (C), and R1P13-2 (D). 2) The relationship between 1/V and 1/[Aniline] of aniline degradation by wild-type (A), and the recombinant strain R1P2-39 (C), and R1P13-2 (D).

The activity of the mutated and wild-type enzyme was assayed by using the reaction mixture as shown in Table 3.3. The mutated enzymes, R1P1-26, R1P2-39, and R1P13-2, were characterized in comparison to the wild-type. The kinetics parameter was shown in Table 4.5. The mutants showed the different value of kinetic parameter of 4CA and aniline conversion. The kinetic parameters of the enzyme were focused on their specificity (K_m) and maximum rate (V_{max}) using various concentrations of 4CA or aniline (Table 4.5). For using aniline as substrate, the mutated enzymes R1P2-39 and R1P13-2, showed the higher K_m than that of wild-type, while the V_{max} of mutants were 2.6 to 4 times higher than wild-type. The K_m of wild-type enzyme toward 4CA was 0.9 mM. The mutants R1P2-39 and R1P13-2 showed the lower K_m than that wild-type, whereas R1P1-26 showed the higher K_m. Apparently, R1P1-26 had lower specificity of 4CA compare with the others, while all of mutants had the maximum rate of 4CA higher than that wild-type.

In total, 833 mutants were screened by using aniline as substrate. R1P1-26, R1P2-39 and R1P13-2 showed the higher enzyme efficiency (1.5 to 2 fold) than that of wild-type in 4 hours transformation. The V_{max} of mutants (R1P1-26, R1P2-39, and R1P13-2) using aniline as substrate also showed the higher maximum rate, correlated to HPLC analysis. The results of catalytic efficiency by using aniline as substrate indicated that the mutants (R1P2-39 and R1P13-2) are able to degrade aniline faster than that wild-type. For 4CA degradation, the results showed that R1P2-39 and R1P13-2 had lower K_m, whereas R1P1-26 showed high K_m value, compared to wild-type. These results indicated that the mutants (R1P2-39 and R1P13-2) were more

specific with 4CA than that of the wild-type enzyme, while R1P1-26 showed the opposite result.

Table 4.5 Kinetics parameter of mutants and wild type (crude enzyme)

Strains	4CA		Aniline	
	K_m (mM)	V_{max} (mM.min^{-1})	K_m (mM)	V_{max} (mM.min^{-1})
Wild-type	0.9168 ± 0.5658	0.0091 ± 0.0069	0.1294	0.0017
R1P1-26	5.0136	0.0056	NA	NA
R1P2-39	0.3803 ±0.3192	0.0012 ± 0.0003	3.2385	0.0072
R1P13-2	0.1961	0.0013	0.4444	0.0045

NA = not determine

To improve the catalytic efficiency, the obtained mutants are expected to have high affinity (low K_m) and high catalytic rate (high V_{max}), however this study showed different results. The results of kinetic parameter of mutants and the wild-type showed low of K_m and low of V_{max} in mutants (R1P2-39 and R1P13-2) owing to the limitation of enzyme function. Toluene dioxygenase has three main subunits, which are oxygenase, reductase and ferredoxin. The oxygenase subunit (*todC1C2*), which is the targeted gene for mutation is essential for activity and plays a major role in substrate specificity. In consequence, the mutants showed the improvement of catalytic efficiency with higher specificity (low K_m) compared with that wild-type. The catalytic rate (V_{max}) of mutants showed lower than the wild-type possibly due to the limitation of electron transfer of enzyme function. The ferredoxin (*todB*) and reductase (*todA*) is important for electron transfer to catalytic take place (oxygenase

subunit) of enzyme. From this reason, lower of catalytic rate also depend on the reductase and ferredoxin subunit of this enzyme.

For application, the efficiency of the mutated enzyme is one of several factors affecting enzyme bioremediation process. The enzyme with low K_m can be selected in the application due to its high specificity with substrate, solving the problem of substrate inhibition. When, the contaminated field contains many chemicals, the enzyme with low K_m can be functioned with the targeted substrate; while the enzyme with high V_{max} can be selected due to the ability of high catalytic rate with the pollutant substrate.

4.3 Identification and analysis of the point mutation of toluene dioxygenase

4.3.1 Identification the mutated points by sequencing analysis

Comparing with the wild-type, all three selected mutants showed the enhancing catalytic efficiency of toluene dioxygenase with 4CA or aniline. The identified amino acid substitutions in the selected mutants were shown in Table 4.6. From the results of identification of amino acid substitutions by sequencing analysis revealed that the amino acid substitution occurred in 4 positions in *todC1*(i.e. E135, M186, E339, V345) and another position in *todC2* (i.e. Q175). The position at 345 and 339 showed the amino acid substitution was difference in 3 mutants. It acts as substrate binding site. It can be summarized that the different activity is depend on these residues. The three selected mutant are R1P1-26 (E339Q), R1P2-39 (V345G), and R1P13-2 (E339K). Since E339Q, V345G, and E339K are involved in substrate binding site, therefore, the enhancing of enzyme efficiency may associate with charge alterations or interactions between proteins and their function (Kim and Lei, 2008; Shoichet et al., 1996).

The enhancing efficiency of toluene dioxygenase by error prone PCR were reported (Sakamoto et al., 2001; Zhang et al., 2000). Most of mutated positions are in substrate binding site, which is important for enzyme to fit with substrate. Sakamoto and coworker (2001) reported the mutation at position 451, which is a stop codon of *todC1* and found that a new stop codon is generated just after the start codon of *tod*C2. Therefore, the mutated point generating a stop codon leads to the extension of *tod*C1 and the extension of C-terminal which improve the activity of toluene dioxygenase toward the substrate (4-picoline) (Sakamoto et al., 2001).

Table 4.6 The identified amino acid substitutions by sequencing analysis.

Strains	**Mutation position *todC1* (450aa;)**				**Mutation position *todC2* (187aa)**
	135 (404)	**186 (557)**	**339 (1015)**	**345 (1034)**	**175 (524)**
P. putida T57	E (gag)	M (atg)	E (*g*ag)	V (g*t*g)	Q (cag)
P. putida F1	E (gag)	M (atg)	E (*g*ag)	V (g*t*g)	Q (cag)
Wild-type-ref	E (gag)	M (atg)	E (*g*ag)	V (g*t*g)	Q (cag)
R1P1-26	G (g*g*g)	T (a*c*g)	Q (*c*a*a*) insertion a at 1017	FRAME SHIFT	R (c*g*g)
R1P2-39	G (g*g*g)	T (a*c*g)	E (*g*ag)	G (g*g*g)	R (c*g*g)
R1P13-2	G (g*g*g)	T (a*c*g)	K (*a*ag)	V (g*t*g)	R (c*g*g)

4.3.2 The prediction of the mutated points by comparing with the reference structure.

From the random mutagenesis of residues, two mutated points, V345 and E339, which is closer to the active site, were identified. The substitutions in these two positions enhanced aniline or 4CA conversion. The 3EN1 (pdb. files) of *todC1C2* (large α-subunit and small β-subunit) was used as a model structure in this experiment. The location of mutated amino acid was shown in Figure 4.19. The model structure was determined using the Swiss PDB Viewer (spdbv) program. The procedure of Swiss PDB viwer (spdbv) program was shown in Appendix D3. The *todC1* gene was aligned with rieske non-heme oxygenase family, which are napthalene dioxygenase (Kauppi et al., 1998), toluene dioxygenase (Friemann et al.,

2009) and biphenyl dioxygenase (Furusawa et al., 2004). It showed the conserved region of substrate binding site (at 226-384 residues) that involved in the active site at 226-388 residues. The other important residues are 2Fe-2S binding site and Fe binding site were at 100-125 and 233-388 residues, respectively. The results revealed that the mutated points (V345G and E339K) were in the substrate binding site (Figure 4.20), while the M186T and E135G did not relate to any reported important function. Figure 4.21 showed the dissimilarity of mutated amino point in each position. The results revealed that the substituted amino acid at the position of M186 and E135 may involve in hydrogen bonding between amino acid, which may be a reason of improving activity. The *todC2* showed the mutation at the Q175R, which is the β-subunit of oxygenase subunit.

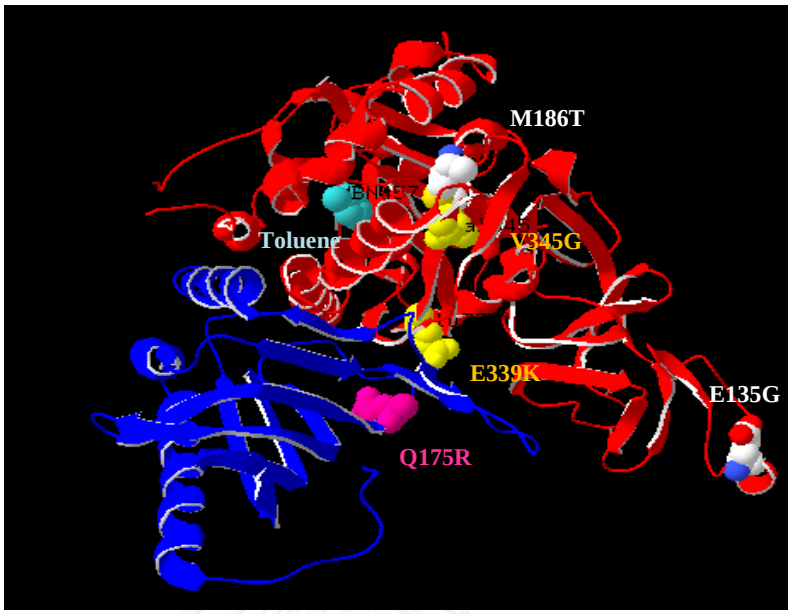


Figure 4.19 Location of residue substitutions in the structure of *todC1*(red)*C2*(blue) subunit (PDB ID:3EN1) (Friemann et al., 2009) The white color (E135G and M186T) represented the mutated point, which is not related to substrate binding site in *todC1* The yellow color (E339K and V345G) represented the mutated point related to substrate binding site in *todC1*. The pink color showed the mutated point in *todC2*. Toluene molecule was shown in blue color.

Figure 4.20 The alignment *todC1* of Rieske non-heme oxygenase family: Napthalene dioxygenase, Toluene dioxygenase and Biphenyl dioxygenase. The color box indicated the function of α- subunit; grey, yellow, and red boxes. The grey, yellow and red box is 2Fe-2S binding site, substrate binding site and the mutated point, respectively (Appendix C4).

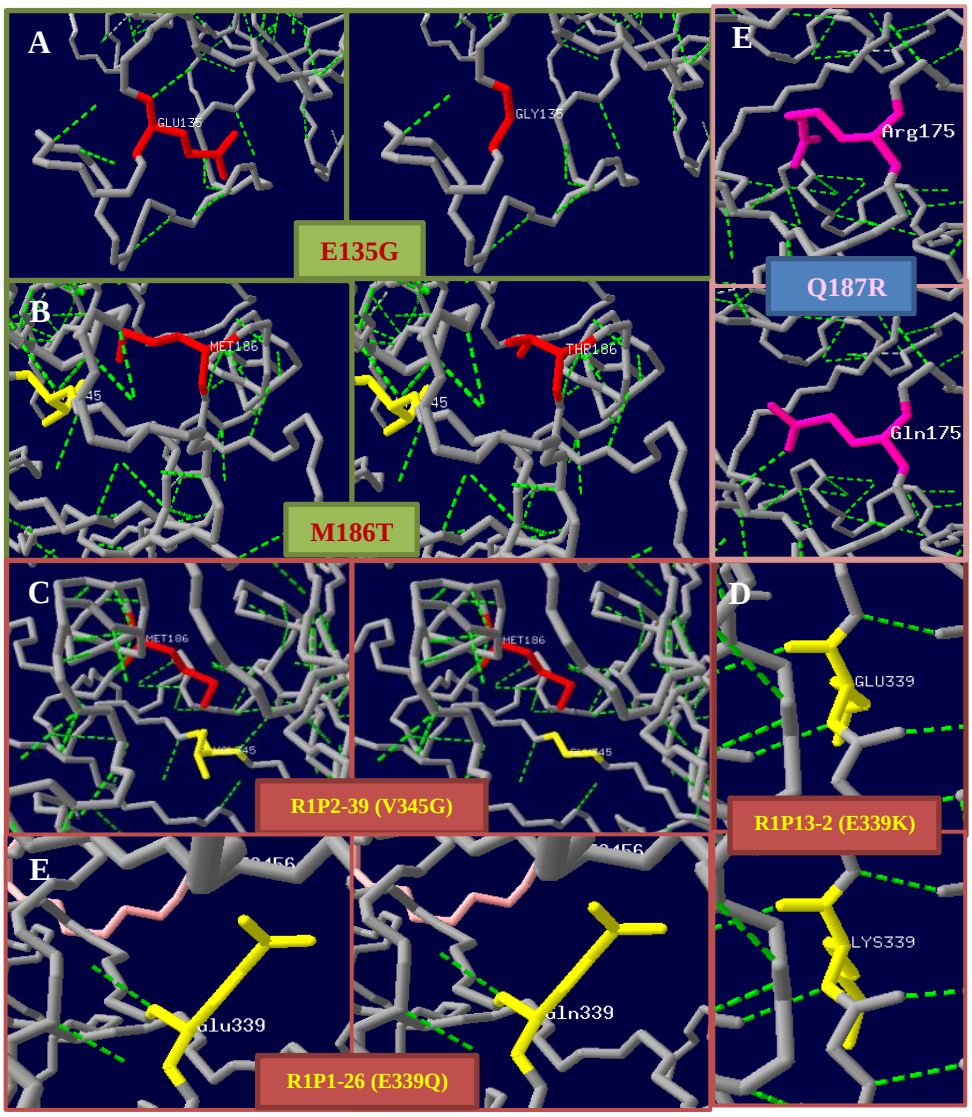


Figure 4.21 Structural model for the three-dimensional structure of the wild-type (1) comparing with mutated point (2) of *todC1C2*. The positions of mutated amino acid are E135G (A), M186T (B), V345G (C), E339K (D), and E339Q (E) in *todC1* and Q187R (E) in *todC2*. The green line showed the predicted H- bond by Swiss PDB Viewer (spdbv) program.

In the first mutant, R1P1-26 (E339Q), glutamate of 339 was substituted by glutamine. The amino acid structure of glutamate and glutamine was similar. It was changed from electrically charge side chain to be polar side chain. From this result, it could be described that the substitution of amino acid may affect the substrate binding site. So, the *Km* of kinetic parameter with 4CA showed higher than that of the wild type. It had the lower affinity with 4CA than the wild-type. In the second mutant, R1P2-39 (V345G), the amino acid substitution was occurred at 345 by changing valine to glycine. From the result of kinetic parameter showed the lower K_m of 4CA and the higher V_{max} of aniline compare with the wild-type. It can demonstrate that R1P2-39 was higher affinity with 4CA, but it had the higher degradation rate of aniline than of the wild-type. The disappearance of amino acid side change may be support the chlorine function of 4CA. In the third mutant, R1P13-2, the amino acid substitution was occurred at 339 by changing glutamate to lysine. The amino acid residue was changed from acidic charged to be a basic charged residue. From the result of kinetic parameter showed that it had the highest specificity with 4CA and showed the higher rate of aniline degradation than wild-type. The amino acid substitution structure could be demonstrated that the longer side chain of amino acid substitution and the electrically charge of residues may be affected with substrate specificity with 4CA. The interaction between substrate and substrate binding site should be more studied by using the complex computational modeling.

Jiang et al,. 1996 reported that the mononuclear iron binding residue is important for toluene dioxygenase activity. The conserved motif Glu-X_{3-4}-Asp-X_2-His-X_{4-5}-His was reported to important for catalytic function. From their results, toluene

dioxygenase activity was completely abolished due to mutation at Glu-214, Asp-219, His-222, or His-228 (Jiang et al., 1996). The directed evolution proposed that mutated position closer to the active site of enzyme may be more enhancer the catalytic efficiency (Morley and Kazlauskas, 2005).

In this study, three mutants were mutated in E339 and V345 in *todC1*, which is involved in substrate binding site. In R1P1-26 (E339Q), one bp was inserted that occurred near the C-terminal region of *todC1* (nucleotide position 1017). The insertion caused in a change of the translational reading frame. As a result, a predicted protein by contained one more amino acid than the wild-type *todC1* (α-subunit). The frame shift did not affect the start codon or reading frame of *todC2*. In the second mutants, R1P2-39, a substitution V345G also occurred within *todC1*. Another mutant (R1P13-2) showed the substitution at E339K of *todC1*. Table 4.7 showed the location of mutated residue in target gene, which has an effect with enzyme activity.

Table 4.7 The functions of mutated residues

Direct evolution	**Mutated points**		**Reference**
	Position	**Function**	
Error prone PCR: Toluene dioxygenase (*todC1*)	L306	Substrate binding site	(Zhang et al., 2000)
	A212	-	
	A234	Substrate binding site	
Error prone PCR: Toluene dioxygenase (*todC1*)	D345	Substrate binding site	(Sakamoto et al., 2001)
	R450	-	
	Stop451	Stop codon	
Error prone PCR: Phytase (*App*A2)	K46	Substrate binding site	(Kim and Lei, 2008)
	K97	involved in hydrogen bonding	
	V227		
	G344	α-helix	
Error prone PCR: Aniline dioxygenase (*Atd*A)	V205	substrate binding	(Ang et al., 2009)
	I248		
	S404	α-helix	
Error prone PCR: styrene monooxygenase (*sty*AB)	R87, R88	-	(Gursky et al., 2010)
	A179, K245	- (far from active site)	
	V303	FAD/NAD(P)H binding	
Error prone PCR: Malic enzyme R221G/K228R/I310V	R221	cofactor binding site	(Morimoto et al., 2014)
	K228	cofactor binding site	
	I310		
Error prone PCR: Toluene dioxygenase (*todC1*) E135/ M186/V345 E135/ M186/ E339	E135, M186	-	This study
	V345, E339	Substrate binding site	
	Q187	β-subunit	

In the three mutants (R1P1-26, R1P2-39, and R1P13-2) the substitution in same position in *todC1* (α-subunit), E135G, M186T, and a single mutation (Q187R) within the *todC2* (β-subunit), were observed. Their positions were reported to be far from active site. The exact effect of these amino acid substitutions is still unclear. However, previous studies found that the amino acid substitution occur outside the active site or substrate binding site of enzymes that have been improved through random mutagenesis (Bornscheuer and Pohl, 2001; Farinas et al., 2001; Glieder et al., 2002). It could be indicated that effects of amino acid changes, which far from active site was difficult to report exactly function, but it could affect enzyme activity.

Moreover, the substitution amino acid residues of mutants (339 and V345) found at 226-388 residues to play an important role in substrate binging site (Friemann et al., 2009; Parales et al., 2000).

CHAPTER 5

CONCLUSIONS AND SUGGESTION

5.1 Conclusions

Due to the increasing accumulation of chloroanilines in environment, especially 4CA, which was reported to accumulate in soil and water during the degradation of pesticides; many microorganisms have been reported to be able to degrade them, while the information of enzyme involved in chloroaniline oxidation is limited. Previously, chloroaniline dioxygenase had been reported to degrade 3CA as a substrate whereas it could not degrade 4CA as substrate. Protein engineering is an efficient technique to improve enzyme activity. The protein engineering of enzyme involved in chloroaniline oxidation is limited. Recently, toluene dioxygenase from *P. putida* T57 showed the capability to degrade 4CA as a substrate. Thus, this study focused on the enhancement of toluene dioxygenase activity toward 4CA by using error prone PCR. Error prone PCR is a common technique to randomly mutate the gene of interest. The *todC1C2* is the interest gene for improving the efficiency of toluene dioxygenase because it is where the catalytic reaction takes place and involves in the substrate binding site. In this study, the modified Gibbs method was used to screen the mutants' library. The three tentative mutants showed higher catechol formation (1.5 to 2.3 folds) than that of the wild-type. The increasing efficiency detected by the modified Gibbs screening method was confirmed by HPLC analysis. The identified amino acid substitutions in the selected mutants were at the position 339 and 345, which were involved in the substrate binding site. This research is an in

depth work of how to improve enzyme involved in bioremediation pathway of toxic pollutant. It is considered the first step of how to develop and improve enzyme for further application of enzyme bioremediation as well as industrial enzyme for waste bioconversion.

In conclusion, the random mutation of toluene dioxygenase using error prone PCR has generated three mutants with the enhanced catalytic activity of enzyme as a biocatalyst by improving its activity toward 4-chloroaniline.

5.2 Suggestions

Based the results obtained in this study, it provided the potency of using error prone PCR technique to enhance the activity of toluene dioxygenase toward 4-chloroaniline degradation. Some recommendations for future study are proposed as follows:

5.2.1. Because of the improved catalytic efficiency of enzyme, the amino acid substitution on protein function should be studied such as the direction of 4CA bind with substrate binding site by using the complex computational modeling.

5.2.2 The interaction of amino acid substitutions should be investigated such as hydrogen bonding and van der Waals, which is played a role in folding and stability of enzyme.

In term of application, this work can be applied in enzyme bioremediation and industrial enzyme for waste bioconversion. The crude enzyme has sufficient activity for treatment in the real site of industrial waste or agricultural field. In term of enzyme production, the purified enzyme should be used to produce the high yield of

product. From the results, each mutant had different efficiency. So, the contamination of chemicals in the real site should be concerned before select the mutated enzyme for bioremediation.

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APPENDIX

Appendix A

Medium preparation, protein analysis and molecular technique

A1. LB (Luria-Bertani) medium (1L)

Tryptone	10	g
NaCl	10	g
Yeast extract	5	g

Autoclaved; 121°C, 15min, 121psi

A2. MSBY medium (1L)

K_2HPO_4	4.3	g
KH_2PO_4	3.4	g
$(NH_4)2SO_4$	2	g
$MgCl_2 \cdot 6H_2O$	0.34	g
$MnCl_2 \cdot 4H_2O$	1	mg
$FeSO_4 \cdot 7H_2O$	6	mg
$CaCl_2 \cdot 2H_2O$	26	mg
$Na_2MoO \cdot 2H_2O$	0.02	mg
$ZnCl_2$	0.1	mg
$CoCl_2 \cdot 6H_2O$	0.01	mg
$CuSO_4$	0.01	mg
$NiSO_4 \cdot 6H_2O$	0.001	mg
Na_2SeO_4	0.0001	mg
Yeast extract	1	g

Autoclaved ; 121°C,15min,121psi

A3. 1M Sodium phosphate buffer stock Solution , pH7 (1L)

1. Solution A:

   Dissolve 138.0 g NaH_2PO_4 in 1 liter distilled water (pH 7.0).

2. Solution B:

   Dissolve 142.0 g Na_2HPO_4 in 1 liter distilled water (pH 7.0).

3. Mix 423 ml Solution A with 577 ml Solution B.

4. Autoclave and store at room temperature.

A4. SDS-PAGE

- Stock solution

  2.0 M Tris-HCl pH 8.8

  Tris (hyddroxymethyl) aminomethane 24.2 g

  Adjust pH to 8.8 with 1 M HCl and adjust volume with distilled water to 100 ml.

  1.0 M Tris-HCl pH 6.8

  Tris (hyddroxymethyl) aminomethane 12.1 g

  Adjust pH to 6.8 with 1 M HCl and adjust volume with distilled water to 100 ml.

10% (w/v) SDS

Sodium dodecyl sulfate (SDS)	10 g

Added distilled water to 100 ml of total volume.

50% (v/v) glycerol

100% glycerol	50 ml
Distilled water	50 ml

1% (w/v) bromophenol blue

bromophenol blue	100 mg

Added distilled water to 10 ml of total volume and stir until dissolve.

- Working solution

Solution A (30% (w/v) Acrylamide,0.8% (w/v) bis-acrylamide)

Acrylamide	29.2 g
N,N'-methyl-bis-acrylamide	0.8g

Adjust distilled water to 100ml of total volume, store in dark at 4°C.

Solution B (1.5M Tris-HCl, pH 8.8 and 0.4% SDS)

2.0 M Tris-HCl pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (0.5 M Tris-HCl,pH 6.8 and 0.4% SDS)

1.0M Tris-HCl pH6.8	50 ml
10% SDS	4 ml
Distilled water	46 ml

10% (w/v) ammonium persulfate

Ammonium per sulfate	0.5 g
Distilled water	5.0 ml

Electrophoresis buffer

Tris	3.0 g
Glycine	14.4 g
SDS	1 g

Dissolved and adjusted to total volume 1 L with distilled water (final pH should be 8.3)

5X Sample buffer

1M Tris-HCl (pH 6.8)	0.6 ml
50% glycerol	5 ml
10% SDS	2 ml
2-mercaptoethanol	0.5 ml
1% bromophenol blue	1 ml
Distilled water	0.9 ml

- Separating gel 12.5%

Solution A	3.33 ml
Solution B	2.5 ml
Distilled water	4.17 ml
10% Ammonium persulfate	50 µl
TEMED	5 µl

- Stacking gel 5%

Solution A	0.67 ml
Solution C	1.0 ml
Distilled water	2.3 ml
10% Ammonium persulfate	30 µl
TEMED	5 µl

A5. Plasmid DNA extraction: Alkaline lysis with SDS (mini preparation)

- Working solution

Alkaline lysis solution I (GTE buffer)
50 mM Glucose
25 mM Tris-HCl
10 mM EDTA (pH.8)
Stored at 4°C until use.

Alkaline lysis solution II (1 ml)

1 N NaOH	200 µl
10% SDS	100 µl
Distilled water	700 µl

Alkaline lysis solution III (pH 5.2)

5 M potassium acretate	60.0 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

Stored at 4°C until use

A6. Preparation of *E. coli* competent cell for heat shock method

- Stock solution

0.5 M HEPES pH 6.7, 100ml

HEPES	15.1 g

Adjust pH to 6.7 with 5 M NaOH and adjust volume with distilled water to 100 ml. Sterile the solution by filtration through a filter (0.2 µm pore size) and then stored frozen at -20°C

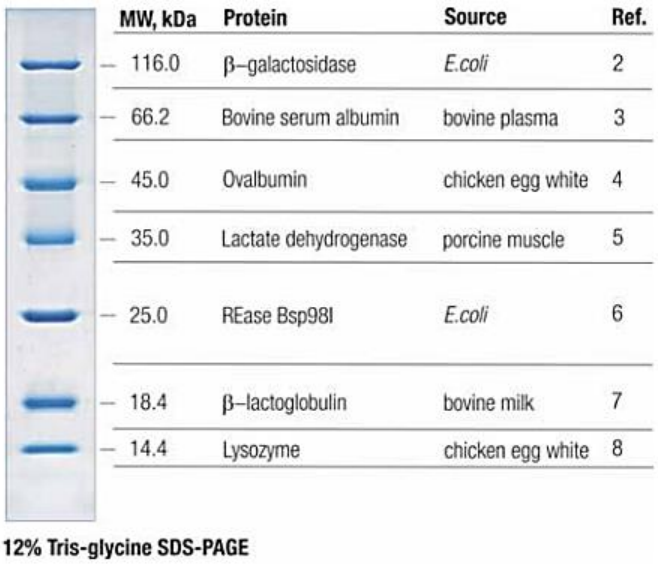
- Working solution

  Inove transformation buffer (1L)

$MnCl_2 \cdot 4H_2O$	10.88 g
$CaCl_2 \cdot 2H_2O$	2.20 g
KCl	18.65 g
HEPES (0.5 M HEPES, pH 6.7)	20 ml

  Adjust the volume to 1L with pure water

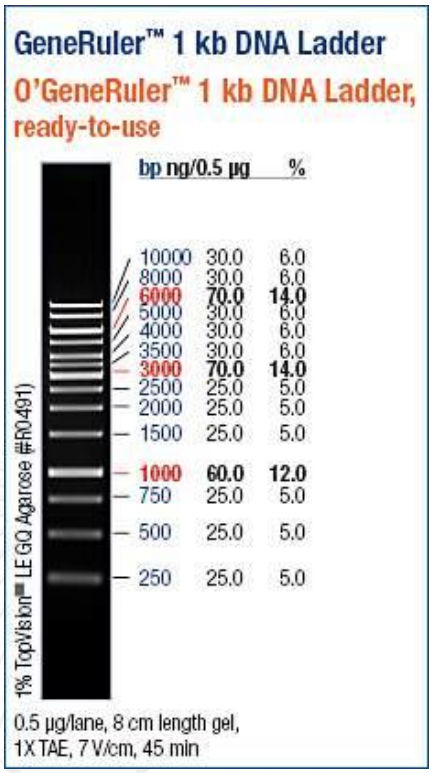
A7. Unstained Protein Molecular Weight Marker #SM0431 (Fermentus, USA)



MW, kDa	Protein	Source	Ref.
116.0	β–galactosidase	*E.coli*	2
66.2	Bovine serum albumin	bovine plasma	3
45.0	Ovalbumin	chicken egg white	4
35.0	Lactate dehydrogenase	porcine muscle	5
25.0	REase Bsp98I	*E.coli*	6
18.4	β–lactoglobulin	bovine milk	7
14.4	Lysozyme	chicken egg white	8

12% Tris-glycine SDS-PAGE

A8. Gene Ruler™ 1 kb DNA Ladder (Thermo Scientific, USA)



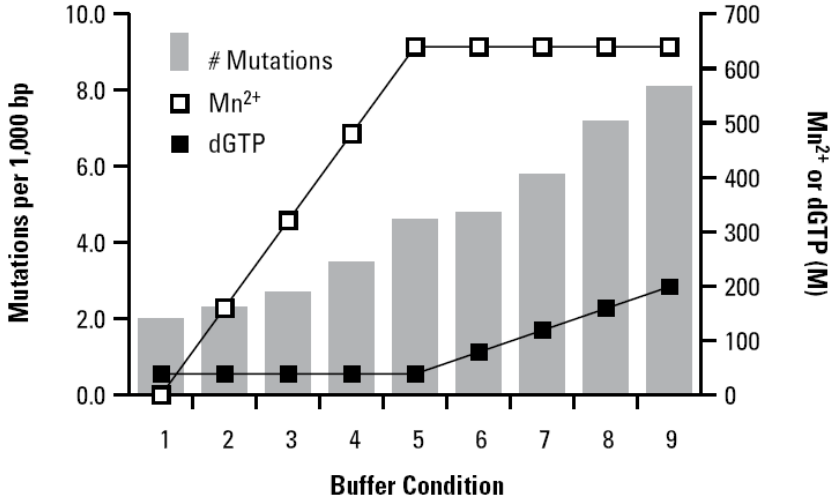
A9. Diversify® PCR Random Mutagenesis Kit (Clontech, USA)

- **Random mutagenesis reactions**

	Volumes by Buffer Condition (µl)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Std.[a]**
Mutations per 1,000 bp	2.0	2.3	2.7	3.5	4.6	4.8	5.8	7.2	8.1	–
PCR Grade Water	40	39	38	37	36	35	34	33	32	41
10X TITANIUM *Taq* Buffer	5	5	5	5	5	5	5	5	5	5
$MnSO_4$ (8 mM)	0	1	2	3	4	4	4	4	4	0
dGTP (2 mM)	1	1	1	1	1	2	3	4	5	0
50X Diversify dNTP Mix	1	1	1	1	1	1	1	1	1	0
50X dNTP Mix	0	0	0	0	0	0	0	0	0	1
Primer mix[b]	1	1	1	1	1	1	1	1	1	1
Template DNA[c]	1	1	1	1	1	1	1	1	1	1
TITANIUM *Taq* Polym.	1	1	1	1	1	1	1	1	1	1
Total volume	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**

[a] Standard PCR reaction using TITANIUM *Taq* DNA Polymerase
[b] Experimental or Control Primer Mix (10 µM each primer)
[c] Experimental or Control PCR Template (~1 ng/µl)

- **The level of Diversify mutagenesis is controlled by varying the concentrations of manganese and dGTP. The concentrations of other dNTPs are held constant**



- **Diversify mutagenesis sequencing data**

	Buffer Condition		
	1	**5**	**9**
$MnSO_4$ (µM)	0	640	640
dGTP (µM)	40	40	200
Total bp sequenced	18,414	20,705	15,148
Mutations per 1,000 bp	2.0	4.6	8.1
Total mutations found	36	96	123
Type of Mutation (%)			
Ts: A → G or T → C	33.3	42.7	74.0
Ts: G → A or C → T	8.3	11.5	4.9
Tv: A → T or T → A	16.7	26.0	13.8
Tv: A → C or T → G	27.8	8.3	4.1
Tv: G → C or C → G	0.0	0.0	1.6
Tv: G → T or C → A	0.0	6.3	0.8
Insertions	2.8	2.1	0.0
Deletions	11.1	3.1	0.8

Ts = Transition
Tv = Transversion

Appendix B

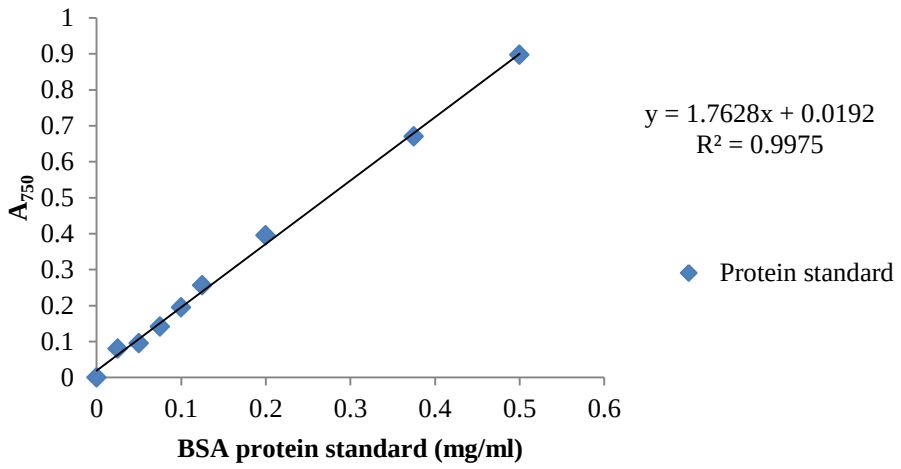
The standard curves

B1. Protein standard curve

- Protein (BSA) standard curve information

BSA Concentration (mg/ml)	A_{750}
0	**0**
0.025	**0.08**
0.05	**0.095**
0.075	**0.141**
0.1	**0.195**
0.125	**0.256**
0.2	**0.395**
0.375	**0.67**
0.5	**0.897**

- Standard curve of Lowry method used to determine cell protein

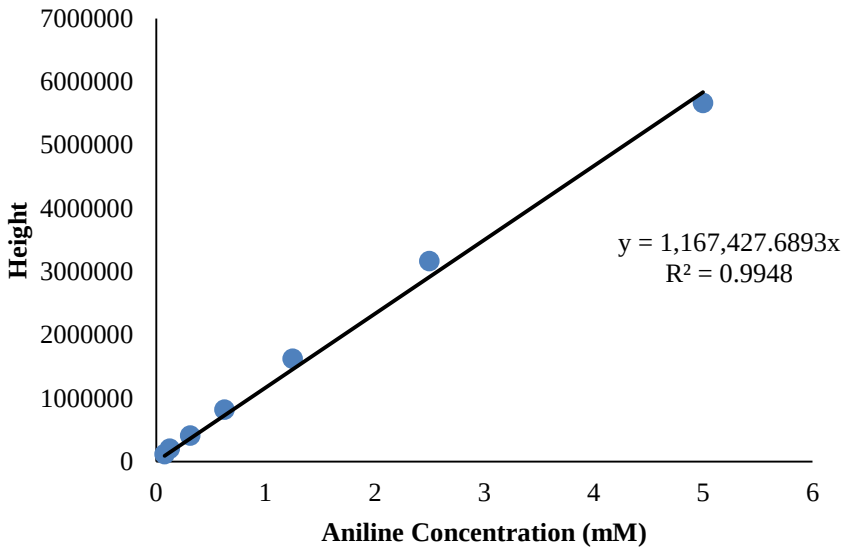


B2. Chemical standard curve for HPLC

- The aniline standard curve for HPLC analysis

Aniline Concentration (mM)	Height	Height*Dilution factor (4)
0.078	28662	114648
0.125	50093	200372
0.3125	101797	407188
0.625	204661	818644
1.25	405042	1620168
2.5	790438	3161752
5	1414956	5659824

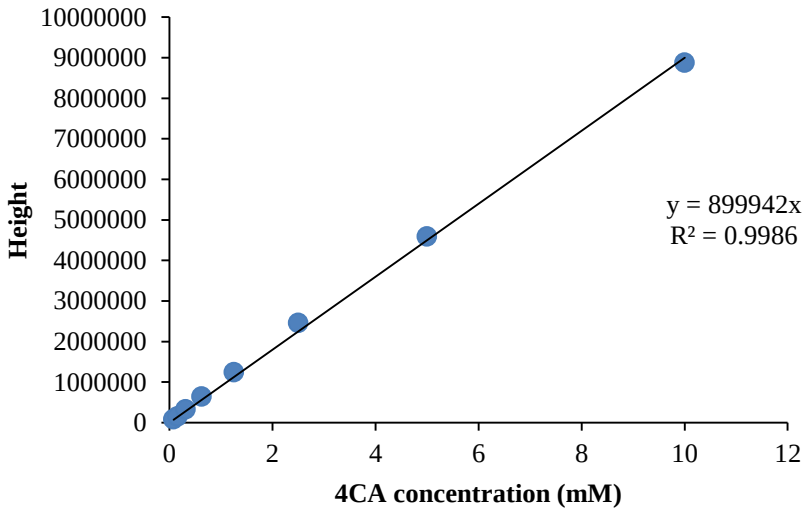
- The aniline standard curve



- The 4CA standard curve for HPLC analysis

4CA Concentration (mM)	Height	Height*Dilution factor (4)
0.078	20677	82708
0.156	39420	157680
0.3125	81504	326016
0.625	160802	643208
1.25	310051	1240204
2.5	614705	2458820
5	1148413	4593652
10	2219746	8878984

- The 4CA standard curve



Appendix C

Raw data of chapter 4

C1. Raw data of kinetic parameter; Michalelis-Menten and the Lineweaver-Burk plot

4CA degradation by crude enzyme

Wild-type

Exp1			
S	**V**	**1/S**	**1/V**
4CA Conc. (mM)	**Specific activity (mM/min/mg.protein)**		
0.0602	0.00006091	16.6129	16416.3712
0.1136	0.00086725	8.7990	1153.0659
0.3225	0.00132319	3.1011	755.7499
0.5566	0.00061249	1.7966	1632.6727
0.7751	0.01082860	1.2902	92.3480
1.0753	0.00236889	0.9300	422.1381
3.1069	0.01675786	0.3219	59.6735
5.4580	0.00365786	0.1832	273.3836
Exp2			
S	**V**	**1/S**	**1/V**
4CA Conc. (mM)	**Specific activity (mM/min/mg.protein)**		
0.0121	0.00000890	82.7525	112419.3103
0.1037	0.00107165	9.6448	933.1365
0.0823	0.00074867	12.1492	1335.6961
0.4933	0.00197375	2.0270	506.6489
0.9357	0.00544540	1.0688	183.6413
2.6895	0.01493459	0.3718	66.9587
4.5105	0.01529431	0.2217	65.3838
Exp3			
S	**V**	**1/S**	**1/V**
4CA Conc. (mM)	**Specific activity (mM/min/mg.protein)**		
0.0127	0.00007650	78.6666	13072.0128
0.0515	0.00040164	19.4160	2489.7969

0.1027	0.00013862	9.7361	7213.7648
0.3797	0.00111300	2.6337	898.4728
1.1067	-0.00741481	0.9036	-134.8652
2.7629	0.00080337	0.3619	1244.7514
4.8137	0.03396157	0.2077	29.4450

P1-26

Exp1			
S	**V**	**1/S**	**1/V**
4CA Conc. (mM)	**Specific activity (mM/min/mg.protein)**		
0.0122	0.00000263	81.66442831	380271.842
0.0269	0.00003003	37.21228912	33298.76025
0.0512	0.00006885	19.53338253	14525.28044
0.1246	0.00014558	8.028028546	6869.072291
0.5111	0.00055555	1.956752972	1800.018186
0.7818	0.00298381	1.279162521	335.1416654
3.1958	0.00110416	0.312906196	905.6679099
4.8640	0.00502204	0.205592945	199.1223109

P2-39

Exp1			
S	**V**	**1/S**	**1/V**
4CA Conc. (mM)	**Specific activity (mM/min/mg.protein)**		
0.0555	0.00012046	18.0096	8301.5845
0.0863	0.00017515	11.5868	5709.3111
0.3046	0.00013535	3.2835	7388.4102
0.6039	0.00088648	1.6558	1128.0580
0.9527	-0.00117313	1.0496	-852.4229
0.8000	0.00134353	1.2501	744.3074
2.7493	0.00105677	0.3637	946.2774
5.4891	0.00074266	0.1822	1346.5179
Exp2			
S	**V**	**1/S**	**1/V**
4CA Conc. (mM)	**Specific activity (mM/min/mg.protein)**		
0.0129	0.00008070	77.4713	12391.8895

0.0810	0.00008181	12.3521	12223.8411
0.0774	0.00041589	12.9184	2404.4655
0.4527	0.00139931	2.2092	714.6371
0.9007	-0.00456344	1.1103	-219.1330
2.6675	0.00052235	0.3749	1914.4211
4.5542	0.00724018	0.2196	138.1180

P13-2

Exp1			
S	**V**	**1/S**	**1/V**
4CA Conc. (mM)	**Specific activity (mM/min/mg.protein)**		
0.0101	0.00006381	98.67785088	15671.41537
0.0373	0.00040440	26.84470827	2472.769029
0.0576	0.00029497	17.37339768	3390.172421
0.1314	0.00033620	7.612949616	2974.461275
0.5117	0.00171567	1.954152625	582.8623164
0.8073	-0.00303093	1.238750048	-329.9319392
3.0165	0.00287247	0.331506004	348.132131
5.2696	0.00716214	0.189767499	139.6229899

Aniline degradation by crude enzyme

wild-type

Exp1			
S	**V**	**1/S**	**1/V**
Aniline Conc. (mM)	**Specific activity (mM/min/mg.protein)**		
0.0087	0.00010904	114.4537	9170.7029
0.0505	0.00025970	19.7856	3850.6665
0.1091	0.00023167	9.1635	4316.5416
0.5055	0.00372139	1.9782	268.7165
0.9340	0.00403549	1.0707	247.8012
3.1704	0.02224924	0.3154	44.9454
5.3458	0.01105829	0.1871	90.4299

P2-39

Exp1			
S	**V**	**1/S**	**1/V**
Aniline Conc. (mM)	**Specific activity (mM/min/mg.protein)**		
0.0099	-0.000117	101.0235	-8580.5935
0.0488	0.000107	20.4869	9355.0046
0.0747	0.000221	13.3787	4517.9801
0.5356	0.000820	1.8670	1219.1087
0.8712	0.001998	1.1478	500.5121
3.3817	0.004979	0.2957	200.8622
5.4728	0.014322	0.1827	69.8214

P13-2

Exp1			
S	**V**	**1/S**	**1/V**
Aniline Conc. (mM)	**Specific activity (mM/min/mg.protein)**	**1/mM**	
0.0148	0.00002657	67.7634	37636.0638
0.0225	0.00021514	44.5311	4648.1484
0.0463	0.00044386	21.6030	2252.9465
0.9731	0.00221613	1.0277	451.2362
2.7606	-0.00013470	0.3622	-7424.1437
5.0135	0.00609434	0.1995	164.0866

C2. Optimization of modified Gibbs method

- The optimum reaction time

Aniline

	A_{660} of Aniline					
	0	0.1	0.5	0.7	1	3
min	0mM	0.1mM	0.5mM	0.7mM	1mM	3mM
1	0.04535	0.05415	0.0819	0.10665	0.0476	0.2718
2	0.04585	0.0553	0.089	0.11725	0.0655	0.31145
3	0.06205	0.0582	0.1048	0.14335	0.10925	0.40605
5	0.0475	0.06765	0.1474	0.2144	0.23225	0.6792
8	0.04865	0.08095	0.20995	0.3215	0.3609	1.10955
10	0.0528	0.09405	0.2686	0.4224	0.5336	1.4992
12	0.0514	0.1091	0.3389	0.5442	0.80775	1.97725
15	0.06785	0.12485	0.4243	0.68455	1.0669	2.5237
20	0.0641	0.16445	0.6162	1.01865	1.62035	3.5624
40	0.0705	0.321	1.40215	2.41015	3.55875	#DIV/0!

Catechol

Reaction time	Average A_{590} of Catechol					
	0	0.1	0.5	0.7	1	3
min	0mM	0.1mM	0.5mM	0.7mM	1mM	3mM
1	0.06515	0.3951	1.2823	1.91915	2.13835	3.226
2	0.065	0.38735	1.26685	1.9166	2.1626	3.2494
3	0.0677	0.3979	1.24805	1.88715	2.17895	3.1374
5	0.06935	0.4064	1.20335	1.8078	2.1286	3.36375
8	0.0711	0.417	1.1582	1.73125	2.0335	2.82705
10	0.07375	0.42225	1.1245	1.67605	1.9584	2.5372
12	0.0758	0.414	1.0884	1.6196	1.8704	2.23075
15	0.08615	0.39405	1.05645	1.5696	1.7684	1.96755
20	0.09235	0.388	1.00275	1.4945	1.4944	1.6121
40	0.10915	0.36375	0.92855	0.954	0.9067	1.00115

4-chlorocatechol (4CC)

Reaction time	4CC					
min	0 mM	0.1 mM	0.5 mM	0.7 mM	1 mM	3 mM
0	0.04935	0.2066	0.451115	0.59025	0.78505	1.07855
2	0.0494	0.32505	0.800846	1.07855	1.40615	1.7982
3	0.0502	0.3911	1.035423	1.41945	1.8238	2.0503
5	0.0506	0.4214	1.101192	1.53455	1.97765	2.02205
8	0.05285	0.43225	1.086615	1.5424	1.95685	1.93755
10	0.0539	0.42665	1.063577	1.5236	1.9254	1.89655
12	0.055	0.4163	1.047077	1.5114	1.87565	1.83245
15	0.05745	0.4042	1.026462	1.48925	1.8045	1.7688
20	0.05895	0.39575	1.005692	1.459	1.71715	1.68505
40	0.0718	0.37655	0.886423	1.2585	1.54145	1.58935
60	0.09125	0.41195	0.858458	1.1857	1.46045	1.4543

- The optimum of transformation time

The calculation of optimum 4CA transformation time

pHKTodC1C2BA-JM109

Transformation time (hr)	OD.600		A590 (4CC)		A590/OD.600		Average A590/OD.600
0	0.332	0.2388	0.374363	0.276841	1.1276	1.1593	1.14345
2	0.3877	0.3101	0.686888	0.534488	1.7717	1.7236	1.74765
4	0.3765	0.2989	0.716781	0.555954	1.9038	1.86	1.8819
6	0.3249	0.2957	0.46347	0.427967	1.4265	1.4473	1.4369
9	0.3679	0.3188	0.508732	0.411316	1.3828	1.2902	1.3365
12	0.2388	0.3877	0.316792	0.472102	1.3266	1.2177	1.27215
24	0.3101	0.2765	0.411131	0.315708	1.3258	1.1418	1.2338
48	0.2989	0.3249	0.390632	0.360607	1.3069	1.1099	1.2084

negative control pHK-DH5α

Transformation time (hr)	OD.600		A590 (4CC)		A590/OD.600		Average A590/OD.600
0	0.2824	0.2761	0.322586	0.22088	1.1423	0.8	0.97115
2	0.3228	0.2711	0.308274	0.207202	0.955	0.7643	0.85965
4	0.2742	0.2439	0.247575	0.201144	0.9029	0.8247	0.8638
6	0.3293	0.3654	0.335293	0.298897	1.0182	0.818	0.9181
9	0.2502	0.3002	0.248649	0.22587	0.9938	0.7524	0.8731
12	0.3008	0.3081	0.290212	0.232739	0.9648	0.7554	0.8601
24	0.2929	0.2742	0.261296	0.20406	0.8921	0.7442	0.81815
48	0.3075	0.2439	0.302211	0.201437	0.9828	0.8259	0.90435

- Calculation of formation and conversion rate in 4 h.

	OD_{600}	A_{576} Catechol	A_{660} Aniline	Catechol	Aniline	Rate of Catechol (A_{576}/A_{600}/hr)	% formation rate	Rate of Aniline (A_{660}/A_{600}/hr)	% degradation rate
				A_{576}/A_{600}	A_{660}/A_{600}				
P13-2	0.498	0.407	0.558	0.817	1.119				
	0.434	0.329	0.427	0.757	0.983	**P13-2**			
Average	0.466	0.368	0.492	0.787	1.051	0.197	100.14	0.263	116.89
P1-26	0.548	0.32	0.44	0.583	0.802				
	0.505	0.308	0.414	0.609	0.82	**P1-26**			
Average	0.527	0.314	0.427	0.596	0.811	0.149	51.58	0.203	67.31
P2-39	0.484	0.359	0.467	0.742	0.966				
	0.386	0.415	0.543	1.076	1.406	**P2-39**			
Average	0.435	0.387	0.505	0.909	1.186	0.227	131.04	0.297	144.72
Wildtype	0.557	0.201	0.251	0.36	0.45				
	0.332	0.129	0.155	0.389	0.466				
	0.356	0.153	0.192	0.43	0.538	**Wild type**			
Average	0.344	0.161	0.199	0.393	0.485	0.098	0	0.121	0
Neg control	0.061	0.146	0.188	2.404	3.087				
	0.085	0.133	0.166	1.553	1.948				
	0.052	0.145	0.184	2.782	3.531	**Neg control no cell**			
Average	0.057	0.141	0.18	2.246	2.855	0.562		0.714	

C3. Alignment of *todC1C2* of mutants compare with Reference gene

Pseudomonas putida F1 (GenBank ID: J04996.1)

Pseudomonas putida T57 (GenBank ID: AB828709.1)

- *todC1C2*-DNA

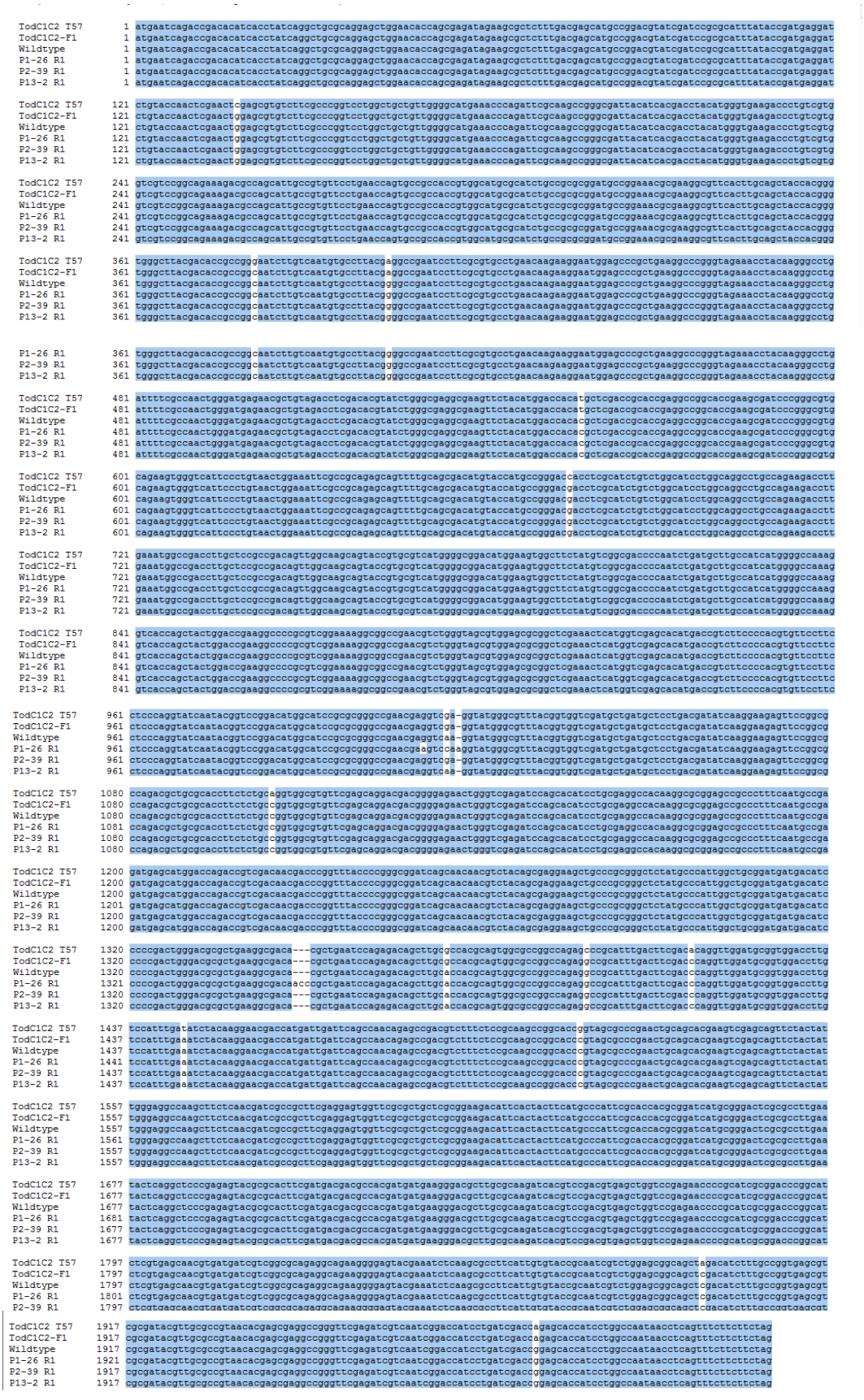
  Alignment: Global DNA alignment against reference molecule

  Parameters: Scoring matrix: Linear (Mismatch 2, OpenGap 4, ExtGap 1)

  Reference molecule: TodC1C2 T57 , Region 1-2027

  Number of sequences to align: 6

Pos	Sequence	Start	End	Length	Matches	%Matches
Ref 1	TodC1C2 T57	1	2027	2027		
2	TodC1C2-F1	1	2027	2027	2018	99
3	Wildtype	1	2027	2027	2013	99
4	P1-26 R1	1	2031	2031	2012	99
5	P2-39 R1	1	2027	2027	2014	99
6	P13-2 R1	1	2027	2027	2013	99



- *todC1C2*-amino acid

Alignment: Global Protein alignment against reference molecule

Parameters: Scoring matrix: BLOSUM 62

Reference molecule: TodC1 T57, Region 1-450

Number of sequences to align: 6

Pos	Sequence	Start	End	Length	Matches	%Matches
Ref 1	TodC1 T57	1	450	450 aa		
2	TodC1 F1 aa	1	450	450 aa	450	100
3	TodC1 wildtype	1	450	450 aa	447	99
4	TodC1 P1-26 R1 aa	1	451	451 aa	357	76
5	TodC1 P2-39 aa	1	450	450 aa	448	99
6	TodC1 P13-2 aa	1	450	450 aa	447	99

```
TodC1 T57          1 mnqtdtspirlrrswntseiealfdehagridpriytdedlyqlelervfarswlllghetqirkpgdyittymgedpvvvvrqkdasiavflnqcrhrg
TodC1 F1 aa        1 mnqtdtspirlrrswntseiealfdehagridpriytdedlyqlelervfarswlllghetqirkpgdyittymgedpvvvvrqkdasiavflnqcrhrg
TodC1 wildty       1 mnqtdtspirlrrswntseiealfdehagridpriytdedlyqlelervfarswlllghetqirkpgdyittymgedpvvvvrqkdasiavflnqcrhrg
P1-26 R1 aa        1 mnqtdtspirlrrswntseiealfdehagridpriytdedlyqlelervfarswlllghetqirkpgdyittymgedpvvvvrqkdasiavflnqcrhrg
TodC1 P2-39        1 mnqtdtspirlrrswntseiealfdehagridpriytdedlyqlelervfarswlllghetqirkpgdyittymgedpvvvvrqkdasiavflnqcrhrg
TodC1 P13-2        1 mnqtdtspirlrrswntseiealfdehagridpriytdedlyqlelervfarswlllghetqirkpgdyittymgedpvvvvrqkdasiavflnqcrhrg

TodC1 T57        101 mricradagnakaftcsyhgwaydtagnlvnvpyeaesfaclnkkewsplkarvetykglifanwdenavdldtylgeakfymdhmldrteagteaipgv
TodC1 F1 aa      101 mricradagnakaftcsyhgwaydtagnlvnvpyeaesfaclnkkewsplkarvetykglifanwdenavdldtylgeakfymdhmldrteagteaipgv
TodC1 wildty     101 mricradagnakaftcsyhgwaydtagnlvnvpygaesfaclnkkewsplkarvetykglifanwdenavdldtylgeakfymdhtldrteagteaipgv
P1-26 R1 aa      101 mricradagnakaftcsyhgwaydtagnlvnvpygaesfaclnkkewsplkarvetykglifanwdenavdldtylgeakfymdhtldrteagteaipgv
TodC1 P2-39      101 mricradagnakaftcsyhgwaydtagnlvnvpygaesfaclnkkewsplkarvetykglifanwdenavdldtylgeakfymdhtldrteagteaipgv
TodC1 P13-2      101 mricradagnakaftcsyhgwaydtagnlvnvpygaesfaclnkkewsplkarvetykglifanwdenavdldtylgeakfymdhtldrteagteaipgv

TodC1 T57        201 qkwvipcnwkfaaeqfcsdmyhagttshlsgilaglpedlemadlapptvgkqyraswgghgsgfyvgdpnlmlaimgpkvtsywtegpasekaaerlgs
TodC1 F1 aa      201 qkwvipcnwkfaaeqfcsdmyhagttshlsgilaglpedlemadlapptvgkqyraswgghgsgfyvgdpnlmlaimgpkvtsywtegpasekaaerlgs
TodC1 wildty     201 qkwvipcnwkfaaeqfcsdmyhagttshlsgilaglpedlemadlapptvgkqyraswgghgsgfyvgdpnlmlaimgpkvtsywtegpasekaaerlgs
P1-26 R1 aa      201 qkwvipcnwkfaaeqfcsdmyhagttshlsgilaglpedlemadlapptvgkqyraswgghgsgfyvgdpnlmlaimgpkvtsywtegpasekaaerlgs
TodC1 P2-39      201 qkwvipcnwkfaaeqfcsdmyhagttshlsgilaglpedlemadlapptvgkqyraswgghgsgfyvgdpnlmlaimgpkvtsywtegpasekaaerlgs
TodC1 P13-2      201 qkwvipcnwkfaaeqfcsdmyhagttshlsgilaglpedlemadlapptvgkqyraswgghgsgfyvgdpnlmlaimgpkvtsywtegpasekaaerlgs

TodC1 T57        301 vergsklmvehmtvfptcsflpgintvrtwhprgpneve---vwaftvvd-----adapddikeefrrqtlrtfsaggvfeqddgenwveiqhilrghka
TodC1 F1 aa      301 vergsklmvehmtvfptcsflpgintvrtwhprgpneve---vwaftvvd-----adapddikeefrrqtlrtfsaggvfeqddgenwveiqhilrghka
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P1-26 R1 aa      301 vergsklmvehmtvfptcsflpgintvrtwhprgpnevqgmgvyggrccsryggrvpap-daahllcrwrvragrrg--------------elgrdpah
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TodC1 T57        393 rsrpfnaemsmdqtvdndp---vypgrisnnvyseeaarglyahwlrmmtspdwdalkatr--------
TodC1 F1 aa      393 rsrpfnaemsmdqtvdndp---vypgrisnnvyseeaarglyahwlrmmtspdwdalkatr--------
TodC1 wildty     393 rsrpfnaemsmdqtvdndp---vypgrisnnvyseeaarglyahwlrmmtspdwdalkatr--------
P1-26 R1 aa      385 parpqgaeppfqcrdehgpdrrqrpg-lpradqqqrlqrgscpralcplaadd-diprlgraegdnplx
```

C4. Amino acid sequence of Rieske non-heme oxygenase family

Biphenyl dioxygenase [*Burkholderia xenovorans* LB400]

GenBank: AAB63425.1

>gi|151084|gb|AAB63425.1| biphenyl dioxygenase [Burkholderia xenovorans LB400]

MSSAIKEVQGAPVKWVTNWTPEAIRGLVDQEKGLLDPRIYADQSLYELELERVFGRSWLLLG
HESHVPETGDFLATYMGEDPVVMVRQKDKSIKVFLNQCRHRGMRICRSDAGNAKAFTCSYHG
WAYDIAGKLVNVPFEKEAFCDKKEGDCGFDKAEWGPLQARVATYKGLVFANWDVQAPDLETY
LGDARPYMDVMLDRTPAGTVAIGGMQKWVIPCNWKFAAEQFCSDMYHAGTTTHLSGILAGIP
PEMDLSQAQIPTKGNQFRAAWGGHGSGWYVDEPGSLLAVMGPKVTQYWTEGPAAELAEQRLG
HTGMPVRRMVGQHMTIFPTCSFLPTFNNIRIWHPRGPNEIEVWAFTLVDADAPAEIKEEYRR
HNIRNFSAGGVFEQDDGENWVEIQKGLRGYKAKSQPLNAQMGLGRSQTGHPDFPGNVGYVYA
EEAARGMYHHWMRMMSEPSWATLKP

Biphenyl dioxygenase [*Rhodococcus* sp. RHA1]

GenBank: BAA06868.1

>gi|510285|dbj|BAA06868.1| biphenyl dioxygenase [*Rhodococcus jostii* RHA1]

MTDVQCEPALAGRKPKWADADIAELVDERTGRLDPRIYTDEALYEQELERIFGRSWLLMGHE
TQIPKAGDFMTNYMGEDPVMVVRQKNGEIRVFLNQCRHRGMRICRADGGNAKSFTCSYHGWA
YDTGGNLVSVPFEEQAFPGLRKEDWGPLQARVETYKGLIFANWDADAPDLDTYLGEAKFYMD
HMLDRTEAGTEAIPGIQKWVIPCNWKFAAEQFCSDMYHAGTTSHLSGILAGLPDGVDLSELA
PPTEGIQYRATWGGHGSGFYIGDPNLLLAIMGPKVTEYWTQGPAAEKASERLGSTERGQQLM
AQHMTIFPTCSFLPGINTIRAWHPRGPNEIEVWAFTVVDADAPEEMKEEYRQQTLRTFSAGG
VFEQDDGENWVEIQQVLRGHKARSRPFNAEMGLGQTDSDNPDYPGTISYVYSEEAARGLYTQ
WVRMMTSPDWAALDATRPAVSESTHT

Naphthalene 1,2-dioxygenase iron sulfur protein component large subunit (plasmid) [*Pseudomonas putida*]

NCBI Reference Sequence: NP_863072.1

GenPept Identical Proteins Graphics

>gi|32469898|ref|NP_863072.1| naphthalene 1,2-dioxygenase iron sulfur protein component large subunit (plasmid) [*Pseudomonas putida*]

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EVIVSRQNDGSIRAFLNVCRHRGKTLVSVEAGNAKGFVCSYHGWGFGSNGELQSVPFEKDLY
GESLNKKCLGLKEVARVESFHGFIYGCFDQEAPPLMDYLGDAAWYLEPMFKHSGGLELVGPP
GKVVIKANWKAPAENFVGDAYHVGWTHASSLRSGESIFSSLAGNAALPPEGAGLQMTSKYGS
GMGVLWDGYSGVHSADLVPELMAFGGAKQERLNKEIGDVRARIYRSHLNCTVFPNNSMLTCS
GVFKVWNPIDANTTEVWTYAIVEKDMPEDLKRRLADSVQRTFGPAGFWESDDNDNMETASQN
GKKYQSRDSDLLSNLGFGEDVYGDAVYPGVVGKSAIGETSYRGFYRAYQAHVSSSNWAEFEH
ASSTWHTELTKTTDR

Toluene dioxygenase large subunit [*Pseudomonas putida* T57]

GenBank: BAN59728.1

>gi|519662006|dbj|BAN59728.1| toluene dioxygenase large subunit [*Pseudomonas putida*]

MNQTDTSPIRLRRSWNTSEIEALFDEHAGRIDPRIYTDEDLYQLELERVFARSWLLLGHETQ
IRKPGDYTTYMGEDPVVVVRQKDASIAVFLNQCRHRGMRICRADAGNAKAFTCSYHGWAYDT
AGNLVNVPYEAESFACLNKKEWSPLKARVETYKGLIFANWDENAVDLDTYLGEAKFYMDHML
DRTEAGTEAIPGVQKWVIPCNWKFAAEQFCSDMYHAGTTSHLSGILAGLPEDLEMADLAPPT
VGKQYRASWGGHGSGFYVGDPNLMLAIMGPKVTSYWTEGPASEKAAERLGSVERGSKLMVEH
MTVFPTCSFLPGINTVRTWHPRGPNEVEVWAFTVVDADAPDDIKEEFRRQTLRTFSAGGVFE
QDDGENWVEIQHILRGHKARSRPFNAEMSMDQTVDNDPVYPGRISNNVYSEEAARGLYAHWL
RMMTSPDWDALKATR

C5. The amino acid table

First Position	Second Position: U code	U Amio Acid	C code	C Amio Acid	A code	A Amio Acid	G code	G Amio Acid	Third Position
U	UUU	phe	UCU	ser	UAU	tyr	UGU	cys	U
	UUC		UCC		UAC		UGC		C
	UUA	leu	UCA		UAA	STOP	UGA	STOP	A
	UUG		UCG		UAG	STOP	UGG	trp	G
C	CUU	leu	CCU	pro	CAU	his	CGU	arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	ile	ACU	thr	AAU	asn	AGU	ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	lys	AGA	arg	A
	AUG	met	ACG		AAG		AGG		G
G	GUU	val	GCU	ala	GAU	asp	GGU	gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	glu	GGA		A
	GUG		GCG		GAG		GGG		G

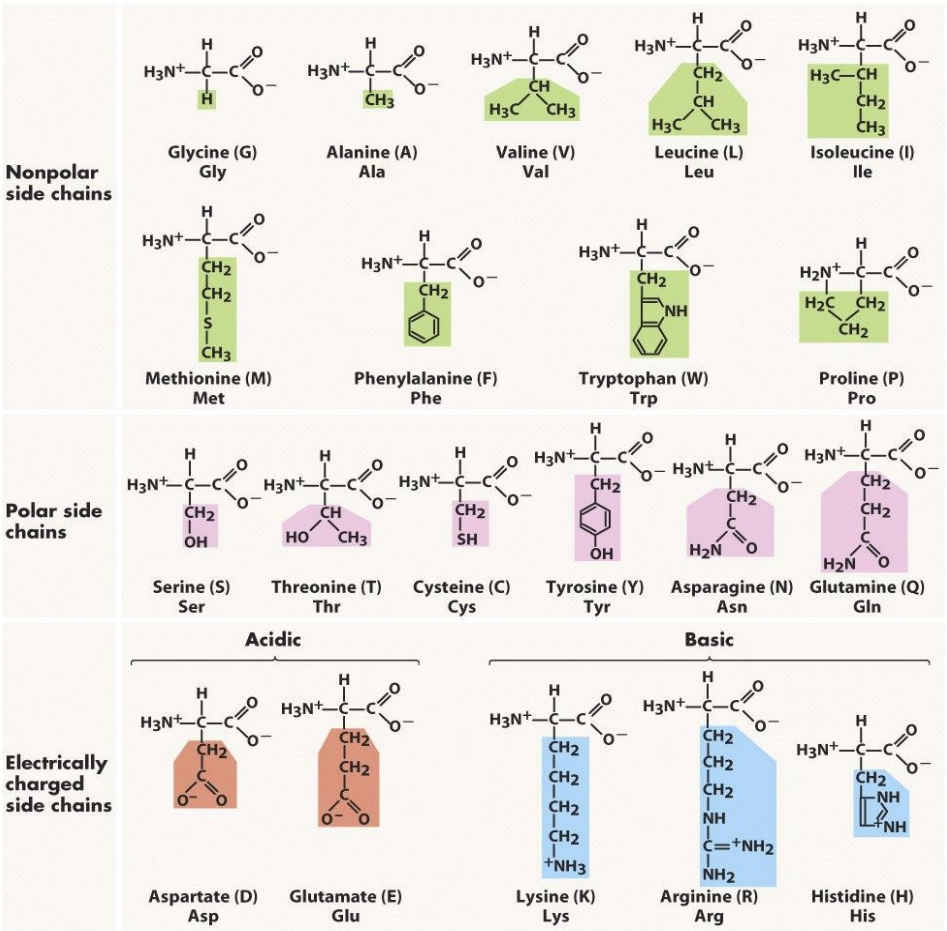
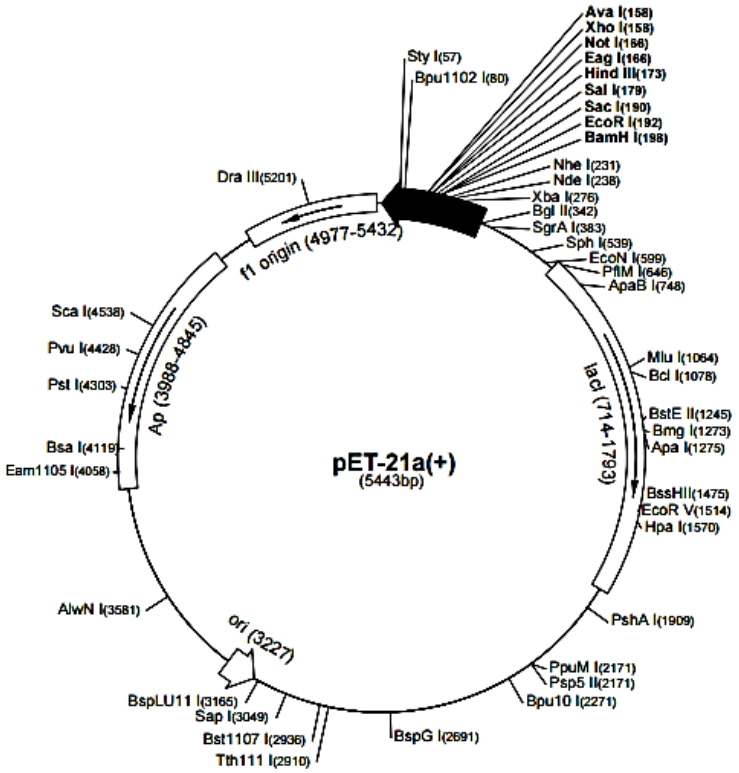


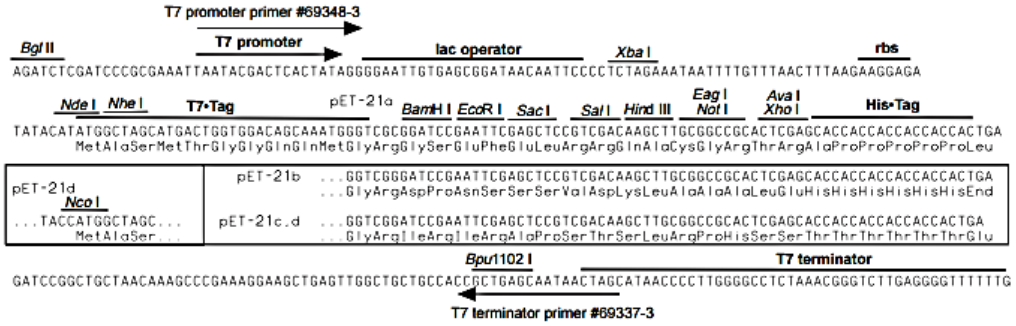
Figure 3-5 Biological Science, 2/e

Appendix D

Restriction map of vector and designed primer

D1. pET21a





pET-21a-d(+) cloning/expression region

D2. Primer: EPPCR R1 for construct pET21a*todC1C2BA*

TodC1 F-*Bam*HI	ATCGGGATCCATGAATCAGACCGACACATC
TodC2 R-*Eco*RI	ATCGGAATTCCTAGAAGAAGAAACTGAGGTTAT
TodB F-*Eco*RI	ATCGGAATTCATGACTTGGACATACATATTGCGG
TodA R-*Sac*I	ATCGGAGCTCTCACGTTAGGTCTCCTTCATT

Pseudomonas putida DNA, toluene dioxygenase (tod) operon, complete sequence, strain: T57 (5,877bp)

GenBank: AB828709.1

>gi|519662005|dbj|AB828709.1| *Pseudomonas putida* DNA, toluene dioxygenase (tod) operon, complete sequence, strain: T57

TodC1F TodC2R TodBF TodAR

GATCGAGCAAAACCGGGGCTTTATCCGCTTGGTCAACGATTTTCTTGCCGCGGAGGACTGAT
CGCAAAAACGGGAATGACCATCCGTTCTGAAAGCACGTCATCGGCAATTGCCTGCCAAGTAC
CCGCCATCCACTACCTTGAAAAGTGAGAAGACAATGAATCAGACCGACACATCACCTATCAG
GCTGCGCAGGAGCTGGAACACCAGCGAGATAGAAGCGCTCTTTGACGAGCATGCCGGACGTA
TCGATCCGCGCATTTATACCGATGAGGATCTGTACCAACTCGAACTCGAGCGTGTCTTCGCC
CGGTCCTGGCTGCTGTTGGGGCATGAAACCCAGATTCGCAAGCCGGGCGATTACATCACGAC
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CCTCCCAGGTATCAATACGGTCCGGACATGGCATCCGCGCGGGCCGAACGAGGTCGAGGTAT
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CGCGCTGAAGGCGACACGCTGAATCCAGAGACAGCTTGCGCCACGCAGTGGCGCCGGCCAGA
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CGCCACTGGTCGGTGGTGCGCTACGACCGTATCAGCATCTGGGGACACAAGTTCCAGGCGCC
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CCTGGTTGGACGCAAGATTGGGCTGACCTCTGTCGCTGTACAGAAGCAGCTCGGAGTGGAAC
AGCCCGACTATGGCATGTTGTTCGCAGACATGGCGCGTACCGAGGGGGA

The size of each gene; *todC1*, *todC2*, *todB*, *todA*

todC1= 1,353 bp

todC2 = 564 bp

todB = 324 bp

todA = 1,233 bp

The expected size of PCR product

PCR product *todC1C2* = 2,027 bp

PCR product *todBA*= 1,556 bp

D3. The prediction of mutated points by comparing with the reference structure

Swiss-Pdb Viewer was used to analyze the amino acid substitution in *todC1C2*. The 3EN1-PDB File (Text) was downloaded from http://www.rcsb.org/pdb/explore/explore.do?structureId=3EN1 used as a reference structure of toluene dioxygenase subunit alpha and beta (*todC1C2*).

The procedure of determination of amino acid substitution in *todC1C2* was shown as follows:

1. Opening the files

   To open a PDB file into Deep View simply select File → Open from the File menu, and select the file. 3EN1-PDB File (Text) was input as *todC1C2* reference structure.

2. Most of the manipulation of your structure will take place in the Control Panel. The label is the option on will place a small floating label next to the residue. The mutated point was labeled; it showed the amino acid name and position of mutated point.

3. Mutation of amino acid residues

   - The mutant of R1P1-26, the glutamate of 339 was substituted by glutamine by the command of mutate on the main toolbar. The structure was changed from glutamate to be glutamine. Then, the results could be saved as image and PDB files.

- The mutant of R1P2-39 was mutated by using the procedure as described above. The amino acid substitution was occurred at 345 by changing valine to be glycine and the results could be saved as image and PDB files.
- The mutant of R1P2-39 was mutated by using the procedure as described above. The amino acid substitution was occurred at 339 by changing glutamate to be lysine and the results could be saved as image and PDB files.

4. Saving the files

Select *File* > *Save*: this command offers a submenu to save data and images. The current layer was saved the currently active layer in PDB format and the images were saved an exact copy of the current Graphic window contents.

VITA

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Publications and conference presentations status

Poster presentation

1. Chanikan Laohajinda, and Alisa S. Vangnai., (2014). "Toluene dioxygenase-based whole-cell and enzymatic degradation of 4-chloroaniline." The 26th Annual Meeting of the Thai society for Biotechnology and International Conference between 26th and 29th November, 2014, Chiang Rai, Thailand

2. Chanikan Laohajinda, and Alisa S. Vangnai., (2015). "Toluene dioxygenase based biodegradation of 4-chloroaniline" The 3rd Joint Symposium CU-NUT, 5 January, 2015, Chulalongkorn University, Bangkok, Thailand.

3. Chanikan Laohajinda, and Alisa S. Vangnai., (2015). "The oxidation of 4-chloroaniline by Pseudomonas putida T57's toluene dioxygenase and reaction monitoring by Gibbs's test" Industrial and Hazardous Waste Conference 2015, 31 January, 2015, Bangkok International Trade & Exhibition Centre (BITEC), Thailand

4. Chanikan Laohajinda, and Alisa S. Vangnai., (2015). "The enhancement of toluene dioxygenase to degrade 4-chloroaniline by error prone PCR technique" The 27th Annual Meeting of the Thai society for Biotechnology and International Conference between 17th and 20th November, 2015, Bangkok, Thailand