PRODUCTION OF 1, 12- DODECANEDIOIC ACID BY RECOMBINANT MICROBIAL CYTOCHROME P450 IN *Pichia pastoris* AND *Saccharomyces cerevisiae*

Miss Phawadee Buathong

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
Common Course
Faculty of Science
Chulalongkorn University
Academic Year 2018

การผลิต 1, 12- โดเดคคานีดิโออิกแอซิดโดยไซโตโครม P450 ลูกผสมจากจุลินทรีย์ใน *Pichia pastoris* และ *Saccharomyces cerevisiae*

น.ส.ภาวดี บัวทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ ไม่สังกัดภาควิชา/เทียบเท่า
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	PRODUCTION OF 1, 12- DODECANEDIOIC ACID BY RECOMBINANT MICROBIAL CYTOCHROME P450 IN *Pichia pastoris* AND *Saccharomyces cerevisiae*
By	Miss {Phawadee Buathong
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Warawut Chulalaksananukul, Ph.D.
Thesis Co Advisor	Gilles Truan, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the Faculty of Science
(Professor Polkit Sangvanich, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman
(Associate Professor Supachitra Chadchawan, Ph.D.)

..... Thesis Advisor
(Associate Professor Warawut Chulalaksananukul, Ph.D.)

..... Thesis Co-Advisor
(Gilles Truan, Ph.D.)

..... Examiner
(Assistant Professor Chompunuch Glinwong, Ph.D.)

..... Examiner
(Associate Professor Suchada Chanprateep Napathorn, Ph.D.)

..... External Examiner
(Nassapat Boonvitthya, Ph.D.)

ภาวดี บัวทอง : การผลิต 1, 12- โดเดคคานีดิโออิกแอซิดโดยไซโตโครม P450 ลูกผสมจากจุลินทรีย์ใน *Pichia pastoris* และ *Saccharomyces cerevisiae* . (PRODUCTION OF 1, 12-DODECANEDIOIC ACID BY RECOMBINANT MICROBIAL CYTOCHROME P450 IN *Pichia pastoris* AND *Saccharomyces cerevisiae*) อ.ที่ปรึกษาหลัก : รศ. ดร.วรวุฒิ จุฬาลักษณานุกูล, อ.ที่ปรึกษาร่วม : กิลล์ ตรวน

1,12-Dodecanedioic acid (DDA) คือกรดไดคาร์บอกซิลิกที่เป็นสารตัวกลางสำคัญในการผลิตสารเคมีที่มีมูลค่าหลายชนิด โดยทั่วไปสารนี้สามารถสังเคราะห์ได้ด้วยวิธีการทางเคมี แต่พบว่ามีข้อเสียหลายประการ ดังนั้นจึงมีความสนใจที่จะผลิตสารนี้ด้วยวิธีทางชีวภาพ โดยการใช้จุลินทรีย์ที่มีการพัฒนาสายพันธุ์โดยการเหนี่ยวนำให้เกิดการกลายด้วยเทคนิคทางพันธุศาสตร์ วัตถุประสงค์ของงานวิจัยนี้ คือ การโคลนยีน (r) CYP52A17 จาก *Candida tropicalis* ที่มีความสามารถในการออกซิไดซ์กรดไขมันไปเป็นไฮดรอกซีของกรดไขมันและกรดไดคาร์บอกซิลิก ซึ่งเรียกกระบวนการนี้ว่า ω-ออกซิเดชัน โดยทำให้ยีน rCYP52A17 เกิดการกลายจากการเปลี่ยนกรดแอมิโนตัวที่ 261 และ 490 จากลิวซีนไปเป็นเซอรีน (L261S/L490S) แล้วเรียกยีนกลายนี้ว่า rCYP52A17mut จากนั้นนำยีน rCYP52A17 และ rCYP52A17mut ไปแสดงออกใน *Pichia pastoris* และ *Saccharomyces cerevisiae* เพื่อใช้ในการผลิต12-hydroxydodecanoic acid (HDDA) และ 1,12-DDA จากสารตั้งต้นชนิดกรดลอริก โดยที่ *S. cerevisiae* นั้นมีการแสดงออกของ NADPH cytochrome P450 reductase ร่วมด้วย พบว่าริคอมบิแนนท์ *Pichia* ที่มีการแสดงออกของ rCYP52A17 และ rCYP52A17mut ภายใต้การควบคุมของโพรโมเตอร์ที่มีการแสดงออกแบบเหนี่ยวนำ (*P. pastoris*/pPICZA-CYP52A17 และ *P. pastoris*/pPICZA-CYP52A17mut) มีปริมาณ P450 เท่ากับ 0.2 นาโนโมลต่อมิลลิกรัมโปรตีน โดยทั้งสองสายพันธุ์นี้มีความสามารถในการออกซิไดซ์กรดลอริกได้เพียงเล็กน้อย นอกจากนี้ยังพบว่า *P. pastoris*/pPICZA-CYP52A17mut สามารถผลิต 12-HDDA ได้สูงที่สุดเท่ากับ 0.8 ไมโครโมลาร์ภายใน 24 ชั่วโมง ในขณะที่ริคอมบิแนนท์ *Saccharomyces* ที่มีการแสดงออกของ rCYP52A17 และ rCYP52A17mut ภายใต้การควบคุมของโพรโมเตอร์ที่มีการแสดงออกแบบเหนี่ยวนำ (*S. cerevisiae* BY(2R)/pYeDP60-CYP52A17 และ *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut) มีปริมาณ P450 เท่ากับ 0.13 และ 0.28 นาโนโมลต่อมิลลิกรัม ตามลำดับ นอกจากนี้ *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17 และ *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut สามารถออกซิไดซ์กรดลอริกไปเป็น 12-HDDA ได้เท่ากับ 4 และ 12 ไมโครโมลาร์ตามลำดับ เมื่อนำ *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut มาเลี้ยงในอาหารที่มีกรดลอริก พบว่าสามารถผลิต 12-HDDA ได้สูงที่สุดเท่ากับ 45.8 ไมโครโมลาร์ ภายใน 24 ชั่วโมง และยังสามารถออกซิไดซ์ 12-HDDA ไปเป็น 1,12-DDA ได้เท่ากับ 20.8 ไมโครโมลาร์ภายใน 72 ชั่วโมง โดยความเข้มข้นของกรดลอริกที่เหมาะสมต่อการผลิต1,12-DDA ด้วยสายพันธุ์กลายนี้คือ 500 ไมโครโมลาร์ นอกจากนี้เมื่อนำสายพันธุ์กลายนี้มาเลี้ยงในถังหมัก 5 ลิตรพบว่าปริมาณ 12-HDDA และ 1,12-DDA ที่ผลิตได้ไม่มีความแตกต่างกันจากการเลี้ยงแบบ shake flasks สุดท้ายได้นำ *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut มาประยุกต์ใช้ในการผลิต 1,12-DDA จากน้ำเสียของโรงงานผลิตกะทิ พบว่ายีสต์สายพันธุ์กลายนี้สามารถเจริญในอาหาร YPGE และผลิต 1,12-DDA ได้สูงที่สุด ภายใน 24 ชั่วโมง จากการทดลองจะเห็นได้ว่า *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut มีความสามารถในการเร่งปฏิกิริยาω-ออกซิเดชันได้ทั้งสองขั้นตอน จึงแสดงให้เห็นว่าสายพันธุ์กลายนี้สามารถนำมาใช้เป็นจุลินทรีย์ทางเลือกในการผลิต 1,12-DDA ในอุตสาหกรรมได้

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต .....
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		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5373943523 : MAJOR BIOTECHNOLOGY
KEYWORD: ω-oxidation, CYP52A17, 12-Hydroxydodecanoic acid, Dodecanedioic acid, Pichia pastoris, Saccharomyces cerevisiae, Lauric acid
Phawadee Buathong : PRODUCTION OF 1, 12- DODECANEDIOIC ACID BY RECOMBINANT MICROBIAL CYTOCHROME P450 IN *Pichia pastoris* AN D *Saccharomyces cerevisiae* . Advisor: Assoc. Prof. Warawut Chulalaksananukul, Ph.D. Co-advisor: Gilles Truan, Ph.D.

1,12-Dodecanedioic acid (DDA) is a primary compound which is used as an intermediate precursor for production of valuable chemical products. Biosynthesis of 1,12-DDA by recombinant (r) microorganisms can be used as an alternative method to compensate several disadvantages from chemical synthesis. The purpose of this study was to clone a CYP52A17 from *Candida tropicalis* to produce cytochrome P450 which can terminally oxidize fatty acids to hydroxy-fatty acids and further to dicarboxylic acids (ω-oxidation). The wild type rCYP52A17 and its engineered L261S/L490S (leucine changed to encode for serine) form (rCYP52A17mut) were expressed in *Pichia pastoris* and *Saccharomyces cerevisiae* which coexpressing the yeast NADPH cytochrome P450 reductase in order to produce 12-hydroxydodecanoic acid (HDDA) and, potentially, 1,12-DDA from lauric acid. The P450 contents of microsomes from *P. pastoris*/pPICZA-CYP52A17 and *P. pastoris*/pPICZA-CYP52A17mut were 0.2 nmol/mg of protein. *P. pastoris*/pPICZA-CYP52A17 and CYP52A17mut presented a few the oxidation ability. The recombinant *P. pastoris*/pPICZA-CYP52A17mut accumulated the highest level of 12-HDDA (0.8 µM) within 24 h. The recombinant *S. cerevisiae* expressing rCYP52A17 showed higher P450 contents than the others. The P450 content of microsomes from *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17 and *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut were 0.13 and 0.28 nmol/mg of protein, where lauric acid was oxidized to provide approximately 4 and 12 µM of 12-HDDA, respectively. Moreover, biotransformation of lauric acid by *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut showed the highest level of HDDA (45.8 µM) at 24 h, which was oxidized to yield 20.8 µM 1,12-DDA at 72 h. The optimal lauric concentration of biotransformation was 500 µM, while the levels of HDDA and 1,12-DDA production in bioreactor were almost similar to the shake flask cultivation. The recombinant yeast cells which initially cultured in YPGE can be able to produce the highest 1,12- DDA from coconut milk wastewater in 24 h. From this study, *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut demonstrated as a promising source to produce potential 1,12-DDA and can be applied for industrial applications.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

First, I would like to express my deepest gratitude to my advisor Associate Professor Warawut Chulalaksananukul, Ph.D., my co-advisor Dr. Gilles Truan, and Dr. Nassapat Boonvitthya, for his good advice, a powerful support, guidance throughout the process of this research, providing a nice working environment, and for spending time discussing on various points.

I would like to thank Associate Professor Supachitra Chadchawan, Ph.D., Assistant Professor Chompunuch Glinwong, Ph.D., Associate Professor Suchada Chanprateep Napathorn, Ph.D., and Nassapat Boonvitthya, Ph.D., the member of thesis committee, for their useful comments.

I would to acknowledge my scholarship the Royal Golden Jubilee, Thailand Research Fund for their financial support during my thesis and the French embassy for their 6 months financial support to do the research in France and extend my thankful to Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés (LISBP) de l'INSA de Toulouse, France and Department of Botany, Faculty of Science, Chulalongkorn University

for facilitate experiments and equipments in laboratory.

I would like to thank all member in EAD11, LISBP, INSA, Toulouse, France. Especially, Hery, Robert, Arthur who made a nice working environment and friendship. Specially, Sara and Mathieu who give me a help, a kind assistance, friendship throughout during my stay in France.

I would like to express a special thanks to all members of Biofuel by Biocatalyst Research Unit, Chulalongkorn University and my friends for their friendship, understanding, kind assistance and a kind support.

Special thanks are also extending to Bara Scientific co., ltd. for providing the HPLC-MS, LCMS -8030 and Mr. Jatuporn Auttsinthong, Bara Scientific co., ltd. for a powerful encourage to analyze the products and a helpful training for HPLC-MS.

Finally, I would like to thank my family for their unlimited love, a huge support, understanding, staying beside my side through the long thesis journey.

Phawadee Buathong

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BMGY	buffered complex glycerol medium
BMMY	buffered complex methanol medium
BSA	bovine serum albumin
CO	carbon monoxide
CPR	cytochrome P450 reductase
CSM	complete supplement mixture
CYP	cytochrome P450
DCA	dicarboxylic acid
DNA	deoxyribonucleic Acid
GAL	galactose
LiAc	lithium acetate
NADPH	nicotinamide adenine dinucleotide phosphate
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
TBME	*tert*-butyl methyl ether
TEAA	triethylamine acetate
YNB	yeast Nitrogen Base
YPDS	yeast extract peptone dextrose sucrose medium
YPGE	yeast extract peptone glucose ethanol medium

CHAPTER I

INTRODUCTION

Introduction

α, ω- Dicarboxylic acids (DCAs) are important versatile chemical intermediates for the preparation of nylon and other polyamides, polyesters, perfumes, polymer, lubricants, cosmetic ingredients and coatings. For example, dodecanedioic acid (DDA) is the precursor of polyamide 6,12 (nylon 6,12) (Funk et al., 2017; Huf et al., 2011; Waché, 2013). Currently, DCA production is mainly performed via chemical synthesis, but this has limitations and drawbacks, such as a risky byproduct, the cost of removal of the byproduct and the use of non-renewable petrochemical feedstocks. As a potential alternative, the production of DCA from biological conversion is a fascinating commercial opportunity (Craft et al., 2003; Lu et al., 2010; Sathesh-Prabu and Lee, 2015). Cytochromes P450 (P450s or CYPs) are heme-thiolate proteins that play key roles in the hydroxylation of a wide range of primary and secondary metabolites (Brocca et al., 1998; Tamaki et al., 2005). The hydroxylation mechanisms of CYPs consists of splitting an oxygen molecule, one oxygen atom being transferred to the substrate while the other is reduced to H_2O. In addition, different CYP isoforms differ in the position of the hydroxylation and the subterminal or terminal (ω) ending they perform (Brocca et al., 1998; Farinas et al., 2001; Lee et al., 2016). The CYPs can oxidize fatty acids at the omega position to generate ω-hydroxy fatty acids and then further to α, ω-DCAs (Lu et al., 2010; Seo et al., 2015). Normally the oxidization of alkanes and fatty acids via oxidation requires cytochrome

P450 reductase (CPR) to transfer an electron from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to the CYP.

The CYPs belonging to the CYP52 (P450Alk) family are found in many yeast species, such as *Candida tropicalis, C. maltosa, C. albicans, Starmerella bombicola* and *Yarrowia lipolytica* (Iwama et al., 2016; McLean et al., 2015). *C. tropicalis* can catabolize saturated and unsaturated fatty acids (SFAs and UFAs, respectively) efficiently through a ω-oxidation pathway (Craft et al., 2003; Črešnar and Petrič, 2011; Eschenfeldt et al., 2003). Craft *et al.* reported on the production of α, ω-DCAs by *C. tropicalis* ATCC 20336. The alkane-inducible CYP P450 genes were isolated from *C. tropicalis* ATCC 20336 and found to demonstrate different patterns of expression depending on the fatty acid used in the culture medium as the inducer (Craft et al., 2003). Eschenfeldt et al. (2003) demonstrated that CYP52A13 and CYP52A17 could be expressed in insect cells where they showed the highest ability to oxidize not only SFAs but also UFAs efficiently into DCAs. However, the production of DCAs from SFAs only occurred with CYP52A17.

Although *C. tropicalis* can directly oxidize fatty acids, it is a prevalent pathogenic yeast (Eirich et al., 2004) and as such cannot be used directly as an industrial strain. Additionally, although the ω-hydroxylation of fatty acids by CYP52A17 has already been described (Črešnar and Petrič, 2011), there is no report of the use of CYP52A17 to produce, in an industrially compatible microorganism, 1,12-DDA from lauric acid.

This study presents the cloning and expression of CYP52A17 in *Pichia pastoris* and *Saccharomyces cerevisiae*, as well as the use of both genetically modified strains to oxidize lauric acid and produce 12-hydroxydodecanoic acid (HDDA) as well as 1,12-DDA.

Objectives

1. To clone *CYP52A17* gene in *P. pastoris* and *S. cerevisiae* and synthesize α, ω- dicarboxylic acid from lauric acid using recombinant cytochrome P450.

2. To apply the recombinant *S. cerevisiae* expressing cytochrome P450 for bioproduction of α, ω- dicarboxylic acid from coconut milk wastewater.

Scope of study

The *CYP52A17* gene of *C. tropicalis* was cloned and expressed into *P. pastoris* and *S. cerevisiae* systems (inducible promoter). The *CYP52A17* gene has not been studied in *P. pastoris* and *S. cerevisiae* systems before; therefore, the CYP52A17 was expressed in *P. pastoris* and *S. cerevisiae*. The recombinant CYP52A17 produced from *P. pastoris* and *S. cerevisiae* systems were determined the enzyme activity. Finally, the recombinant *S. cerevisiae* producing the highest CYP52A17 activity would be selected for application in 1,12-dodecanedioic acid production and production of 1,12-DDA from lauric acid-containing wastewater. The coconut milk wastewater was used in this experiment.

Expected results

The expression of CYP52A17 in *P. pastoris* and *S. cerevisiae* systems could produce more CYP52A17 than wild-type. The recombinant *P. pastoris* and *S. cerevisiae* can have the potential to be used as an alternative microorganism for 1,12-dodecanedioic production and application in 1,12-DDA production from lauric acid-containing wastewater.

CHAPTER II

LITERATURE REVIEW

Dicarboxylic acids

α, ω-dicarboxylic acids (DCAs) are served as a building blocks for synthesize plastics, polyamides, polyesters, perfumes, lubricants, adhesives, and greases (Funk et al., 2017; Lu et al., 2010; Seo et al., 2015). The industrial application of medium-chain primary alcohol and α, ω-diols is presented as Table II-1. Most of DCAs are synthesized by chemical conversion that comes with many disadvantages such as by-product and the operation under high pressure and temperature (Funk et al., 2017). Dodecanedioic acid is synthesized by a four-step process involving a nickel-catalyzed cyclic trimerization of butadiene, then hydrogenation to cyclododecane, followed by air oxidation to a mixture of cyclododecanone and cyclododecanol, and finally, nitric acid oxidation to dodecanedioic acid (Yang et al., 2017). The chemical production of DCAs can be led to many drawbacks such as high pressure, temperature, and strong acids (Seo et al., 2015), high energy-demanding processes and bring about unwanted by-products which are difficult to purify. Biological production of DCAs is more interesting and important to replace the chemical route which make efficient and more greener processes. α, ω-Dicarboxylic acids can be produced through an ω-oxidation pathway (Yang et al., 2017).

Table II- 1 Industrial applications of medium-chain primary alcohol and α, ω-diols (Honda Malca, 2013)

Product	Industrial use
1-alcohols	
C_6	Hexylglycol, UV-absorber, solvent in the production of trimethylquinone
C_8	Octylamine, plasticizer, catalyst
C_6-C_{10}	Plasticizers, surfactants, solvents, coalescent aids
C_{12}-C_{17}	Lubricant oils, alcohol ethoxylates, alcohol ethoxysulfates
α, ω-diols	
C_4-C_{12}	Coatings, adhesives, plasticizers, polyesters, polyamides, polyurethanes, poly (diol co-citrates)

ω-oxidation pathway

Many ω-oxidizing microorganisms and their enzymes have been researched over the last 20 years. In 1932, Verkade and his co-worker discovered ω-oxidation in Netherlands where dogs were fed with fatty acids in different chain lengths resulting urinary dicarboxylic acids (Kancherla, 2016). Although ω-oxidation is referred to oxygenation of the terminal C-H bond of a hydrocarbon chain, P450 enzymes preferentially more catalyze (ω-1) than ω-hydroxylation reaction (Johnston et al., 2011; Seo et al., 2015). Cytochrome P450 takes part in the steps continuing β-oxidation, but P450s do not mediate in fatty acids β-oxidation (Van Bogaert et al., 2010). There are three steps in ω-oxidation for fatty acids. First, fatty acids are oxidized to ω-hydroxy fatty acids by cytochrome P450 monooxygenase and a NADPH: cytochrome P450 oxidoreductase complex in endoplasmic reticulum membrane (Eschenfeldt et al., 2003). Second, fatty alcohol oxidase (FAO) converted ω-hydroxy fatty acids in the first step to an aldehyde. Finally, fatty acid aldehyde was further catalyzed to dicarboxylic acids by fatty aldehyde dehydrogenase (FAHDH) (Eirich et al., 2004). It had proved in *C. tropicalis* ATCC 20336 was blocked in β-oxidation but it can convert fatty acids to long-chain α, ω-dicarboxylic acids. Because *C. tropicalis* ATCC 20336 contains two strong cytochrome P450s. This strain can be able to perform all steps in ω- oxidation to produce DCA (Eschenfeldt et al., 2003). In 2004, Eirich and co-workers reported that *C. tropicalis* ATCC 20336 can produce long chain dicarboxylic acids via ω-hydroxy fatty and ω-aldehydes by a fatty alcohol oxidase gene (FAO). This strain β- oxidation is blocked and three FAO genes (FAO1 and FAO2a and FAO2b) have been cloned (Eirich et al., 2004). The ω-oxidizing microorganisms is presented in Table II-2.

Table II- 2 Enzyme classes involved in the oxidation of alkanes and fatty acids (Honda Malca, 2013; Van Beilen et al., 2003).

Enzyme class	**Strain(s)**	**Composition and cofactors**	**Substrate length**	**Remarks**
Eukaryotic P450 (CYP52, class II))	*Candida apicola*, *Candida maltosa*, *Candida tropicalis*, *Yarrowia lipolytica*	Microsomal oxygenase: P450 heme reductase: FAD, FMN, NADPH	C_{10}-C_{16} *n*-alkanes and fatty acids	1-alcohols, ω-OHFAs and α, ω-DCAs as products
Bacterial P450 oxygenase systems (CYP153, class I) **Bacterial P450 oxygenase systems (CYP153, class I)**	*Rhodococcus rhodochrous* 7E1C *Acinetobacter* sp. EB104, OC4 *Mycobacterium* sp. HXN-1500 *Alcanivorax borkumensis* SK2	P450 oxygenase: P450 heme ferredoxin: iron-sulfur ferredoxin reductase: FAD, NADH	C4-C16 *n*-alkanes, cycloalkanes, 1-alcohols,	95% ω-regioselectivity
Butane monooxygenase (similar to pMMO)	Short-chain alkane oxidizers, *Nocardioides* sp. CF8 *Methylocystis* sp.	Two polypeptides: copper, iron quinone oxidoreductase: FAD, NADH	Probably C_4-C_{10} alkanes	regioselectivity depends on the chain length of the substrate (27% ω-OH from C_5 alkanes; 63% ω-OH from C_6 alkanes)
Butane monooxygenase (similar to sMMO)	*Pseudomonas butanovora*	α2β2γ2 hydroxylase: bi-nuclear iron	C_2-C_8 *n*-alkanes	

Enzyme class	Strain(s)	Composition and cofactors	Substrate length	Remarks
		reductase: [2Fe-2S], FAD, NADH regulatory subunit		
AlkB-related alkane hydroxylases	*Acinetobacter*, *Alcanivorax*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, *etc.*	Membrane hydroxylase: bi-nuclear iron rubredoxin: iron rubredoxin reductase: FAD, NADH	C_5-C_{16} *n*-alkanes	95% ω-regioselectivity
CHA0	*Pseudomonas fluorescens* CHA0	Inferred alkane oxygenase	C_{18}-C_{28} alkanes	
AlkM	*Acinetobacter* sp.	Integral membrane copper dioxygenase Rubredoxin Rubredoxin reductase	C_{10}-C_{44} alkanes and alkanes	Strongly inhibited by Fe^{2+} and Zn^{2+}

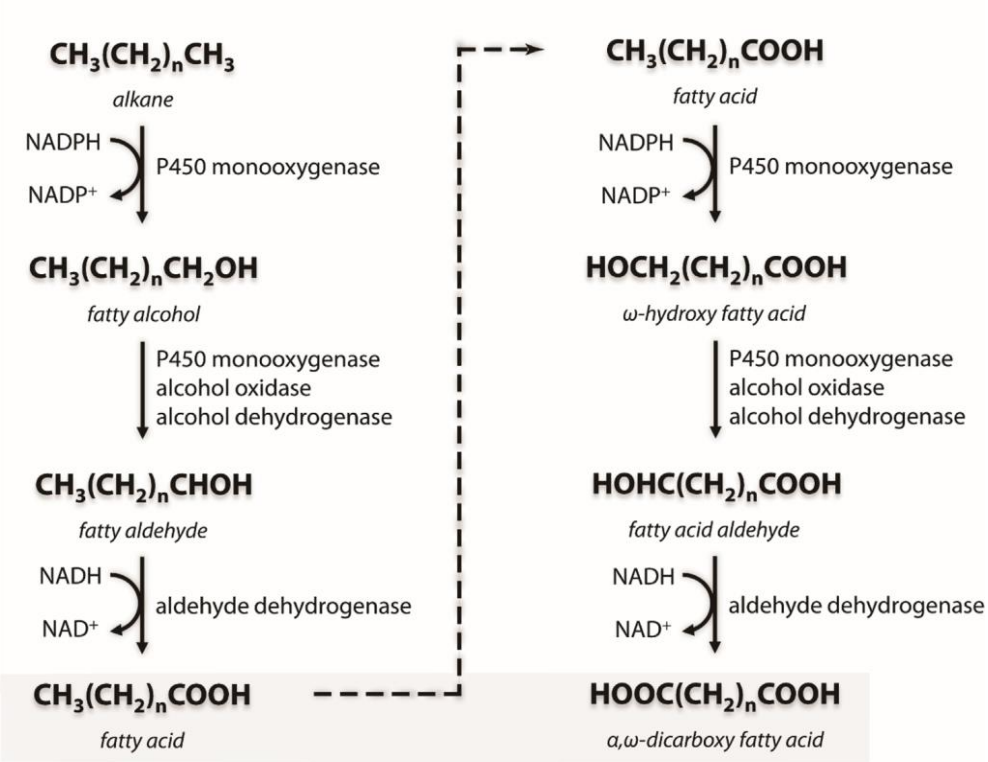


Figure II-1 Fatty acid degradation pathway of yeasts (Seo et al., 2015)

Cytochromes P450 (P450, CYPs)

Cytochrome P450 enzymes belong to the class of monooxygenases (EC 1.14.x.y) and are implicated as key enzymes in primary and secondary metabolism as well as in the detoxification of xenobiotic compounds (Črešnar and Petrič, 2011; Maurer and Schmid, 2005). Cytochrome P450 enzymes are heme-thiolate proteins and incorporate one atom of oxygen into a substrate whereas the other oxygen molecule is reduced to water. The reaction cycle of P450 monooxygenases is presented in figure II-3. The reducing equivalents are transferred by the cofactor NAD(P)H. P450s can be both as epoxidases or as hydroxylases of fatty acids which can be found in all organisms (prokaryotes (archaea, bacteria) and lower eukaryotes

(fungi and insects) to higher eukaryotes (plants, animals, and humans) (Sono et al., 1996).

Generally, cytochromes P450s are active when they interact with one or more cofactors and redox proteins. Depending on the architecture of the heme and the electron transfer (reductase) system, which delivers their reducing equivalents from the cofactors NAD(P)H to the redox protein and the heme iron catalytic site, CYPs have been divided into four classes: (O'Reilly et al., 2011; Roberts et al., 2002)

Class I: Electrons are supplied from a flavoprotein reductase via a 2Fe-2S iron-sulfur protein to the P450. This type of P450s can often occur in mitochondrial systems.

Class II: Electrons are transferred by a single FAD/FMN containing reductase. This type can be mainly founded in the endoplasmic reticulum.

Class III: The reduction equivalents are not necessary, because they use peroxygenated substrates which have already incorporated “activated” oxygen.

Class IV Electrons have directly obtained from NADH. This class is presented by a single member and nitric oxide synthase (Maurer and Schmid, 2005). (Urlacher and Girhard, 2012)

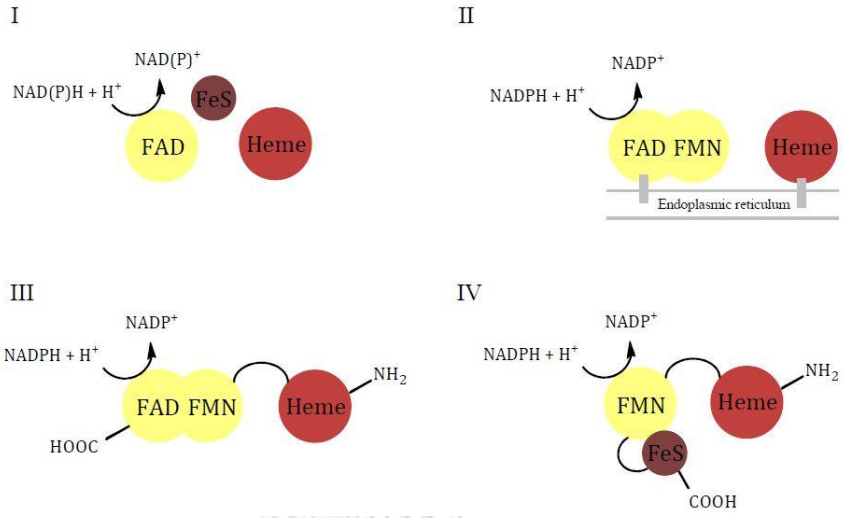


Figure II- 2 The overall architecture of P450 enzymes (Honda Malca, 2013).

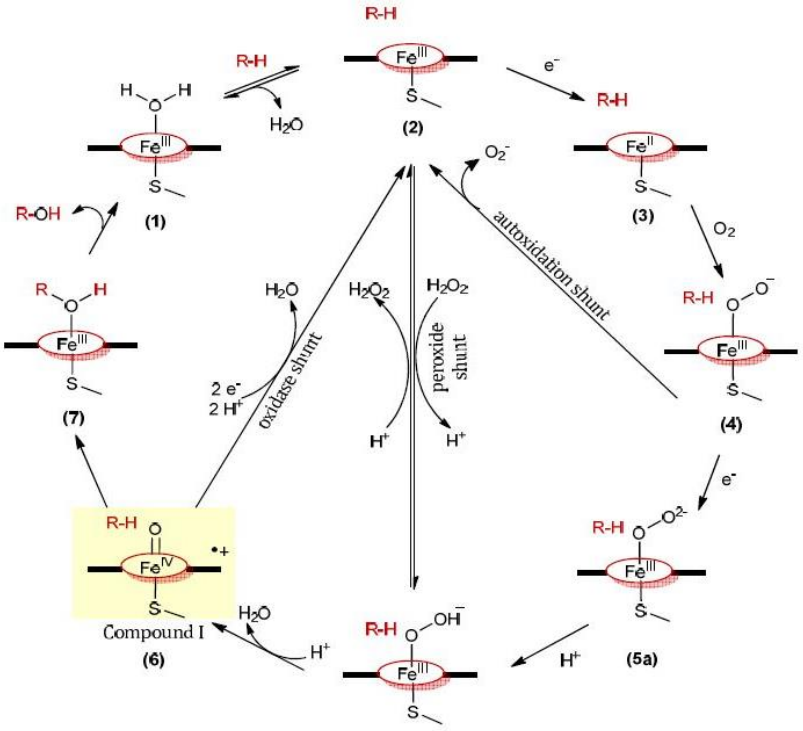


Figure II- 3 The reaction cycle of P450 monooxygenases (Denisov et al., 2005).

CYP52 family

Terminal oxidation can be occurred in all organisms. The CYP52 family (CYP52s, P450 Alk) are involved in the *n*-alkane and fatty acid degradation pathway of yeasts (Fukuda and Ohta, 2017). CYP52s have ability to catalyze *n*-alkane and fatty acid into α, ω -dicarboxylic acids where terminal hydroxylation of *n*-alkanes is rate-limiting, and ω-hydroxylation of fatty acids (Fig.1) (Maurer and Schmid, 2005). CYP52 enzymes, membrane-bound microsomal monooxygenase, belongs to class II CYPs which contained by CYP protein and NADPH-dependent FAD/FMN-containing cytochrome P450 reductase (CPR). CYP52-family P450s have been identified in various *n*-alkane-assimilating yeasts, including *C. tropicalis*, *C. maltose*, *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, *Debaryomyces hansenii*, *Lodderomyces elongisporus*, *Starmerella bombicola*, and *Y. lipolytica* (Fukuda and Ohta, 2017).

CYP52A3, CYP52A4, CYP52A5 and CYP52A9 have been already studies and characterized as well as CYP52 at least 8 genes were identified from *C. maltose*. CYP52A3 showed efficiently the hydroxylation activity and preferred dodecane and hexadecane, yielding the corresponding aldehyde, ω-OHFA and α, ω-DCA (Honda Malca, 2013; Zimmer et al., 1996). CYP52A4 significant preferred dodecanoic acid than the other fatty acids, potentially, ω-OHFA. Ten members of CYP52 family of *C. tropicalis* have been studies (Craft et al., 2003). Both CYP52A13 and CYP52A17 are strongly induced by oleic acid. CYP52A13 potentially converted oleic acid and other unsaturated acids to ω-hydroxy fatty acids. Although CYP52A17 preferentially oxidized oleic to ω-hydroxy fatty acids and α, ω-diacid, shorter saturated fatty acids as myristic acid are efficiently oxidized. Furthermore, CYP52A17 showed higher diacid formation (Eschenfeldt et al., 2003). *C. tropicalis* strain AR40 successfully

oxidized C_{12} and C_{20} of *n*-alkanes and fatty acids as well (Yang et al., 2009). CaAlk8 is one of CYP52 members from *C. albicans* (CYP52A21) and showed the hydroxylase activity on lauric, myristic and palmitic (Kim et al., 2007). *Y. lipolytica* contains twelve *ALK* genes in the CYP52family that involved in ω-oxidation. Alk4p, Alk5p and Alk7p efficiently hydroxylate at the ω-terminal end of dodecanoic acid and Alk3p and Alk6p also have ability to oxidize *n*-alkanes and dodecanoic acid (Iwama et al., 2016).

Biotransformation of *n*-alkanes and fatty acids to α, ω-diacid using yeast CYP52 enzymes is presently the basis of the bio-based production. Metabolic engineering is an important technique for improvement of diacid production. *C. tropicalis* converted oleic acid to a maximum yield of 1, 18-*cis*-9-octadecenedioic after 48 h (Yang et al., 2009). *Y. lipolytica* mutant lacking its twelve constitutive CYP52 gene isoforms as a host for studies of specific CYP52 characterization and applications of biotechnology. A constructed *Y. lipolytica* mutant lacking its four peroxisomal acyl-CoA oxidases (POX genes) accumulated 20 g/L of α, ω-DCA from 23 g/L of dodecane after 50 h (Smit et al., 2005). The accumulation of 174 g/L ω-OHFA and 6 g/L α, ω-DCA from 200 g/L methyl tetradecanoate after 148 h of fermentation was performed by an improved *C. tropicalis* strain. Moreover, the over-oxidation product, 1, 14-tetradecanedioic acid, was obtained up to 6.1 g/L. The codon-optimized of CYP52A17 was overexpressed in *C. tropicalis* under the control of the fatty acid-inducible isocitrate lyase (ICL) promoter. To study ω- hydroxyfatty acid production and specificity of substrates on the production, 16 genes (6 cytochrome P450s, 4 fatty acid alcohol oxidases and 6 alcohol dehydrogenases) were identified and deleted (Lu et al., 2010).

Whole-cell biotransformation with recombinant P450s

The use of whole-cell biocatalysts in biocatalytic reactions are preferred over isolated monooxygenase enzymes because of regenerating NAD(P)H capacity of cells. It is better for organization of membrane-bound enzymes and may give a higher stability by supplying a protected compartment. For industrial purposes, the isolated enzyme is not practical due to it is not stable under process conditions, contain many components and need high-priced cofactors. Whole-cell biocatalyst can be conducted in two phases as many P450 substrates and products are hydrocarbon and not water-soluble. Nevertheless, the other factors such as cofactor supply, host physiology, strain stability, substrate or product toxicity, product transport, substrate uptake, byproduct formation and limited oxygen transfer have to be overcome (Honda Malca, 2013; Urlacher and Girhard, 2012).

Yeast expression system

Bacterial system is still the great choice for expression of heterologous protein, but the expression of eukaryotic genes is difficult in this system. As *E. coli* is prokaryote, lack of the correction of folding the foreign protein and carrying out other post-translation modifications limits the types of protein (Daly and Hearn, 2004). Then, heterologous protein expressions have been developed in eukaryotic hosts such as yeast, insects, filamentous fungi, and mammalian cells. Yeasts are attractive host because of simplicity in genetic manipulation, rapidly growth in simple media to obtain high cell density and ability of post-translational modifications (Juturu and Wu, 2012). Furthermore, many yeast strains are considered as non- pathogenic and generally recognized as safe (GRAS) by the Food and Drug Administration (FDA,

USA). Yeasts are also provided low-cost screening and production systems for authentically processed and modified compounds (Gellissen et al., 2005). Generally, *S. cerevisiae, P. pastoris, Hansenula polymorpha, Kluyveromyces lactis* and *Yarrowia lipolytica* are used for heterologous production systems (Buckholz and Gleeson, 1991).

Pichia pastoris

In 1969**,** Koichi Ogata initially discovered *Pichia pastoris*. *P. pastoris* belongs to the methylotrophic yeast, because it has ability to utilize methanol as a sole carbon source in order to produce single-cell protein. *P. pastoris* is an attractive host for the expression of recombinant proteins which has rapidly become a widely popular host. The Phillips Petroleum collaborated with the Salk Institute Biotechnology/Indrustrial Associates, Inc. (SIBIA) and they have initially developed the protocols and media to grow *P. pastoris* on methanol in continuous culture, since the 1980s this yeast was widely popular for protein production. *P. pastoris* were studied and developed as a heterologous protein expression system, including its vectors and methods (Cereghino and Cregg, 2000). Afterwards in 1993, *P. pastoris* expression system was developed and created as a kit which is available from Invitrogen Corporation (Carlsbad, CA, USA). This yeast expression system based on the strong regulated *AOX*1 (alcohol oxidase 1) gene promoter were explained in several studies (Macauley-Patrick et al., 2005). Furthermore, it has widely used because of its ability in high expression of heterologous protein, high cell densities, high secretion efficiency, growing in the simple conditions, ease of scaling up in industrial level, and low level secretion of native protein (Barr et al., 1992; Macauley-Patrick et al., 2005).

P. pastoris expression platform

Characteristics

The methylotrophic yeast, *P. pastoris*, was considered as a potential host for heterologous proteins production. The advantages are provided (Ferrer et al., 2009; Li et al., 2007; Macauley-Patrick et al., 2005) :

1) Rapid growth rate (> 130 g/L dry cell weight) in minimal medium

2) High level of heterologous protein expression

3) Ease of genetic manipulation

4) Efficient host and vector system

5) Existence of the methods to tightly regulate foreign gene expression

6) Addition of method as an inducer for gene expression.

7) Easy purification due to low level of endogenous proteins.

P. pastoris is, a methylotrophic yeast, a well-known host to produce recombinant soluble as well as membrane proteins with correct post-translational modifications (Wriessnegger et al., 2016). Moreover, a successful cloning and expression of heterologous proteins in *P. pastoris* should be considered in promoter, terminator, selection markers and suitable vector system for either intracellular or secreted expression.

Expression vectors

The expression vectors are set up as a bi-functional system enabling in both of *E. coli* and *P. pastoris* which can replicate in *E. coli* and propagate in *P. pastoris* using selective markers, either auxotrophic markers or genes resistance to drugs (Ahmad et al., 2014; Cereghino et al., 2002). The most expression vectors are composed of an origin of replication, multi-cloning sites (MCS) for insertion of the foreign coding sequence, a promoter to drive the expression of the interested gene, terminator and selectable markers. Expression vectors of *P. pastoris* for heterologous protein expression is represented as Table II-3. Some expression vectors, such as pPICZ series (Figure II-4), contain the *Sh ble* gene from *Streptoalloteichus hindustanus* as the selectable marker for both *E. coli* and *P. pastoris* and the construction of expression vectors is facilitated with multiple expression cassette copies (Li et al., 2007).

Integration of expression vectors into the *P. pastoris* genome to generate stable transformants can be done via homologous recombination. The *P. pastoris* genome are homologous to the regions of vector/expression cassette and then the integration can be presented *via* gene insertion or gene replacement (Figure II-5). Tandem multiple integration can be occurred by gene insertion, because of repeated recombination events. Whereas gene replacement usually resulted in single copy transformants, transformants are generally more stable. Gene replacement is provided by digestion of expression vector at 5' and 3' ends resulting in the 5' and 3' *AOX1* regions of the *AOX1* chromosomal locus. Then transformation presents site-specific replacement of the *AOX1* gene. In addition, gene insertion at the *his 4* or *AOX1* locus

can provide transformants of either His^+ conversions or of the Mut^+ phenotype (Cregg et al., 2000; Daly and Hearn, 2005)

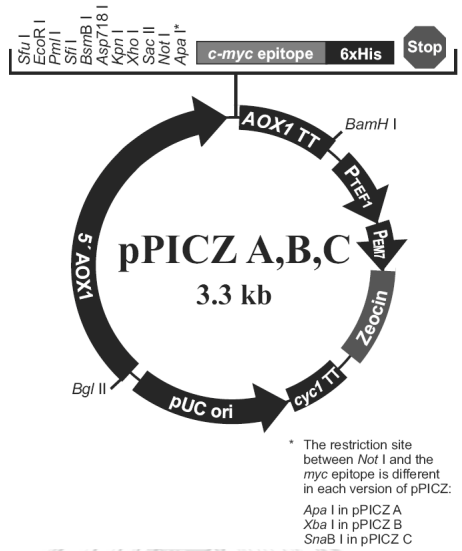


Figure II- 4 Schematic map of the pPICZ expression vector for *P. pastoris*.

Promoters

P. pastoris is a potential host for the heterologous protein expression. Basically, there are two promoters in *P. pastoris* expression system. Alcohol oxidase is an important enzyme for the first step of the methanol utilization pathway (Egli et al., 1980). There are *AOX1* and *AOX2* genes encoding alcohol oxidase (AOX) but the main of alcohol oxidase activity in the cell is responsible by *AOX1* gene (Cregg et al., 1993). 5% of $polyA^+$ RNA of the *AOX1* gene is detected in cells grown on methanol, but there is no expression in cell grown on glucose, glycerol and ethanol as a sole carbon source (Cregg et al., 2000). Although an expression vector p*AOX2* of alcohol oxidase II has been studied, expression level of recombinant protein under the control of p*AOX1* was higher than p*AOX2* (Zhang et al., 2009). AOX1 promoter is, a strong

inducible promoter, controlled and induced by methanol for the expression of recombinant protein. pPICZ and pHIL-D2 vectors contain p*AOX1* that have been successfully expressed cytochrome P450 from many organisms. Dietrich and co-workers reported that pPICZA-CPR-CYP2D6 have been expressed into *P. pastoris* under the control of p*AOX1*. The dogfish shark cytochrome P450c17 (17α-hydroxylase/$C_{17,20}$-lyase) were inserted in pHIL-D2 and expressed in *P. pastoris* in order to overproduce a bioactive enzyme (Trant, 1996). Furthermore, eukaryotic CYPs of spiny lobster and cassava have been expressed in *P. pastoris*. In 2007, Kolar et al. presented the first report showing the heterologous expression of a fully functional human steroidogenic cytochrome P450 enzyme in *P. pastoris* (Kolar et al., 2007). Wriessnegger et al. reported that *RAD52* overexpression appear to be generally applicable for enhanced hydroxylation of hydrophobic compounds in *P. pastoris* (Wriessnegger et al., 2016).

P. pastoris glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) promoter is, a key gene in glycolysis, is strong constitutive expressed on glucose or glycerol (Waterham et al., 1997). Using the GAP promoter is not necessary to transfer the cultures from one carbon source to the other, non-requirement of methanol for induction of expression and it is better for large -scale production (Cereghino and Cregg, 2000; Zhang et al., 2009).

Selection markers

There are several selectable marker genes, used for the molecular genetic manipulation of recombinant genes of *P. pastoris*. The selectable markers are divided into two groups for *P. pastoris* and the other yeasts: biosynthetic pathway genes and drug resistance gene. Selectable markers were based on biosynthetic pathway genes including *ARG4* (argininosuccinate lyase gene) from *S. cerevisiae* and *HIS4* (histidinol dehydrogenase gene) from either *P. pastoris* or *S. cerevisiae* and the *Sh ble* gene from *Streptoalloteichus hindustanus* which confers resistance to the bleomycin-related drug zeocin (Cregg et al., 1989; Higgins and Cregg, 1998).

Table II- 3 Common *P. pastoris* expression vectors (Cregg et al., 2000)

Vector name	Targeting	Selectable markers	Features	References
pHIL-D2	Intracellular	*HIS4*	*Not*I sites for *AOX1* gene replacement	Sreekrishna, personal comm.
pAO815	Intracellular	*HIS4*	Expression cassette sites bounded by *Bam*HI and *Bgl*II for generation of multicopy expression vector	2
pPIC3K	Intracellular	*HIS4* and *kan*^{r}	Multiple cloning sites; for insertion of foreign genes; G418 selection for multicopy strains	
pPICZ	Intracellular	*ble*^{r}	Multiple cloning sites for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags	10
pHWO10	Intracellular	*HIS4*	Expression controlled by constitutive *GAPp*	29
pGAPZ	Intracellular	*ble*^{r}	Expression controlled by constitutive *GAPp*; multiple cloning site for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags	Invitrogen, Carlsbad, CA
pHIL-S1	Secreted	*HIS4*	*AOX1p* fused to *PHO1* secretion signal; *Xho*I, *Eco*RI, and *Bam*HI sites available for insertion of foreign genes	Sreekrishna, personal comm: Invitrogen. Carlsbad, CA
pPIC9K	Secreted	*HIS4* and *kan*^{r}	*AOX1p* fused to α-MF prepro signal sequence; *Xho*I (not unique), *Eco*RI, *Not*I, *Sna*BI, and *Avr*II sites available for insertion of foreign genes; G418 selection for multicopy strains	32
pPICZα	Secreted	*ble*^{r}	*AOX1p* fused to α-MF prepro signal sequence; multiple cloning site for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags	10
pGAPZα	Secreted	*ble*^{r}	Expression controlled by constitutive *GAPp*; *GAPp* fused to α-MF prepro signal sequence: multiple cloning site for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags	Invitrogen, Carlsbad, CA

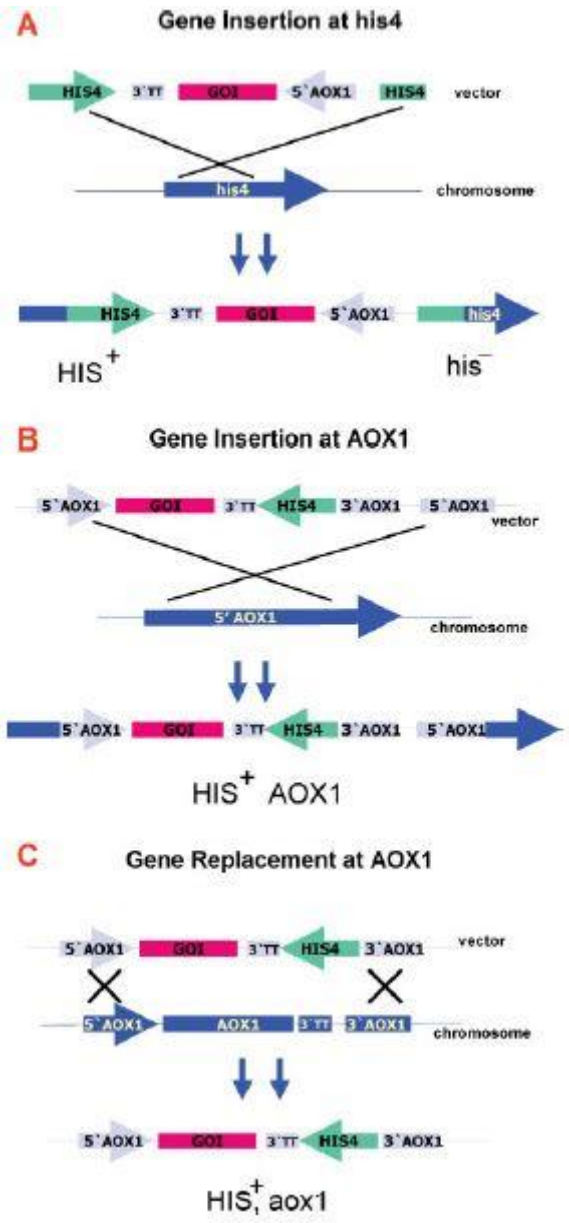


Figure II- 5 Mechanisms of integration of expression cassettes in *P. pastoris* genome by gene insertion (A, B) and gene replacement (C) (Daly and Hearn, 2005).

Saccharomyces cerevisiae

The most well -known yeast species in the laboratory are baker's yeast *S. cerevisiae*. *S. cerevisiae* (baker's yeast) was the basis for the development of yeast system in order to heterologous protein expression. This yeast is an ideal experimental organism for genetic manipulation due to a useful of information is mostly available, its physiology, genetics, biochemistry, fermentation and metabolism knowledge were accumulated, and it has been recognized as generally regarded as safe (GRAS) yeast (Çelik and Çalık, 2012; Zhang and An, 2010). Moreover, the genome sequence and thousand genes of *S. cerevisiae* have already been determined. Therefore, *S. cerevisiae* is one of the most extensively used as a microbial host for foreign gene expression for research and industrial applications (Strausberg and Strausberg, 1995). Many laboratories have studied heterologous gene expression in *S. cerevisiae* for a various protein derived from fungal and mammalian species based on a considerable of vectors and host strains. The group of Ohkawa (Sumitomo Chemical Co., Japan) have already been successful in coexpression of a membrane-bound P450 with CPR in *S. cerevisiae*. Most expression of various microsomal P450s involved in the degradation of xenobiotics or biotransformation of drugs in *S. cerevisiae*, *Y. lipolytica* or *P. pastoris*. Bioconversion of 11-deoxycortisol into cortisol using recombinant CYP11B1 expressed in *Schizosaccharomyces pombe* showed that the production level is lower than CYP11B1 expressed in *S. cerevisiae*.

Saccharomyces cerevisiae expression platform

Characteristics

S. cerevisiae, the traditional biotechnological organism, has been widely utilized for a variety of recombinant protein expression. The advantages of this yeast system are listed (Čepononytė et al., 2008; Liu et al., 2012)

1) Low-cost culture and easy to grow

2) Existence of well-developed tools for manipulation of yeast DNA, including expression vectors and host strains

3) Secretion protein to the extracellular medium

4) No risk for human virus infection

5) Production of protein with appropriate post-translational modifications

6) Extensive track record as a safe organism.

The *S. cerevisiae* yeast has been primarily used as a host for various recombinant protein production (Vieira Gomes et al., 2018). Engineering of *S. cerevisiae* vectors and host strain should be developed for the increasing product yield and quality.

Expression vectors

Generally, expression vectors of *S. cerevisiae* are shuttle vector which contain sequences for propagation and selectable markers in both *E. coli* and *S. cerevisiae.* There are three types of vectors: integration plasmids (YIp), episomal plasmids (YEp), and centromeric plasmids (YCp). Integration plasmids of *S. cerevisiae* contain

selectable genes and have no *ARS* sequence (Zhang and An, 2010). Their integration is homologous recombination between sequences carried on the plasmid and the homologous complementary in the *S. cerevisiae* genome. Although The benefit of integration vectors is highly stable, they are usually presented at low copy numbers (Gellissen and Hollenberg, 1997). The genome integrated expression cassette can be stable under selection. Nevertheless, copy numbers of the integrated expression cassette was decreased when it was maintained in nonselective culture conditions. YEps are the most popular episomal and the best-developed vectors that derived from the endogenous 2μ origin of replication maintained in high-copy number inside the cell (average 40 copies/cell). These vectors present higher stability than the YRp and YCp vectors. However, the cells can be forced by it as a consequence of an increase plasmid variability. YEp vectors are useful for regulation of toxic protein expression. Almost 80 compact expression vectors have been invented originating the pRS series of the centromeric and and 2μ plasmids (Strausberg and Strausberg, 1995). Many companies have developed these type vectors. For example, the GATEWAY vectors (Invitrogen Life Technologies, Carlsbad, CA) have been improved their characteristics to be fast cloning and transferring of genes to several vectors for recombinant protein expression of various *S. cerevisiae* strains. YCPs are vector type derived from a combination of autonomously replicating sequence (ARS) and *S. cerevisiae* centromeric sequence (CEN) which plasmid stability have been improved. Although YCp vectors exhibit low-copy numbers (1–2 copies) which have higher stability, limitation of usage is lower gene expression (Zhang and An, 2010).

Table II- 4 Vector systems used for heterologous protein expression in *S. cerevisiae* (Zhang and An, 2010)

Vector	Copy number per cell
Episomal	
Yep: 2μ-based	25-200
YCp: centromere	1-2
YRp: replicating	1-20
Regulated copy number	3-100
Integrating	
YIp	>1
rDNA-integrating	100-200
Transplacement	1

Promotors

Many promoters have been utilized for heterologous gene expression in *S. cerevisiae*. Promoters are characterized and engineered genetic part in order to providing strong constitutive or inducible promoters with good transcriptional activity which aspired to overexpression of heterologous proteins. Generally, constitutive promoters are commonly simple and high-level of expression, whereas inducible promoters provided an accurate control of expression, are likely to deal with unintended selection of non-recombinant cells and regularly used for separation between growth and protein production part (Čepononytė et al., 2008). There are several constitutive promoters derived from genes such as translation elongation factor 1 (*TEF1*), alcohol dehydrogenase (*ADH*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and phosphoglycerate kinase (Vieira Gomes et al., 2018; Zhang and An, 2010). However, the utilization of promoters must be careful. Their expression is up-regulated by glucose (an ordinary carbon source in the media) (Strausberg and Strausberg, 1995). The galactose-induced *GAL1* and *GAL10* promoters are often used in *S. cerevisiae*. Gene expression can be controlled by a presence of its inductor molecule in the growth medium such as galactose (*GAL1*) (repression by glucose), phosphate (*PHO5*), and copper (*CUP1*). In some inducible promoters always function well in shake flask scale but do not work well in large-scale fermentation, because of limitation of the process (Strausberg and Strausberg, 1995; Vieira Gomes et al., 2018). The example utilized promoters in yeast *S. cerevisiae* are listed in Table II-5.

Selectable marker

One of the important development of expression vectors is the choice of selectable marker. Selectable marker can affect to yeast transformation, copy number and stability of the expression cassette. The marker genes provide successful transformants that contain the interesting gene and markers such as dominant, auxotrophic and autoselective markers. Dominant markers contain genes which resist to copper or appropriate antibiotics such as chloramphenicol, G418, hygromycin, and zeocin. These markers are preferred when an auxotrophic yeast does not present, or a rich medium is used. However, the con of using dominant markers involved in the degradation or inactivation of the antibiotic and higher cost than the others. Auxotrophic markers (*HIS3*, *HIS4*, *LEU2*, *LYS2*, *TRP1*, *URA3*) are the most popular selection markers which have counterpart with a specific auxotrophic mutation in yeast host (Vieira Gomes et al., 2018). For example, the *LEU2* and *URA3* genes utilized in strains that are leu2 or ura3, respectively. The assemblage of host strains allows high stability of these mutant loci. The *LEU2* gene and the *G418* resistance gene are the most widely used selection markers for *S. cerevisiae* (Strausberg and Strausberg, 1995). Autoselection systems depend on the expression of important activity in host strains without such an activity. Moreover, the plasmid should maintain in order to confirm the yeast survival not influenced by the culture conditions.

Table II- 5 Example of promotors available for *S. cerevisiae* (Darby et al., 2012)

Vector	Promotor	Selection marker	Expression	Episomal or integrative
pYX212	*TPI1*	*URA3*	Constitutive	Episomal
pYX222	*TPI1*	*HIS3*	Constitutive	Episomal
pYES2	*GAL1*	*URA3*	Inducible	Episomal
pVTU260	*ADH1*	*URA3*	Constitutive	Episomal
YEpCTHS	*CUP1*	*Ampicillin*	Inducible	Episomal

CHAPTER III

MATERIAL AND METHODS

Part I Cloning and expression of CYP52A17 in *Pichia pastoris*

1. Strains, culture conditions and plasmid

E. coli DH5α (Gibco, USA) and *Pichia pastoris* X-33 (Invitrogen, USA) were used as host cells for plasmid propagation and expression, respectively, of the target gene. *P. pastoris* was used in all experiments. This strain was grown in YPD (10 g yeast extract/L, 20 g peptone/L, and 20 g glucose/L), and incubated at 30 °C with shaking at 200 rpm for 3-5 days. The transformants were selected onto YPDS agar (YPD with 1 M sorbitol) containing 100-2,000 µg/mL zeocin. *E. coli* DH5α was grown at 37 °C in Luria-Bertani (LB) medium (10 g tryptone/L, 5 g yeast extract/L, 10 g NaCl/L) with all appropriate antibiotic for maintenance of plasmids. pPICZA vector (Invitrogen, USA) was used for CYP52A17 expression and maintained by zeocin 100 µg/mL. Kanamycin (50 µg/mL) was added as a selective agent for pUC57-Kan selection. Primers used for gene construction and cloning are shown in Table III-1.

2. DNA manipulations

A codon-optimized *Candida tropicalis* ATCC 20336 CYP52A17 gene was synthesized and introduced into pUC57-Kan vector by GenScript (USA) base on the nucleotide database (GenBank: accession no. AY230504) but with additional 5’

sequences containing *Eco*RI and *Not*I to facilitate in frame directional cloning (Eschenfeldt et al., 2003). The synthetic *CYP52A17* gene was propagated by transformation into *E. coli* DH5α and digested with the appropriate restriction enzymes *Eco*RI and *Not*I and then ligated into similarly digested expression vector pPICZA (p*AOX1*) as shown in Figure III-1. The ligation products were used to transform *E. coli* DH5α competent cells. The constructed plasmid designated as pPICZA-CYP52A17 was selected on low-salt LB agar medium (10 g tryptone/L, 5 g yeast extract/L, 5 g NaCl/L and 15 g agar/L) containing 100 µg/mL zeocin to screen the presented recombinant plasmid. To verify the nucleotide sequence of the CYP52A17 gene, the insertion was checked by double restriction enzyme digestion and plasmid DNAs extracted from the selected clones were sequenced by Eurofins (Toulouse, France) using 5'AOX1 (5' -GACTGGTTCCAATTGACAAGC- 3') and 3' AOX (5' -GCAAATGGCATTCTGACATCC- 3') sequencing primer.

3. Site-directed mutagenesis of the recombinant CYP52A17

The TTG codon encoding leucine at amino acid positions 261 and 490 of CYP52A17 was changed to UCU (TCT) to encode for serine (L261S and L490S) using a site-directed mutagenesis kit (Agilent Technologies, Inc.) as per the manufacturer's instruction and previous report by Eschenfeldt et al., (2003) to yield rCYP52A17mut. The rpPICZA-CYP52A17mut plasmid was transformed into *P. pastoris* X-33 and transformants selected and sequenced to confirm the insert as described above for rCYP52A17

4. *P. pastoris* transformation

The recombinant pPICZA-CYP52A17 plasmid was linearized with *Hin*dIII for intergration into *P. pastoris* genome and then the recombinant pPICZA-CYP52A17 was expressed into *P. pastoris* X-33. The competent *P. pastoris* cells were prepared as described by Invitrogen protocol. The pPICZA-CYP52A17 was transformed into 90 µl of the competent *P. pastoris* X-33 using the lithium acetate and dithiothreitol method by electroporation and 1 mL of 1 M of sorbitol was rapidly added with incubating at 30 °C, 200 rpm for 2 h. Transformants were selected on YPDS agar plates (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of glucose and 1 M of D-sorbitol) containing zeocin at concentration of 100, 500, 1,000, or 2,000 µg/mL and incubated at 30 °C for 3-4 days. The transformants of sequences were checked by colony PCR technique using specific primers (5' and 3' *AOX*) and DNAs extracted from selected transformants were sequenced.

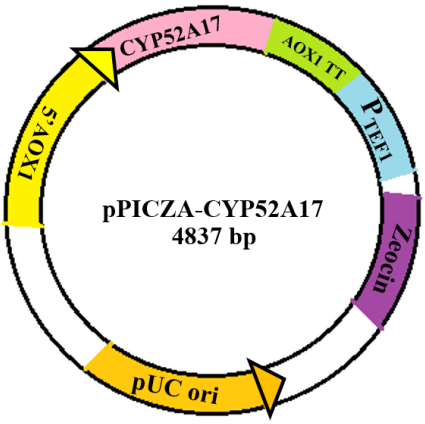


Figure III- 1 Schematic diagram of the expression vector for *CYP52A17* gene insertion. The vector contains *AOX1* promoter for controlling the expression of protein from *Pichia* and zeocin resistance gene for selection.

5. Expression of CYP52A17 in *P. pastoris*

The heterologous expression of CYP52A17 in *P. pastoris* was accomplished according to the supplier's instructions (Invitrogen). The *P. pastoris* with methanol-inducible expression (under *AOX*) was cultured in 50 mL of buffered glycerol-complex medium (BMGY: 10 g yeast extract/L, 20 g peptone/L, 100 mM KH_2PO_4/KOH (pH 6.0), 13.4 g yeast nitrogen base, 4 x 10^{-5} g biotin/L and 10% v/v glycerol) at 30 °C, 200 rpm for 18 h and inoculated into 200 mL of buffered minimal-methanol medium (BMMY: 10 g yeast extract/L, 20 g peptone/L, 100 mM KH_2PO_4/KOH (pH 6), 13.4 g yeast nitrogen base/l, 4 x 10^{-5} g biotin/L and 0.5% v/v methanol instead of glycerol) shaking at 200 rpm, 30 °C for 48 h and then the cultures were induced by 0.5% methanol overnight. The cultures were harvested by centrifugation at 6,000 g for 5 min, 4 °C. Cells were resuspended in TES buffer. The cells were mixed with an equal volume of 0.4-mm acid washed glass beads and then the pelleted cells were broken by manual shaking to prepare the microsomes as reported (Lautier et al., 2016) which were then kept at -80 °C until used.

6. CYP52A17 activity

Protein concentrations were determined by Pierce BCA protein assay kit according to supplier's recommendation. BSA was used as standard. CYP52A17 concentrations in microsomes were detected by reduced carbon monoxide (CO) spectra as follows: 100 µL of microsome were dissolve in 50 mM TE buffer pH 7.4. Some sodium dithionite was added. The sample solution was transferred into cuvettes and then a reference spectrum was measured by the Varian Cary 100 UV-visible spectrophotometer scanning between the wavelengths 400-500 nm. The solution was

sparged with carbon monoxide for 30 s and the spectrum was determined again. The cytochrome P450 concentration was calculated with an extinction coefficient of 91 mM^{-1} cm^{-1}.

7. NADPH -cytochrome P450 reductase

NADPH -cytochrome P450 reductase activity were measured by the reduction of cytochrome C. The reaction mixtures contained 20 mM TE buffer mixing with 372 mM NaCl, 10 uL of 10 mg/mL cytochrome C, 1 uL of microsome and 10 uL of 10 mg/mL NADPH. The reaction was determined at 550 nm using a UV-visible spectrophotometer. The cytochrome P450 reductase activity was calculated with an extinction coefficient of 21 mM^{-1} cm^{-1}.

8. Biotransformation

8.1 *In vitro*

The reaction mixture contained 100 µL of microsome, 5 µL of 100 mM NADPH, 250 µM lauric acid dissolved in 50 mM K_2CO_3 in a final volume of 1 mL. The reaction was incubated at 35 °C for 7 min and stopped by the adding of 100 µL acetonitrile. 100 µL of samples were taken and centrifuged at 13,000 g for 15 min. The organic layer was afterwards collected for HPLC analysis.

The kinetic parameters (K_m, V_{max} and k_{cat}) of the recombinant CYP52A17 enzyme were determined using different substrate concentrations (5-200 µM lauric acid) with the same method described previously at 35°C for 7 min. Kinetic analyses were performed using Michaelis-Menten.

8.2 *In vivo*

Recombinant *P. pastoris* X-33 was inoculated in 5 ml of BMGY medium shaking at 200 rpm, 30 °C. The overnight grown cell was inoculated into 25 ml of BMMY medium to obtain an OD_{600} of 0.4 and the cells were cultured until reached an OD_{600} of 1.0-1.2. Then 0.5% methanol and 250 µM lauric acid were added. The cells were cultured again for 48 h at 30°C, 200 rpm. The samples were taken for product determination.

9. Fatty acid extraction

1,000 µL of 6 M HCl and 2.5 mL of ethyl acetate were added into 5 ml culture samples, then, they were vortexed at the maximum speed for a few minutes and afterwards left them static for 10-15 min until organic phase and hydrophilic phase were separated. Then, the organic layer was transferred to a new tube. The samples were centrifuged at 13,000 g for 15 min, collected supernatant, and evaporated solvent under nitrogen. The dried samples were dissolved in 200 µl acetonitrile and measured by LC-MS analysis.

10. HPLC analysis

The chromatography analysis was performed at 40 °C on a XTerra® MS C_{18} column (4.6 mm x 100 mm I.D., 3.5 µm particle size, 125 Å pore size). The elution of all analytes was gradient method using mobile phase of 20 mM triethylamine acetate buffer and 100% acetonitrile that was run at a flow rate of 1 mL/min. The total run time was 20 minutes.

Part II Cloning and expression of CYP52A17 in *S. cerevisiae.*

1. Strains, culture conditions and plasmid

Escherichia coli DH5α, *S. cerevisiae* BY(2R), W(R) and pYeDP60 were obtained from Molecular and Metabolic Engineering (EAD 11), LISBP, INSA. *E. coli* DH5α and *S. cerevisiae* BY(2R) and W(R) (based on the BY4741 genetic background and a supplementary copy of the CPR gene under its own promoter) (Truan et al., 1993) were used as the host cells for plasmid propagation and expression of the target gene, respectively. Primers used for gene construction and cloning are shown in Table III-1. Yeast strains were grown in YPD (10 g yeast extract/L, 20 g peptone/L, and 20 g glucose/L), medium at 30 °C, 200 rpm in flasks or in Petri dishes containing YPD adding 15 g agar/L. Transformants of auxotrophic mutant strains were selected onto complete minimal medium (CSM) lacking histidine (0.77 g CSM-His/L, 20 g glucose/L, 6.7 g YNB/L, 5 g ammonium sulfate/L) supplemented with all appropriate amino acids and incubated at 30 °C. Whereas *E. coli* DH5α were grown in Luria-Bertani (LB) broth with all appropriate antibiotics for maintenance of plasmids at 37 °C. pYeDP60 were maintained by ampicillin 100 µg/mL.

2. DNA manipulations and yeast transformation

A codon-optimized *Candida tropicalis* ATCC 20336 CYP52A17 gene based on the nucleotide database (Genbank under accession number AY230504) was synthesized and inserted into the plasmid vector pUC57-Kan (GenScript, USA) but with additional 5’ sequences containing *Eco*RI and *Not*I to facilitate in frame directional cloning (Eschenfeldt et al., 2003). The synthetic CYP52A17 gene was

propagated by cloning into *E. coli* DH5α and digested with the *Eco*RI and *Not*I enzymes and then ligated into similarly digested expression vectors pYeDP60. The ligation products were used to transform *E. coli* DH5α. To verify the nucleotide sequence of the genes, plasmid DNAs extracted from selected clones were sequenced. The recombinant pYeDP60-CYP52A17 were transformed into *S. cerevisiae* BY(2R) and W(R) using the LiAC/single-stranded carrier DNA/PEG method developed by Gietz and Woods (2006) (Gietz and Woods, 2006). Recombinants were selected on complete minimal medium without histidine (CSM-his).

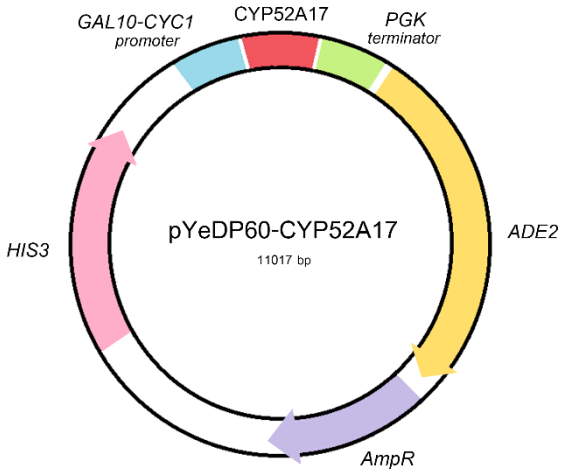


Figure III- 2 Schematic diagram of the expression vector for CYP52A17 gene insertion. The vector contains GAL promoter for regulation of the expression of protein from *Saccharomyces* and ampicillin resistance gene for selection.

3. Site-directed mutagenesis of the recombinant CYP52A17

The TTG codon encoding leucine at amino acid positions 261 and 490 of CYP52A17 was changed to UCU (TCT) to encode for serine (L261S and L490S) using a Site-Directed Mutagenesis Kit (Agilent Technologies, Inc.) as per the manufacturer's instructions and previously reported[14] to yield rCYP52A17mut. The rpYeDP60-CYP52A17mut plasmid was carried out into *S. cerevisiae* by lithium acetate method (Gietz and Woods, 2006) and selected on CSM-histidine agar at 30 ℃ for 3-5 days. Transformants were validated by colony PCR and sequenced for confirmation of the insert gene.

4. Expression of recombinant protein

BY(2R)/pYeDP60-CYP52A17, BY(2R)/pYeDP60-CYP52A17mut, W(R)/ pYeDP60-CYP52A17 and W(R)/ pYeDP60-CYP52A17mut were inoculated in 50 mL of CSM-his, incubated at 30 °C, 200 rpm, and overnight. The preculture were shifted to 200 mL of YPGE medium (10 g yeast extract/L, 20 g peptone/L, 5 g glucose/L and 2% v/v absolute ethanol) and incubated at 30 °C, 200 rpm for 48 h and 20 g galactose/L were added into the cultures for induction of the expression. The cultures were incubated again for 16 h. The cell cultures were harvested by centrifugation at 6,000 g for 5 min. The cell pellets were resuspend in TES buffer and broken by Thermo Scientific FastPrep FP120A Cell Disrupting Homogenizing Isolation System as reported (Lautier et al., 2016). The microsomes were prepared and afterwards kept at -80 °C.

5. CO difference spectra of CYP52A17 activity and protein determination

Protein concentrations were determined by Pierce BCA protein assay kit according to supplier's recommendation. BSA was used as standard. CYP52A17 concentrations in microsomes were detected by reduced carbon monoxide (CO) spectra as follows: 100 µl of microsome were dissolve in 50 mM TE buffer pH 7.4. Some sodium dithionite was added. The sample solution was transferred into cuvettes and then a reference spectrum was measured by the Varian Cary 100 UV-visible spectrophotometer scanning between the wavelengths 400-500 nm. The solution was sparged with carbon monoxide for 30 s and the spectrum was determined again. The cytochrome P450 concentration was calculated with an extinction coefficient of 91 mM^{-1} cm^{-1}.

6. NADPH - cytochrome P450 reductase

NADPH - cytochrome P450 reductase activity were measured by the reduction of cytochrome C. The reaction mixtures contained 20 mM TE buffer mixing with 372 mM NaCl, 10 uL of 10 mg/ml cytochrome C, 1 uL of microsome and 10 uL of 10 mg/mL NADPH. The reaction was determined at 550 nm using a UV-visible spectrophotometer.

7. Biotransformation

7.1 *In vitro*

The mixture contained 100 µL of microsome, 5 µL of 100 mM NADPH, 250 µM lauric acid dissolved in 50 mM K_2CO_3 in a final volume of 1 mL. The reaction was incubated at 35 °C for 7 min and stopped with 100 µL of 100% acetonitrile. 100

µL of Samples were taken and centrifuged at 13,000 g for 15 min. Then, the supernatant was collected for HPLC-MS analysis.

The kinetic parameters (K_m, V_{max} and k_{cat}) of the recombinant CYP52A17 enzyme were determined using different substrate concentrations (5-200 µM lauric acid) with the same method described previously at 35°C for 7 min. All assays were performed in triplicate and the mean values with their standard deviation (SD). Kinetic analyses were performed using Michaelis-Menten plots.

7.2 *In vivo*

BY(2R)/pYeDP60-CYP52A17 was precultured in 5 mL of CSM-his. The overnight grown cell was inoculated into 25 mL of YPGE medium to obtain an OD_{600} of 0.4 and the cells were cultured until reached an OD_{600} of 1.0-1.2. Then 20 g/L galactose and 250 µM lauric acid were added. The cells were cultured again at 30°C, 200 rpm for 120 h.

7.3 Effect of lauric acid concentrations on DCA biotransformation

To examine the effect of lauric acid concentrations on DCA production, the biotransformation was conducted at 500, 600,700 µM of lauric using the recombinant *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut was precultured in 5 mL of YPD. The overnight grown cell of yeast was inoculated into 25 mL of YPGE medium to obtain an OD_{600} of 0.4 and the cells were cultured again until reached an OD_{600} of 1.0-1.2. Then 20 g/L galactose and lauric acid were added. The cells were cultured again for 120 h at 30°C, 200 rpm. 5 ml of samples were collected at different time points, and products were quantified using LC-MS.

7.4 Biotransformation in 5 L bioreactor

BY(2R)/pYeDP60-CYP52A17mut was used for this study which the biotransformation had precise monitoring and control system for pH, temperature, and agitation speed. The conditions and medium were maintained as for the shake flask experiments. The yeast strain was precultured in 30 ml YPD for 18 h and afterwards transferred to 300 mL YPD at 30 ℃, 200 rpm for overnight. The overnight grown cells were inoculated into YPGE medium to obtain an OD_{600} of 0.4 and the cells were cultured again until reached an OD_{600} of 1.0-1.2. Then 20 g/L galactose and 500 µM lauric acid were added. The experiment was conducted at 30 ℃ and agitation speed was 250 rpm. The samples were collected daily for products determination by HPLC-MS (Shimadzu, Japan).

8. Fatty acid extraction

1,000 µL 6 M HCl and 2.5 mL ethyl acetate were added into 5 mL cultures vortexing at the maximum speed for few minutes and left it static for 10-15 min until it separated between organic phase and hydrophilic phase and then transferred the organic phase to a new tube. The samples were dried by nitrogen, dissolved in 200 µL acetonitrile and measured by LC-MS analysis.

9. HPLC analysis

The chromatography analysis was performed at 40 °C on a XTerra® MS C_{18} column (4.6 mm x 100 mm I.D., 3.5 µm particle size, 125 Å pore size) The elution of all analytes was gradient method using mobile phase of 20 mM triethylamine acetate (TEAA) buffer and 100% acetonitrile. A flow rate of 1 mL/min was used. The total run time was 20 minutes.

Table III- 1 Sequence of primer used in PCR reactions.

Purpose	**Primer name**	**Primer sequence**
Generate pYeDP60	Oligo1	5'- ACA CAC ACT AAA TTA CCG AAA TGA TTG AGC AGT TAT TGG AGT ATT GG -3'
	Oligo2	5'- CAT GGG AGA TCC CCC GCT CTA GAA TTT AAC GAT AGC ACC ATC CTG TAA -3'
Expression	Oligo3	5'- ATA CTT CTA TAG ACA CGC AAA CAC AAA TAC ACA CAC TAA ATT ACC -3'
	Oligo4	5'- CCA ATA ATT CCA AAG AAG CAC CAC CAC CAG TAG AGA CAT GGG AGA TCC CCC GC -3'
Genomic DNA	CYP52A17F	5′-ATG ATT GAG CAG TTA TTG GAG TAT TGG -3′
	CYP52A17R	5′-CTA ATT ATT CGA AAC GAT GAT TGA GCA G-3′
Site-directed mutagenesis	782F	5′-CAA CAA AGA GTT TAG AGA TTG TAC AAA GTC TGT TCA CAA GTT TAC C-3′
	782R	5′-GGT AAA CTT GTG AAC AGA CTT TGT ACA ATC TCT AAA CTC TTT GTT G-3′
	1469F	5’- GAG TTT TCA CAC GTC AGA TCT GAT CCA GAC GAA GTT -3’
	1469R	5’ – AAC TTC GTC TGG ATC AGA TCT GAC GTG TGA AAA CTC -3’

Part III The application of recombinant *Saccharomyces cerevisiae* in dodecanedioic acid production of coconut milk wastewater

1. Analytical methods

The coconut milk wastewater used in this study was collected from Chao Koh factory in Nakhon Pathom province, Thailand and kept at -20 ℃ until used. This coconut milk wastewater was mainly produced by the coconut milk process. The sample was characterized for its pH, level of total solids (TS), total suspended solids (TSS), volatile solids (VS), oil and grease, BOD, COD, total Kjeldahl nitrogen, total phosphorus, copper, calcium, magnesium and reducing sugar as outlined in section 1.1.

1.1 Analytical methods

The coconut milk wastewater was analyzed for its pH, TS, TSS, VS, oil and grease, BOD, COD, Kjeldahl nitrogen, total phosphorus, copper, calcium, magnesium and reducing sugar levels according to the standard methods (APHA et al., 2012). Free fatty acids were determined according to AOACS (2009) Aa 6-38 method and reducing sugar were analyzed by AOAC (2016) 968.28 method.

2. Production of dodecanedioic acid using the recombinant *S. cerevisiae*

BY(2R)/pYeDP60-CYP52A17mut was used to determine the dodecanedioic acid production, and BY(2R)/pYeDP60 lacking cytochrome P450 gene was used as a control. Biotransformations were carried out using two different systems, the recombinant *S. cerevisiae* cells were precultured in 10 mL of YPD at 30°C, 200 rpm

for 24 h as an inoculum. (i) the inoculum was cultured in 25 mL of YPGE medium containing 20 g/L of galactose. The flasks were incubated at 30°C for 48 h, with shaking at 200 rpm. Then, the cultures were centrifuged at 3,000 rpm for 5 min to harvest the cells. The cells were transferred to 25 mL of coconut milk wastewater adding 200 g/L galactose, incubated at 30°C for 72 h. (ii) The inoculum was transferred to 25 mL of YPD medium, incubated at 30°C for 48 h. The cells were harvested and further cultured in 25 mL of YPGE medium with adding 20 g/L galactose. The flasks were cultured again at 30 °C for 24 h. Then, the harvested cells were transferred into 25 mL of coconut milk wastewater adding 200 g/L galactose incubating at 30 °C for 72 h. Samples of the culture were taken and treated with 0.5 mL of 6 M HCl and 5 mL of TBME to extract the products. The TBME layer was used for the determination of lauric, 12-hydroxydodecanoic acid and dodecanedioic acid concentrations by HPLC-MS. The HPLC-MS analysis was performed using a Shimadzu LCMS-8030 (Shimadzu, Japan) equipped with a XTerra MS C18 vanguard cart (3.5 µm, 2.1 x 5 mm) and XTerra MS C18 column (3.5 µm, 3.0 x 100 mm; Water, USA) connected to MS. The mobile phase was composed of solution A: 10 mM TEAA and solution B: acetonitrile. A flow rate of 0.3 mL/min and 40°C of column temperature were used.

CHAPTER IV

RESULTS AND DICUSSION

Part I Cloning and expression of CYP52A17 in *Pichia pastoris*

Construction of expression vector, transformation and selection

In order to express and study the biochemical properties of cytochrome P450 rCYP52A17 in *P. pastoris*. The CYP52A17 gene was codon optimized to enhance transcription levels in *P. pastoris*. The gene encoding mature rCYP52A17 was cloned in frame with the C-terminal sequence under the control of *AOX1* promotor by introducing an artificial *Eco*RI restriction site at the 5' end and *Not*I restriction site at the 3' end of this gene. The *P. pastoris* expression vector pPICZA was ligated with the construction at the multiple cloning site to construct the recombinant expression plasmid pPICZA-CYP52A17. Then, the recombinant plasmid was transformed and integrated into *P. pastoris* X-33 genome. The confirmation of the open reading frame and integration of expression cassettes into the yeast genome were validated by PCR analysis and DNA sequencing. A rapid and short way to possibly allow higher protein production, integration of multiple gene copies, was manufactured by selection of the transformants on increasing concentrations of zeocin. In this study, the transformants were plated on YPDS agar containing 100-2,000 µg/ml zeocin. YPDS agar plate containing 1,000 and 2,000 µg/ml zeocin showed none of transformant whereas the highest amount of transformants were showed on YPDS agar containing 100 µg/ml zeocin. The transformant number decreased when the zeocin concentrations increased. The results of a typical PCR analysis of *Pichia pastoris* transformants

presented as figure IV-1. Lane 1-6, 10, 13, 15, 16, and 19 showed the expected size of insert cloned in *P. pastoris* X-33 (277 bp+1551bp) and the wild type *AOX1* gene (2.2 kb).

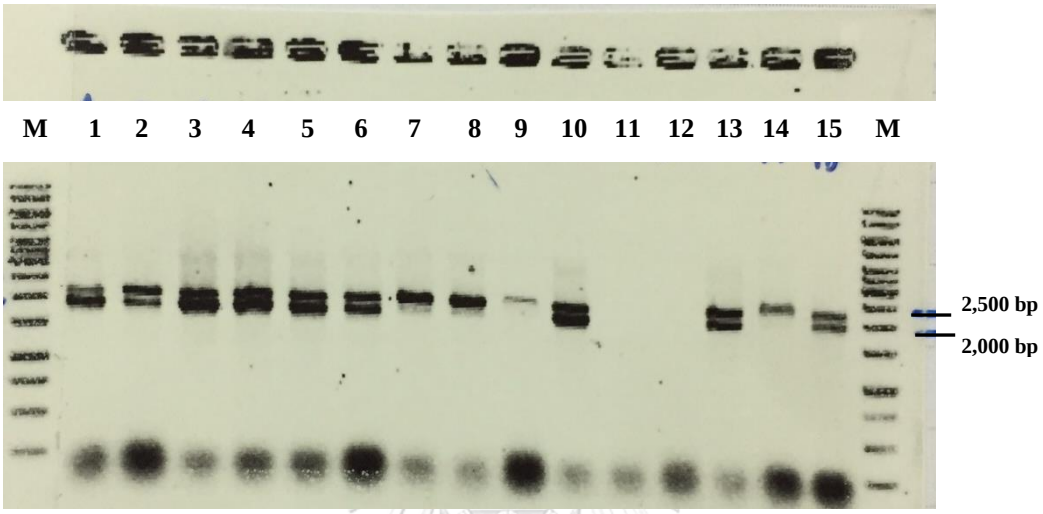


Figure IV- 1 The results of a typical PCR analysis of Pichia pastoris transformants.

Expression of CYP52A17 in *P. pastoris*

Expression levels of rCYP52A17 in *P. pastoris* were tested in BMMY medium and induced daily with 0.5% methanol for inducible expression. CO-differential spectral assay with sodium dithionite was used as a method to determine the concentration of cytochrome P450. This finding indicated that rCYP52A17 failed to express when TTG codons were translated as leucine. Therefore, 2 TTG codon were modified to TCT that translated as serine, yielding rCYP52A17mut, according to CYP52A17 gene in this study was optimized from CYP52A17 of *C. tropicalis* ATCC 20336. Almost species of the genus *Candida* have been reported to modify the universal genetic code which CUG codon encodes serine instead of leucine. So, the

universal leucine CUG codon in *C. tropicalis* is also translated as serine (Blandin et al., 2000). CYP52A17 gene containing CUG codon must confirm having correct amino acid sequence (Eschenfeldt et al., 2003). CUG codons of *Candida* species are translated to $tRNA_{CAG}^{ser}$ and CUA, CUC, and CUU codons for a single $tRNA_{IAG}^{Leu}$ (Butler et al., 2009). In addition, CUG codon is not used as a codon in the cellular mRNAs of these *Candida* species (Tuite and Santos, 1996). Brocca et al., (1998) also reported that codons CTG of *C. rugosa* lipase gene were replaced by other universal codons for serine to facilitate the heterologous expression in yeasts (Brocca et al., 1998).

Table IV- 1 Cytochrome P450 content of microsomes

sample(s)	Total protein concentration (mg/mL)	P450 concentration of microsome (μM)	P450 content (nmol/mg of protein)	cytochrome C reductase (μmol/mg/min)
pPICZA-cyp	46.31±2.44	10.36±0.22	0.22±0.08	0.028±0.01
pPICZA-cypmt	35.81±4.81	7.42±0.113	0.21±0.17	0.026±0.01
pPICZA	84.6±11.3			0.026±0.01

CYP52A17mut sequence were changed TTG encoding amino acid 261 and 490 to TCT which encode serine instead leucine. CYP52A17mut gene were cloned into pPICZA resulting pPICZA-CYP52A17mut. Then they were allowed the expression of protein in *P. pastoris* X-33. Cells precultured to logarithmic phase and were shifted to BMMY medium after that harvested after16-24 h. Microsomes were prepared from these cells and the P450 contents were determined by the CO-difference spectra using the microsomes. No any peak was observed at 450 nm in the microsomes prepared from cells harboring a vector lacking a CYP52A17mut gene as a control (data not shown). The results showed that there was almost no difference of P450 content and cytochrome C reductase (Table IV-1) between *P. pastoris* /pPICZA-CYP52A17mut and *P. pastoris*/pPICZA-CYP52A17. *P. pastoris* does not coexpress with any CPR gene so it must show less CPR activity. Normally, P450s catalyze the introduction of one atom of molecular oxygen into substrate molecules whereas the other oxygen atom is reduced to water. For their catalytic activity, they require electrons which are transfer from NAD(P)H and transferred via various redox partner

proteins. Most eukaryotic P450s obtain electrons from a FAD- and flavin mononucleotide (FMN) containing cytochrome P450 reductase (CPR) (Urlacher and Girhard, 2012). Although microsome of *P. pastoris* /pPICZA-CYP52A17 and *P. pastoris*/pPICZA-CYP52A17mut were higher P450 content than pFBDncp5A containing CYP52A17, there were less cytochrome c reductase activity (Eschenfeldt et al., 2003).

1.3 Oxidation of Lauric acid by recombinant CYP52A17

Microsomes including the recombinant CYP52A17 were examined the oxidation of lauric acid ability. The quantity of P450 of pPICZA-CYP52A17 and pPICZA-CYP52A17mut in the reactions were 0.53 and 0.49 mg/mg protein, respectively. *P. pastoris*/pPICZA-CYP52A17 and CYP52A17mut presented a few the oxidation ability. Microsome of control (without CYP52A17) did not display any oxidation (data not shown)

The results indicated that there was almost no difference of the activity between the *P. pastoris*/pPICZA-CYP52A17 and CYP52A17mut. Whereas *P. pastoris*/pPICZA-CYP52A17 displaying approximately threefold higher V_{max} and k_{cat} values than the other. Its K_m values was not different (Table IV-2). Eschenfeldt et al., (2003) reported CYP52A17 protein can oxidize myristic acid and oleic acid 14.24 and 3.26 nmol/nmol P450/h, respectively. Moreover, *C. tropicalis* expressing CYP52A17 also catalyzed methyl tetradecanoate to 14-hydroxytetradecanoic acid after 148 h and was obtained a few 1,14-tetradecanedioic acid (Lu et al., 2010).

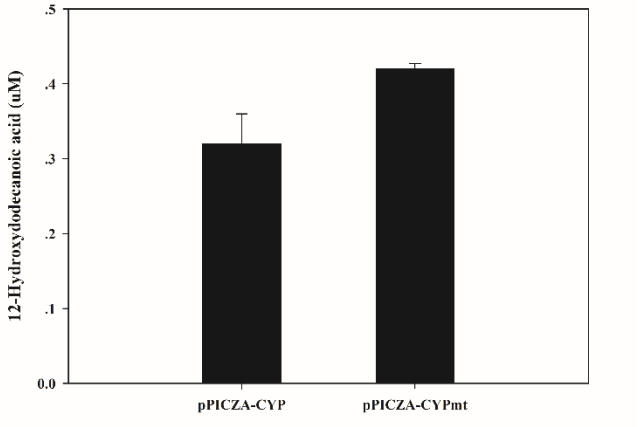


Figure IV- 2 Oxidation of lauric acid to 12-HDDA by microsomes from *P. pastoris* yeasts transformed with rCYP52A17 or rCYP52A17mut. Data are shown as means of three independent experiments and error bars indicate standard deviation.

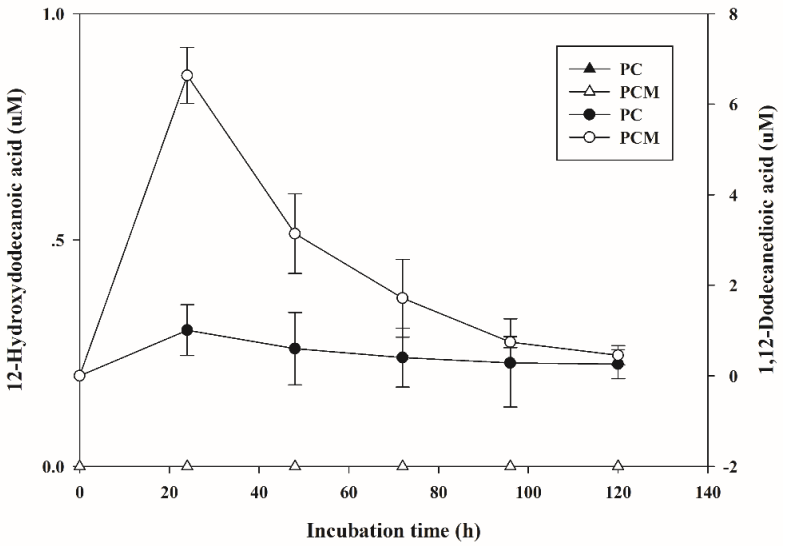
Table IV- 2 Kinetic parameters of the CYP proteins on lauric acid

Protein	V_{max} **(μM /min^{-1})**	K_m **(μM)**	V_{max}/K_m **(min^{-1} μM^{-1})**	k_{cat} **(min^{-1})**	k_{cat}/K_m **(μM^{-1} min^{-1})**
pPICZA-CYP52A17	0.12	50	0.003	1.16	0.023
pPICZA-CYP52A17mut	0.028	60	0.0005	0.38	0.006

1.4 Biotransformation of lauric acid into dodecanedioic acid by the recombinant CYP52A17

Both strains were cultivated in the medium containing lauric acid for determination of the oxidation ability of CYP52A17 (Figure IV-3a, b). The growth of both recombinant *P. pastoris* strains were similar at the beginning stage of cultivation. Recombinant CYP52A17 of *P. pastoris*/pPICZA-CYP52A17 and *P. pastoris*/pPICZA-CYP52A17mut showed low activity. However, the recombinant CYP52A17 from *P. pastoris*/pPICZA-CYP52A17mut accumulated the highest level of 12-hydroxydodecanoic acid, reaching maximum 0.8 µM in 24 h. In fatty acids medium, CYP52A17 gene is transcribed and encoded the known enzyme which oxidize fatty acid to dicarboxylic acids. Moreover, some ω-hydroxyfatty acid can be oxidized by P450 itself (Lu et al., 2010). Oxidation of CYP52A17 have been explained in general (Eschenfeldt et al., 2003). Nevertheless, it did not have special reference for biotransformation of 1,12-dodecanedioic production. Although, there are few studies about CYP52A17 expression. Sathesh-Prabu and Lee, (2015) reported engineered *E. coli* containing engineered CYP153A-NCP produced 1,12-dodecanedioic acid from lauric acid supplementing with co-substrates (glycerol or glucose) (Sathesh-Prabu and Lee, 2015). Furthermore, 56.8 mg/L and 36.0 mg/L of 12-hydroxydodecanoic acid was synthesized in CYP539A7-ScCPR and CYP655C2-ScCPR reconstituted systems (Durairaj et al., 2015). In 2017, Funk and co-workers reported that the whole-cell biotransformation of *C. tropicalis* accumulated 66 g/L of dodecanedioic acid (Funk et al., 2017).

(a)



(b)

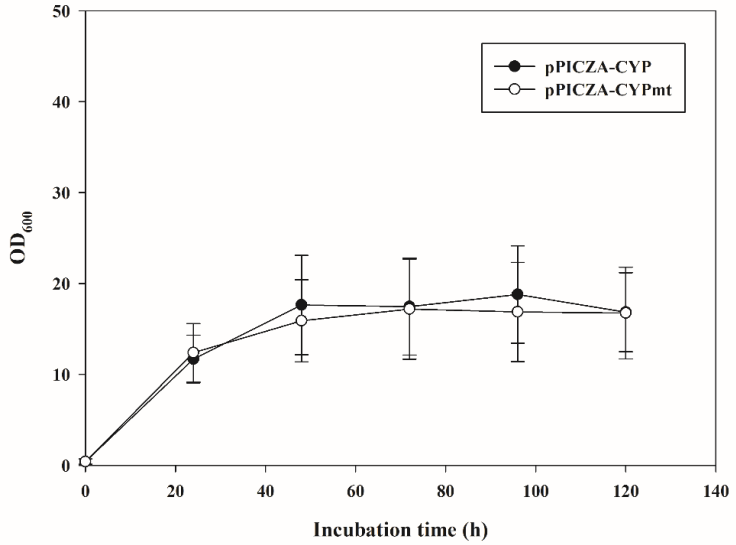


Figure IV- 3 Production of (a) 12-HDDA and 1,12-DDA via biotransformation of rCYP52A17 or rCYP52A17mut into *P. pastoris* X-33 (b) Cell growth, shown as the optical density at 600 nm when the recombinant *P. pastoris* were cultured at 30 ºC, 200 rpm for up to 120 h. Data are shown as means of three independent experiments and error bars indicate standard deviation. The PC and PCM are *P. pastoris* transformed with rCYP52A17 and rCYP52A17mut, respectively.

PART II Cloning and expression of CYP52A17 in *Saccharomyces cerevisiae*

2.1 Cloning of CYP52A17 and expression in *S. cerevisiae* BY(2R) and W(R)

The full-length sequence of CYP52A17 from GenScript (USA) was constructed in the YeDP60 expression vectors prior to transformation into *S. cerevisiae* BY(2R) and W(R) to yield *S. cerevisiae* BY(2R)/YeDP60-CYP52A17 and *S. cerevisiae* W(R)/YeDP60-CYP52A17 and then grown in YPGE medium for microsomal preparation. The reduced CO difference spectrum of the P450 microsomal showed at 450 nm and the expression levels was quite low in the microsomes which prepared from *S. cerevisiae* BY(2R)/YeDP60-CYP52A17 and W(R)/YeDP60-CYP52A17. This result showed that rCYP52A17 was not accomplished to express, when the translation of TTG codons at the position of 261 and 490 were leucine. Therefore, rCYP52A17 was changed to L261S and L490S by site directed mutagenesis (the two TTG codons for leucine were changed to TCT to encode for serine), based upon the codon usage in CYP52A17 of *C. tropicalis* ATCC 20336, to yield CYP52A17mut. Almost all species of the genus *Candida* have been reported to modify the universal genetic code, where the CUG codon is used as serine rather than leucine as a codon in the cellular mRNAs of these *Candida* species (Blandin et al., 2000; Tuite and Santos, 1996). Thus, the CYP52A17 gene containing the CUG codon must confirm to this correct amino acid sequence (Eschenfeldt et al., 2003) where CUG codons of *Candida* species are translated to $tRNA_{CAG}^{ser}$ and CUA, CUC and CUU codons are translated to $tRNA_{IAG}^{Leu}$ (Butler et al., 2009). This confirms the results from Brocca *et al.* which reported that changing the CTG codons in the *C. rugosa* lipase gene to other universal codons for serine to facilitate

heterologous expression in yeasts (Brocca et al., 1998). Ohama et al. examined in the six *Candida* species which CUG is used as serine belong to one distinct group in Hemiascomycetes (Ohama et al., 1993). However, there seems to be no change between the CYP52A17 and CYP52A17mut expression in *P. pastoris* and the average expression level is the same as CYP52A17mut in *S. cerevisiae.*

2.2 Expression of CYP52A17mut in *S. cerevisiae* BY(2R) and W(R)

Table IV- 3 Cytochrome P450 content of microsomes

Sample(s)	**Total protein (mg/mL)**	**P450 concentration of microsome (µM)**	**P450 content (nmol/mg of protein)**	**Cytochrome C reductase (µmol/mg/min)**
BY2R-cyp	20.63±0.74	2.65±1.1	0.13±0.09	0.12±0.01
BY2R-cypmt	44.84±0.8	12.53±1.99	0.28±0.16	0.078±0.01
WR-cyp	42.72±2.8	1.49±0.169	0.031±0.009	0.085±0.047
WR-cypmt	39.90±1.49	9.42±2.7	0.236±0.068	0.101±0.015

BY2R-CYP, BY2R-CYPmut, WR-CYP, and WR-CYPmut are the *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17, *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut, *S. cerevisiae* WR/ pYeDP60-CYP52A17, *S. cerevisiae* WR/ pYeDP60-CYP52A17mut, respectively.

TTG codons of CYP52A17mut (L261S and L490S) sequence were changed to TCT which encode serine instead of leucine, followed by cloning into pYeDP60 resulting pYeDP60-CYP52A17. Then the expression of protein was performed in *S. cerevisiae* BY2R and WR. The recombinant yeast cells were grown in YPGE medium after that harvested at 16-24 h. The yeast microsomes were prepared from these cells

and the P450 were quantified by the absorbance at 450 nm of CO-difference spectra using an extinction coefficient of 91 $mM^{-1}cm^{-1}$. There was none of any peak at 450 nm in the microsomes prepared from yeast cells harboring a vector without a CYP52A17mut gene as a control (data not shown). In contrast, *S. cerevisiae* BY(2R) showed the highest P450 content (nmol/mg of protein) whereas there were no difference of cytochrome C reductase (µmol/mg/min) between *S. cerevisiae* BY(2R) and *S. cerevisiae* W(R) (Table IV-3). P450 content of *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut was about twofold higher than *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17. Microsomes of *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut and W(R)/ pYeDP60-CYP52A17mut showed approximately fourfold higher cytochrome C reductase activity than *P. pastoris* /pPICZA-CYP52A17. Cytochrome C reductase (CPR) is a membrane-bound enzyme that transfer electron from NADPH to molecular oxygen via cytochrome P450 in the endoplasmic reticulum (ER) (de Montellano, 2015). However, in *S. cerevisiae*, the level of increase upon overexpression of the yeast CPR gene is much greater (20X) (Truan et al., 1993). This might indicate a greater level of CPR naturally in *P. pastoris*. In the best case, the cytochrome c activity in BY(2R)and W(R) is 3 times lower than that of the reported expression of NCPA in insect cells (Eschenfeldt et al., 2003). However, the system used in yeast is an integration of one supplementary copy of the yeast CPR gene under the control of the GAL promoter in the chromosome instead of a plasmid in the insect cell expression system. It is therefore possible that the small differences in the reported activities originate from this copy number variation or from a difference between specific activities of the heterologous expressed CPR (*S. cerevisiae* vs. *C. albicans* in the insect cells expression system).

Table IV- 4 Kinetic parameters of the CYP proteins on lauric acid

Protein	V_{max} (μM /min^{-1})	K_m (μM)	V_{max}/K_m (min^{-1} μM^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
BY2R-CYP52A17	0.43	50	0.009	15.03	0.3
BY2R-CYP52A17mut	0.59	50	0.012	4.72	0.09

2.3 Oxidation of Lauric acid by recombinant CYP52A17

Microsomes of the recombinant CYP52A17 were determined the oxidation of lauric acid. The recombinant CYP52A17 enzyme from *S. cerevisiae* BY(2R) and W(R) can oxidize lauric acid to 12-hydroxydodecanoic acid (Figure IV-3), but recombinant CYP52A17 enzyme from *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut showed the highest activity about 11 μM of 12-hydroxydodecanoic acid whereas *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17 was about 4 μM. *S. cerevisiae* W(R)/ pYeDP60-CYP52A17 and CYP52A17mut showed lower oxidation ability than the recombinant *S. cerevisiae* BY(2R). Thus, only the recombinant *S. cerevisiae* BY(2R) were used to perform the further studies.

The quantity of P450 of BY2R-CYP and BY2R-CYPmut in the reactions were 0.33 and 0.66 mg/mg protein, respectively. However, the amount of P450 of BY2R-CYPmut were twice higher than BY2R-CYP that may be encourage its higher activity. The results indicated more clearly the higher activity of the recombinant CYP52A17 enzyme from *S. cerevisiae* BY(2R). There was no difference of K_m, V_{max} and V_{max}/K_m value between the rCYP52A17 and rCYP52A17mut of *S. cerevisiae*. Whereas the rCYP52A17 of *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17 presented

three times higher than the other. The rCYP52A17mut of *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut oxidized lauric acid at 4.1- to 23-fold faster (V_{max} values) than the others, with a K_m value that was equal to that for CYP52A17 in *S. cerevisiae*. Although *S. cerevisiae* BY2R (yeast overexpressing CPR) and *Pichia* showed that there was no difference of P450 content, yeast overexpressing CPR showed higher CPR activity than the others. CPR plays an important role in supporting electron transfer from CYP to P450 (Van Bogaert et al., 2007). Consequently, the oxidation of lauric of *S. cerevisiae* BY2R is better than *Pichia*. P450 of *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17 presented higher k_{cat} and k_{cat}/K_m value than *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut but its oxidation activity was lower than *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut. In contrast, *S. cerevisiae* BY(2R) expressing CYP52A17 displayed higher turnover numbers and k_{cat}/K_m than the recombinant *Pichia*. These results confirmed their oxidation ability in Figure IV-2, 4. Whereas, Eschenfeldt et al. reported that CYP52A17 oxidizes myristic and oleic acids at 14.24 and 3.26 nmol/nmol P450/h, respectively. Huang et al. indicated that K_m values of recombinant CYP52M1 for oleic, linoleic, and arachidonic acids were 40 µM, 68 µM, and 46 µM, respectively (Eschenfeldt et al., 2003; Huang et al., 2014).

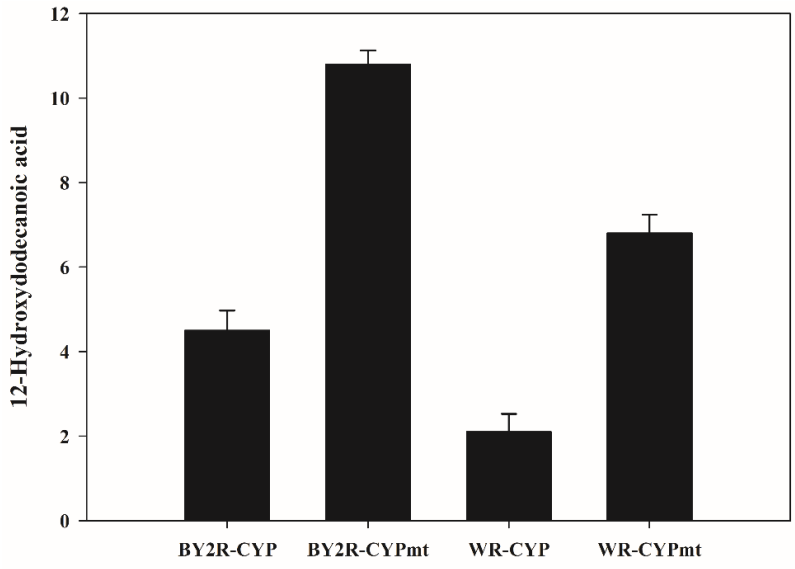


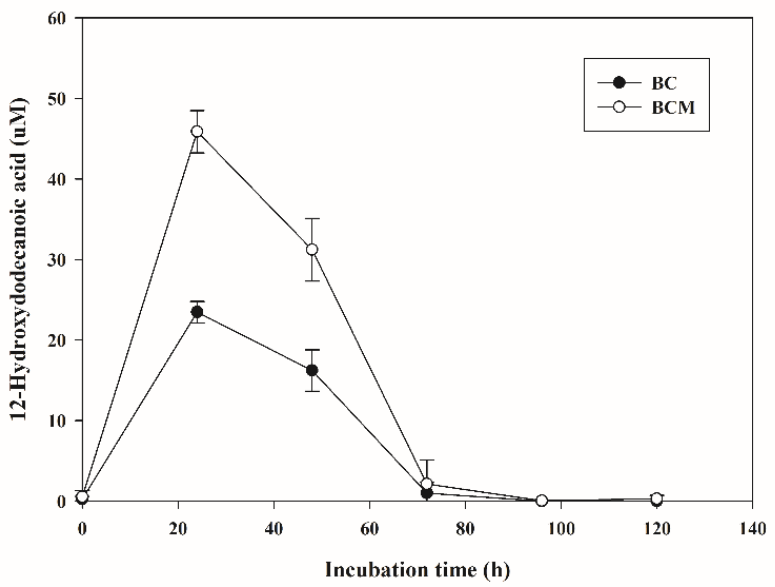
Figure IV- 4 Oxidation of lauric acid to 12-HDDA by microsomes from S. cerevisiae yeasts transformed with rCYP52A17 or rCYP52A17mut. BY2R-CYP, BY2R-CYPmut, WR-CYP, and Wr-CYPmut are the S. cerevisiae BY(2R)/ pYeDP60-CYP52A17, S. cerevisiae BY(2R)/ pYeDP60-CYP52A17mut, S. cerevisiae WR/ pYeDP60-CYP52A17, S. cerevisiae WR/ pYeDP60-CYP52A17mut, respectively. Data are shown as means of three independent experiments and error bars indicate standard deviation.

2.4 Biotransformation of 1,12-dodecanedioic acid by recombinant *S. cerevisiae*

Both strains were cultivated in the medium complement with lauric acid as a substrate for determination of the oxidation ability of CYP52A17 (Figure IV-5a, b). The cell biomass of *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut was nearly identical to that of *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17 on days 2-6 (Figure IV-6). However, *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut cells grew better than *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17 cells at the early of fermentation, obtaining high biomass in just 1 day. Conversely, the growth of

recombinant *P. pastoris* X-33 strains were slightly retarded when compared to both recombinant *S. cerevisiae* BY(2R) strains, especially at the beginning stage of cultivation. Recombinant CYP52A17 of *P. pastoris*/pPICZA-CYP52A17 and *P. pastoris*/pPICZA-CYP52A17mut showed a low activity. Whereas the recombinant CYP52A17 from *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut accumulated the highest level of 12-hydroxydodecanoic acid, up to 45 µM in 24 h. The recombinant *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut oxidized initially 12-hydroxydodecanoic acid corresponding to 1,12-dodecanedioic acid, reaching maximum 21.3 µM in 72 h. In contrast, the recombinant *P. pastoris*/pPICZA-CYP52A17 showed less concentration of 12-hydroxydodecanoic acid and 1,12-dodecanedioic acid than the others. CYP52A17 gene can be transcribed and encoded the known enzyme which oxidize fatty acid to DCAs when fatty acids appear in the medium. Moreover, CYP52A17 is strongly induced by long -chain fatty (Funk et al., 2017). Some ω-hydroxyfatty acid can be oxidized by P450 itself (Lu et al., 2010). Although there are few studies about CYP52A17 expression, *C. tropicalis* strains DP428 carrying CYP52A17 produced over 160 g/L 14-hydroxytetradecanoic acid (Lu et al., 2010). Werner et al., (2017) reported that Cgu *CYP52A12* was overexpress in *C. guilliermondii* and showed an increase in DCA production after 72 h (Werner et al., 2017) The engineered *E. coli* expressing CYP153A-NCP produced 1,12-dodecanedioic acid from lauric acid adding glycerol or glucose as cosubstrates (Sathesh-Prabu and Lee, 2015). CYP539A7-ScCPR and CYP655C2-ScCPR reconstituted systems can produced 12-hydroxydodecanoic acid 56.8 and 36.0 mg/L, respectively (Durairaj et al., 2015).

(a)



(b)

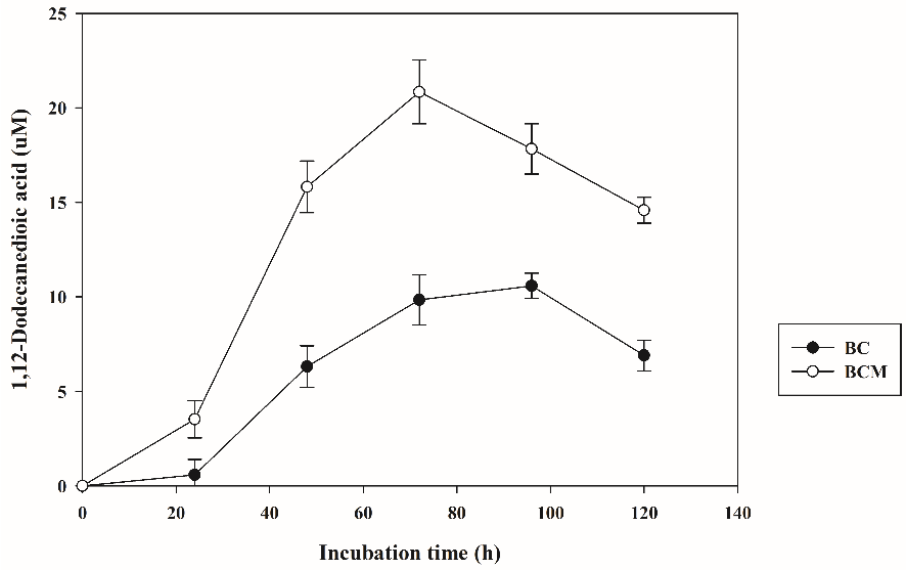


Figure IV- 5 Production of (a) 12-HDDA and (b) 1,12-DDA via biotransformation of rCYP52A17 or rCYP52A17mut into *S. cerevisiae* BY(2R). Data are shown as means of three independent experiments and error bars indicate standard deviation. The BC and BCM are *S. cerevisiae* transformed with rCYP52A17 and rCYP52A17mut, respectively.

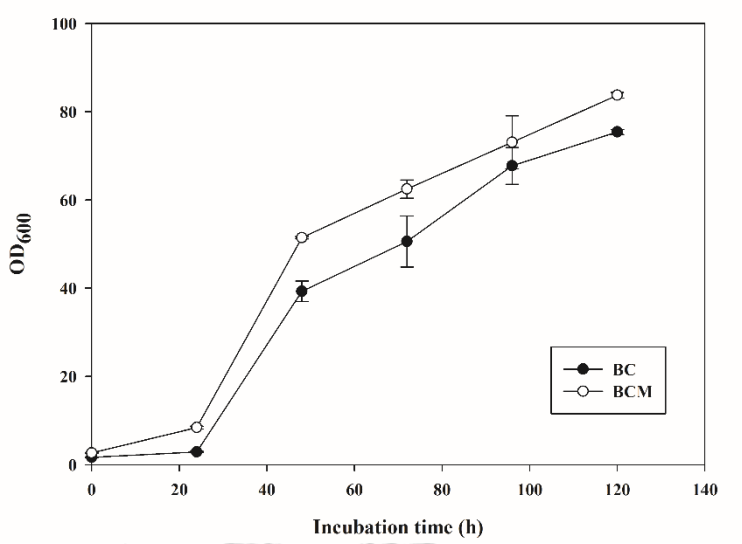


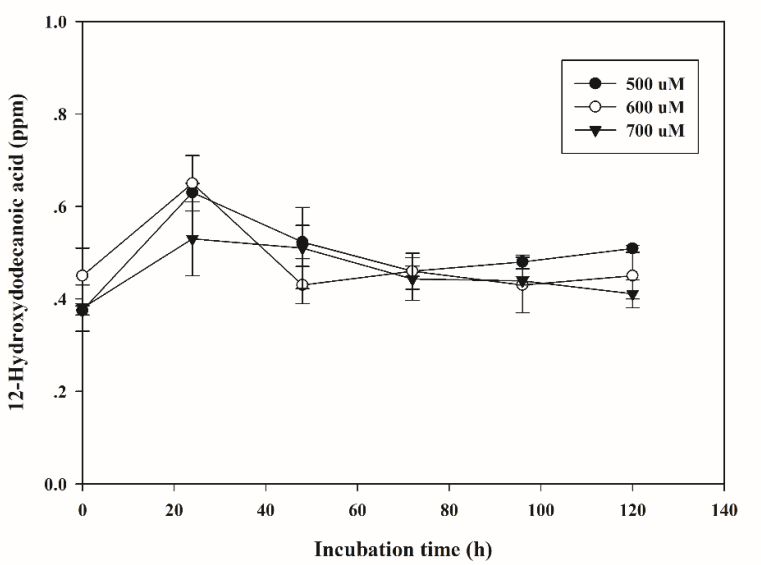
Figure IV- 6 Cell growth, shown as the optical density at 600 nm, of the *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17 and *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut cultures when cultured at 30 ºC, 200 rpm for up to 120 h. Data are shown as means of three independent experiments and error bars indicate standard deviation.

2.5 Effect of lauric acid concentration on biotransformation

The *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut were used to produce dodecanedioic acid (DDA) with up to 700 µM concentrations of lauric acid (C12) as a substrate. After the addition of lauric acid, the growth was not different between the rCYP52A17mut cultures which were cultured in YPGE containing 500 and 600 µM. However, only the grown cultures in the medium adding 700 µM of lauric were slightly slower than the others (Figure IV-8). In biotransformation, the results presented in Figure IV-6a, b 500 µM of lauric acid was the optimum concentration for biotransformation, producing the maximum DDA in 48 h of biotransformation. Although the lauric concentrations were increased, the HDDA production does not affect much and there was no

significant variation in the production. In *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut, the presence of 500 µM of lauric in medium produced 16.9% and 31.76% more DDA than in the presence of 600 and 700 µM of lauric acid, respectively during 48 h of biotransformation (Figure IV-7). The product formation was almost similar for 500-700 µM lauric concentrations, maybe because of the *Km* value of the enzyme being 50 µM, and then an increase in the substrate concentrations does not greatly have an effect on the product formation. These results support the reports of Jeon et al. showing that the increase in the concentration of benzoic acid does not influence the formation of 4-hydroxybenzoic acid by FoCYP53A19 (Jeon et al., 2016).

(a)



(b)

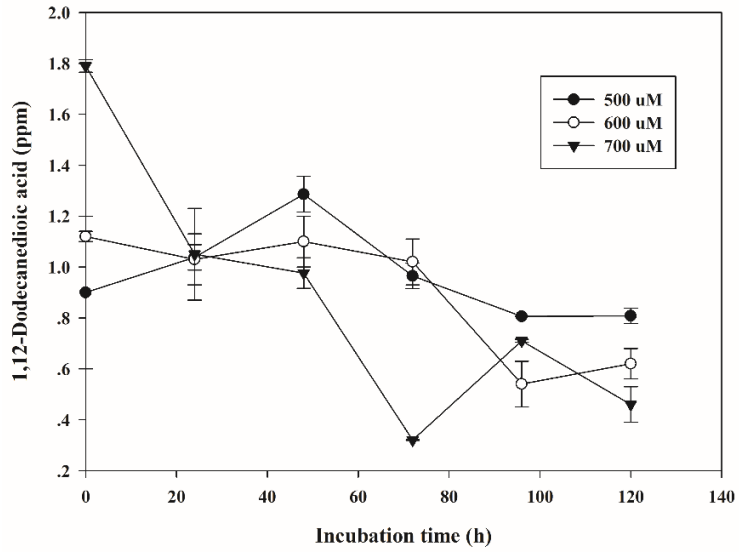


Figure IV- 7 Production of (a) 12-HDDA and (b) 1,12-DDA via biotransformation of rCYP52A17mut into *S. cerevisiae* BY(2R). The cultures of *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut were cultured in YPGE medium containing the difference concentrations of lauric acid as 500, 600, and 700 µM. Data are shown as means of three independent experiments and error bars indicate standard deviation.

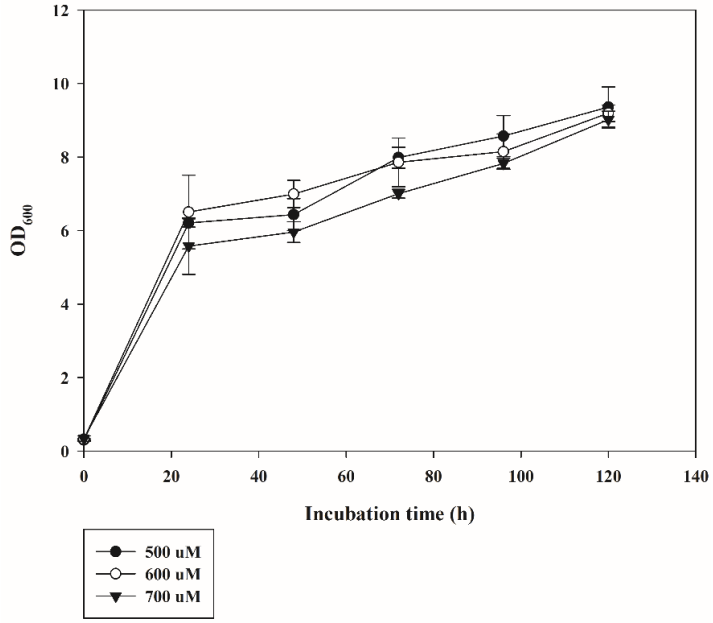


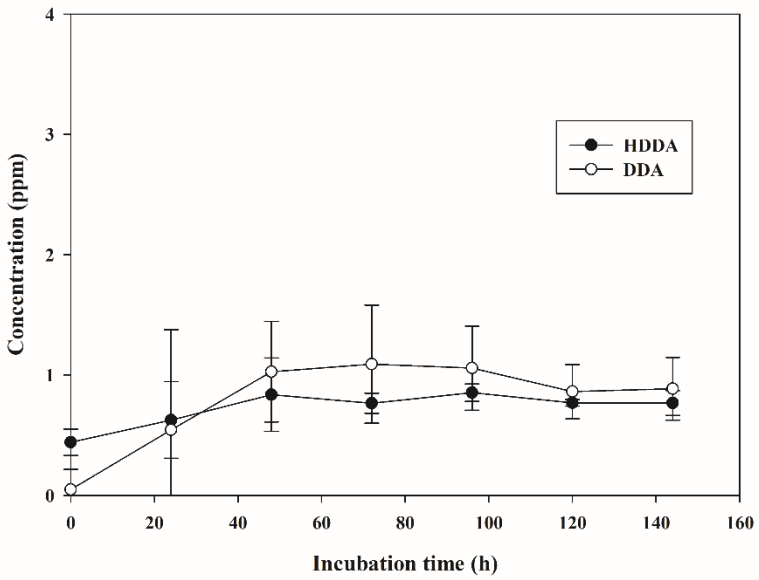
Figure IV- 8 Cell growth, shown as the optical density at 600 nm, of the *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut cultures when cultured in YPGE medium adding 500, 600, and 700 µM of lauric acid at 30 ºC, 200 rpm for up to 120 h. Data are shown as means of three independent experiments and error bars indicate standard deviation.

2.6 Biotransformation in 5 L fermenter

To increase productivity, the biotransformations were performed in a 5 L bioreactor, providing potential mixing with an impeller. The experiments were made in *S. cerevisiae* as production host without further strain optimizations. Transportation of the fatty acid into the cells are possibly disrupted by the formation of fatty acid droplets (Jeon et al., 2016). The efficient mixing can be improved fatty acid transport and spreading fatty acid droplets (Sathesh-Prabu and

Lee, 2015). The results showed that the transformation rate was rapid and the level of HDDA and DDA production stabilized after 72 h (Figure IV-9). Nevertheless, the level of HDDA and DDA production were almost similar to the shake flask condition, perhaps due to adding the antifoam during the biotransformation. Moreover, the high agitation speed can produce a lot of foams, then a higher speed cannot be used. Because strong foaming leads to interference of the bioprocess and the loss of the fermentation medium. Moreover, foaming is related to aeration rates and the pH of medium. It is not quite easy task to transfer the shake flask culture conditions to fermenters due to concerns of substrate solubility and foaming during the fermentation. Higher ω-hydroxyfatty acid production can indicate the utilization of higher fatty acid concentrations. In shake flasks used 1 g/L lauric acid in contrast to the bioreactor used at least 5 - 10 g/L lauric acid. Furthermore, To increase higher products, the biotransformation conditions in bioreactor should be optimized to receive higher products (Scheps, 2013).

(a)



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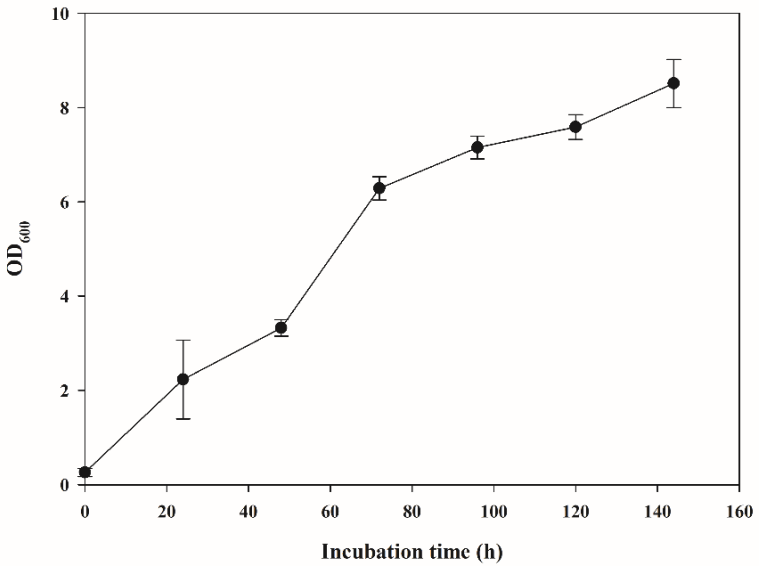

Figure IV- 9 Production of (a) 12-HDDA and 1,12-DDA in 5 L bioreactor by *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut. (b) Cell growth, shown as the optical density at 600 nm. Temperature was maintained at 30 ℃ and agitation speed was maintained at 250 rpm throughout the biotransformation process. Data are shown as means of three independent experiments and error bars indicate standard deviation.

PART III The application of recombinant *Saccharomyces cerevisiae* in dodecanedioic acid production from coconut milk wastewater

3.1 Characteristics of coconut milk wastewater used in this sample

Free fatty acids were determined according to AOACS (2009) Aa 6-38 method and reducing sugar were analyzed by AOAC (2016) 968.28 method. The basic analysis of the coconut milk wastewater used in this study is summarized in Table IV-5, where it clearly had free fatty acids, oil and grease content.

Table IV- 5 Initial characteristics of the coconut milk wastewater used in this study.

Parameter (mg/l)	Value	Method
pH	5.5	
TS	5,836	
TSS	5,270	the standard methods
TKN	83.6	(APHA et al., 2012)
VS	5,172	
Oil and grease	1,994	
BOD	3,516	
COD	7,982	
Sulfate	1	
Total phosphorus	1.31	
Copper	0.06	
Calcium	3.24	
Magnesium	19.3	
Reducing sugar (%)	0.02	
Free fatty acids (%)	0.21	

TS, total solids; TSS, total suspended solids; VS, volatile solids; BOD, biochemical oxygen demand, COD; chemical oxygen demand; TKN, total Kjeldahl Nitrogen.

The coconut milk wastewater used in this study which contained 1,994 mg/l of oil and grease and 0.21% of free fatty acids (Table IV-5), was sterilized at 121 ℃ for 15 min and used as a substrate for this biotransformation.

3.2 Dodecanedioic acid production from coconut milk wastewater using the recombinant *S. cerevisiae*

Biotransformation of fatty acids from renewable resources to specialty chemical is an interesting and attractive opportunity for industry. Oils and fatty acids from wastewater and sustainable resources could be able to use for dodecanedioic acid production (Sathesh-Prabu and Lee, 2015). In this study, coconut milk wastewater was used as a substrate to produce dodecanedioic acid using the recombinant *S. cerevisiae*. The main purpose of using coconut milk wastewater was this wastewater contained oil and various fatty acids, especially lauric acid. Lu and co-workers showed that recombinant cytochrome P450 can oxidize lauric acid and synthesize dodecanedioic acid from cost-effective renewable plant oils (Lu et al., 2010).

From the previous studies, BY(2)R /pYeDP60 -CYP52A17mut showed the highest P450 content of 0.314 nmol/mg of protein that is higher than the P450 content produced *P. pastoris*/pPICZA-CYP52A17mut (0.227 nmol/mg of protein). For this reason, the ability of BY(2)R /pYeDP60 -CYP52A17mut to produce dodecanedioic acid from coconut milk wastewater was tested. The results showed that there was almost no difference between growing in YPD and YPGE of yeast cells in the coconut milk wastewater (Figure IV-10). OD_{600} of the cultures were slowly increased in 24 h and afterwards decreased in 72 h. Thus, this yeast was able to use coconut milk

wastewater as a sole nutrient source. Although the coconut milk wastewater has a high level of COD (7,982 mg/l) and oil and grease (1,994 mg/l) concentrations, this recombinant *Saccharomyces* was ably grown in the coconut milk wastewater without adding nutrient supplements. It is possible that this wastewater may have proper nutrients as well as metabolizable sugar and minerals for growth (Table IV-5). Furthermore, the pH of wastewater was 5.5 which is the optimal pH for *S. cerevisiae* cell growth and the biotransformation reaction carried out in pH 5.5 showed a notable increase in the production rate (Durairaj et al., 2015). Whereas, many previous studies have diluted the wastewater for reduction of any unpleasant substances and satisfaction of the nutrient requirements for the microorganism used such as the addition of ammonium nitrate, yeast extract, dipotassium monohydrogen phosphate, and ammonium chloride (Theerachat et al., 2017).

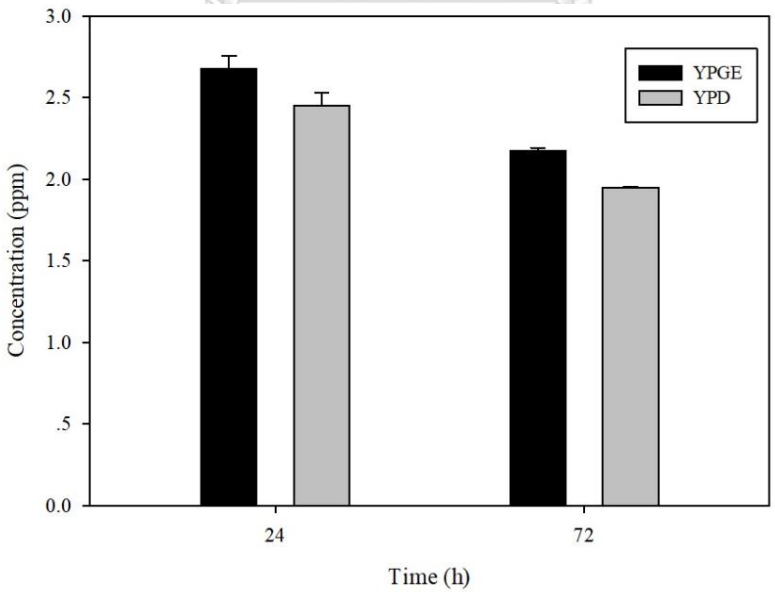

Figure IV- 10 Comparative analysis of biotransformations with recombinant yeast cultured in differential media.

A remarkable bioconversion of coconut milk wastewater using the recombinant *Saccharomyces* was observed in 24 and 72 h of cultivation. Although the growth of yeast cells cultured in YPD were slightly higher than in YPGE, the YPGE cultured cells produced more 12-hydroxydodecanoic acid than YPD cultured cells (Figure IV-9) in 24 h of biotransformation. Furthermore, only the yeast cells were initially cultured in YPGE were able to produce dodecanedioic acid (0.19 ppm) in 24 h. Whereas, culturing the yeast cells initially in YPD media did not show any dodecanedioic acid. Therefore, the recombinant *S. cerevisiae* could be used for the production of dodecanedioic acid from coconut milk wastewater. When growing-cells were cultured in the medium containing glucose, this yeast reconstituted system showed less product conversion due to glucose in the medium affects to the expression of recombinant enzymes and the biotransformation in yeast whole-cell reaction under a galactose promotor (Jeon et al., 2016). This result suggests that glucose and glycerol had repression effects on the induction alkane-fatty acid degrading and DCA producing enzymes in yeasts (Sathesh-Prabu and Lee, 2015).

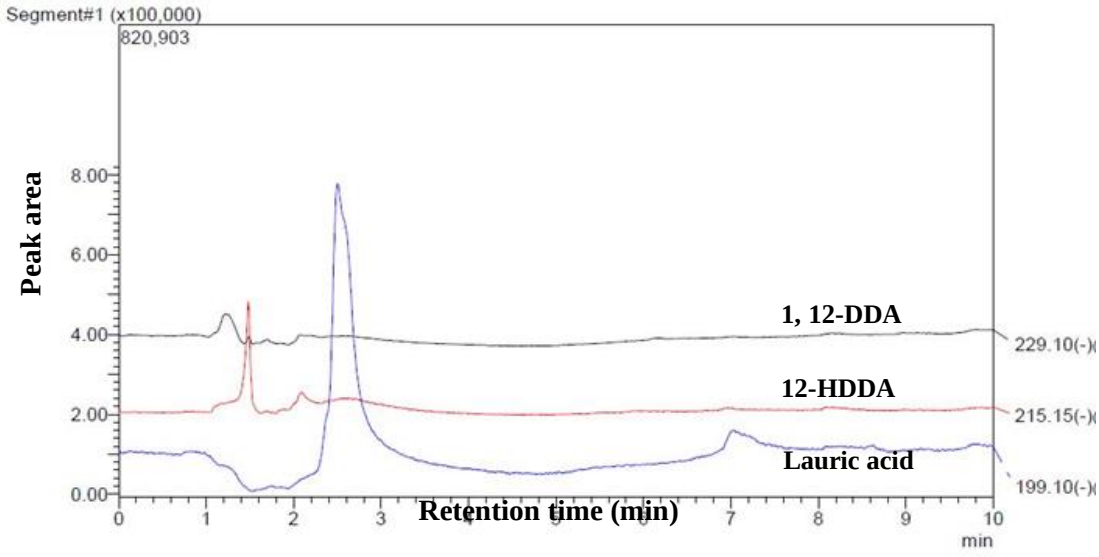

Figure IV- 11 HPLC-MS analysis patterns of different products produced from coconut milk wastewater using *S. cerevisiae* BY(2)R /pYeDP60 -CYP52A17mut. The cultures were incubated at 30 ℃ 200 rpm for 120 h.

CHAPTER V

CONCLUSIONS AND FUTURE PERSPECTIVES

A codon optimized (r)CYP52A17 gene from *C. tropicalis* and its L261S/L490S altered rCYP52A17mut allele were successfully cloned in pPICZ vector and expressed in *P. pastoris*. Recombinant CYP52A17 in an inducible expression system (*P. pastoris*/pPICZ-CYP52A17mut) showed 0.2 nmol/mg of protein of the P450 content. The oxidation activity of rCYP52A17 between the *P. pastoris*/pPICZA-CYP52A17 and CYP52A17mut was not different. *P. pastoris*/pPICZA-CYP52A17mut accumulated the highest level of 12-hydroxydodecanoic acid, reaching maximum 0.8 µM in 24 h.

The rCYP52A17 gene in the pYeDP60 expression vector and its L261S/L490S altered rCYP52A17mut allele were successfully expressed in *S. cerevisiae*. The expressed rCYP52A17mut exhibited a higher production of ω-hydroxy fatty acids and DCAs than the rCYP52A17, and so could be a good candidate for application in the production of α, ω-DCAs, including 1,12-DDA. The rCYP52A17 from *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut accumulated the highest level of 12-HDDA, up to 45 µM in 24 h. The recombinant *S. cerevisiae* BY(2R)/ pYeDP60-CYP52A17mut oxidized initially 12-HDDA corresponding to 1,12-DDA, reaching maximum 21.3 µM in 72 h. The optimal lauric concentration for the biotransformation was 500 µM. The level of HDDA and DDA production in bioreactor were almost similar to the shake flask cultivation. The yeast cells were initially cultured in YPGE medium were able to produce the highest dodecanedioic acid from coconut milk wastewater in 24 h.

S. cerevisiae BY(2R)/pYeDP60-CYP52A17mut is expected to be useful for application in dodecanedioic production. It may be possible to develop this organism into an aerobic wastewater treatment system. *S. cerevisiae* BY(2R)/pYeDP60-CYP52A17mut can be used as a whole-cell biocatalyst in the system without CYP52A17 separation step from yeast cell before using. It is better than the use of CYP52A17 alone in the production.

The recombinant *S. cerevisiae* can grow and produce CYP52A17 simultaneously in wastewater. For the future research, the optimum cultivation conditions and low-cost medium should be studied to reduce the cost of dodecanedioic acid production and fatty acid degradation in wastewater. In addition, fatty acid-containing wastewater from restaurants should be applied in the experiment to analyze other parameters. The co-cultivation of recombinant *S. cerevisiae* expressing CYP52A17 with other microorganisms having ability to produce other enzymes like lipase, protease, amylase, and cellulase is an interesting aspect for application in wastewater pretreatment in the future, potentially, dodecanedioic production.

APPENDIX

Candida tropicalis CYP52A17 ORF from the GenBank sequence AY230504.1. In bold and red are the leucine residues that were modified to serine.

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   1  atgattgaacaactcctagaatattggtatgtcgttgtgccagtgttgtacatcatcaaa   60
   1  M  I  E  Q  L  L  E  Y  W  Y  V  V  V  P  V  L  Y  I  I  K    20
  61  caactccttgcatacacaaagactcgcgtcttgatgaaaaagttgggtgctgctccagtc  120
  21  Q  L  L  A  Y  T  K  T  R  V  L  M  K  K  L  G  A  A  P  V    40
 121  acaaacaagttgtacgacaacgctttcggtatcgtcaatggatggaaggctctccagttc  180
  41  T  N  K  L  Y  D  N  A  F  G  I  V  N  G  W  K  A  L  Q  F    60
 181  aagaaagagggcagggctcaagagtacaacgattacaagtttgaccactccaagaaccca  240
  61  K  K  E  G  R  A  Q  E  Y  N  D  Y  K  F  D  H  S  K  N  P    80
 241  agcgtgggcacctacgtcagtattcttttcggcaccaggatcgtcgtgaccaaagatcca  300
  81  S  V  G  T  Y  V  S  I  L  F  G  T  R  I  V  V  T  K  D  P   100
 301  gagaatatcaaagctattttggcaacccagtttggtgatttttctttgggcaagaggcac  360
 101  E  N  I  K  A  I  L  A  T  Q  F  G  D  F  S  L  G  K  R  H   120
 361  actctttttaagcctttgttaggtgatgggatcttcacattggacggcgaaggctggaag  420
 121  T  L  F  K  P  L  L  G  D  G  I  F  T  L  D  G  E  G  W  K   140
 421  cacagcagagccatgttgagaccacagtttgccagagaacaagttgctcatgtgacgtcg  480
 141  H  S  R  A  M  L  R  P  Q  F  A  R  E  Q  V  A  H  V  T  S   160
 481  ttggaaccacacttccagttgttgaagaagcatattcttaagcacaagggtgaatacttt  540
 161  L  E  P  H  F  Q  L  L  K  K  H  I  L  K  H  K  G  E  Y  F   180
 541  gatatccaggaattgttctttagatttaccgttgattcggccacggagttcttatttggt  600
 181  D  I  Q  E  L  F  F  R  F  T  V  D  S  A  T  E  F  L  F  G   200
 601  gagtccgtgcactccttaaaggacgaatctattggtatcaaccaagacgatatagatttt  660
 201  E  S  V  H  S  L  K  D  E  S  I  G  I  N  Q  D  D  I  D  F   220
 661  gctggtagaaaggactttgctgagtcgttcaacaaagcccaggaatacttggctattaga  720
 221  A  G  R  K  D  F  A  E  S  F  N  K  A  Q  E  Y  L  A  I  R   240
 721  accttggtgcagacgttctactggttggtcaacaacaaggagtttagagactgtaccaag  780
 241  T  L  V  Q  T  F  Y  W  L  V  N  N  K  E  F  R  D  C  T  K   260
 781  ctggtgcacaagttcaccaactactatgttcagaaagctttggatgctagcccagaagag  840
 261  L  V  H  K  F  T  N  Y  Y  V  Q  K  A  L  D  A  S  P  E  E   280
 841  cttgaaaagcaaagtgggtatgtgttcttgtacgagcttgtcaagcagacaagagacccc  900
 281  L  E  K  Q  S  G  Y  V  F  L  Y  E  L  V  K  Q  T  R  D  P   300
 901  aatgtgttgcgtgaccagtctttgaacatcttgttggccggaagagacaccactgctggg  960
 301  N  V  L  R  D  Q  S  L  N  I  L  L  A  G  R  D  T  T  A  G   320
 961  ttgttgtcgtttgctgtctttgagttggccagacacccagagatctgggccaagttgaga 1020
 321  L  L  S  F  A  V  F  E  L  A  R  H  P  E  I  W  A  K  L  R   340
1021  gaggaaattgaacaacagtttggtcttggagaagactctcgtgttgaagagattaccttt 1080
 341  E  E  I  E  Q  Q  F  G  L  G  E  D  S  R  V  E  E  I  T  F   360
1081  gagagcttgaagagatgtgagtacttgaaagcgttccttaatgaaaccttgcgtatttac 1140
 361  E  S  L  K  R  C  E  Y  L  K  A  F  L  N  E  T  L  R  I  Y   380
1141  ccaagtgtcccaagaaacttcagaatcgccaccaagaacacgacattgccaaggggcggt 1200
 381  P  S  V  P  R  N  F  R  I  A  T  K  N  T  T  L  P  R  G  G   400
1201  ggttcagacggtacctcgccaatcttgatccaaaagggagaagctgtgtcgtatggtatc 1260
 401  G  S  D  G  T  S  P  I  L  I  Q  K  G  E  A  V  S  Y  G  I   420
1261  aactctactcatttggaccctgtctattacggccctgatgctgctgagttcagaccagag 1320
 421  N  S  T  H  L  D  P  V  Y  Y  G  P  D  A  A  E  F  R  P  E   440
1321  agatggtttgagccatcaaccaaaaagctcggctgggcttacttgccattcaacggtggt 1380
 441  R  W  F  E  P  S  T  K  K  L  G  W  A  Y  L  P  F  N  G  G   460
1381  ccaagaatctgtttgggtcagcagtttgccttgacggaagctggctatgtgttggttaga 1440
 461  P  R  I  C  L  G  Q  Q  F  A  L  T  E  A  G  Y  V  L  V  R   480
1441  ttggtgcaagagttctcccacgttaggctggacccagacgaggtgtacccgccaaagagg 1500
 481  L  V  Q  E  F  S  H  V  R  L  D  P  D  E  V  Y  P  P  K  R   500
1501  ttgaccaacttgaccatgtgtttgcaggatggtgctattgtcaagtttgactag        1554
 501  L  T  N  L  T  M  C  L  Q  D  G  A  I  V  K  F  D  *         517
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VITA

NAME	Phawadee Buathong
DATE OF BIRTH	2 October 1987
PLACE OF BIRTH	Ratchaburi, Thailand
INSTITUTIONS ATTENDED	She is a Ph.D. scholarship student in the Royal Golden Jubilee (RGJ) Ph.D. program. She got a Bacherlor's Degree of Science in Biological Science (Second Class Honors) from Faculty of Liberal Arts and Science, Kasetsart University in 2010. Then, she studied in Program in Biotechnology, Faculty of Science, Chulalongkorn University, with financial support from RGJ and French embassy to do a research for one year and a half under co-supervisor of Professor Gilles Truan at Laboratory of Biological system engineering and Bioprocess (LISBP), Institut National des Sciences Appliquées (INSA), Toulouse, France.
HOME ADDRESS	1 Moo 20, Thapa, Banpong, Ratchaburi, 70110
PUBLICATION	Buathong, P., Boonvitthya, N., Truan, G., and Chulalaksananukul, W. 2018. Biotransformation of lauric acid into 1,12-dodecanedioic acid using recombinant cytochrome P450 expressed in Saccharomyces cerevisiae. (submitted in RSC Advances) Poster: Buathong, P., Boonvitthya, N., Truan, G., and Chulalaksananukul, W. 2017. Production of recombinant microbial cytochrome P450 in Pichia pastoris and Saccharomyces cerevisiae. RGJ. -PH.D. Congress 18 The Thailand Research Fund, Nonthaburi, 8-10 July 2017. Thailand.