THE CONSTRUCTION OF *PICHIA PASTORIS* TO PRODUCE POLYUNSATURATED FATTY ACIDS (PUFAs)

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การสร้าง *Pichia pastoris* ให้ผลิตกรดไขมันที่มีความไม่อิ่มตัวสูง

นางสาวโชติกา โกศัลวิตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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โชติกา โกศัลวิตร : การสร้าง *Pichia pastoris* ให้ผลิตกรดไขมันที่มีความไม่อิ่ม ตัวสูง (THE CONSTRUCTION OF *PICHIA PASTORIS* TO PRODUCE POLYUNSATURATED FATTY ACIDS (PUFAs) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 79 หน้า

กรดไขมันที่มีความไม่อิ่มตัวสูง (polyunsaturated fatty acids; PUFAs) ถูกสร้างขึ้นโดยอาศัยเอนไซม์ desaturase และ elongase หลายชนิด เช่น Δ5 desaturase (Δ5) Δ6 desaturase (Δ6) Δ5 elongase (E5) และ Δ6 elongase (E6) เอนไซม์ทั้งสองกลุ่มนี้ พบได้ในสัตว์มีกระดูกสันหลังทั่วไปรวมถึงปลาด้วย ในการศึกษานี้ได้ทำการสกัดยีน Δ6 จากปลานิล และยีน Δ6&Δ5 E5 และ E6 จากปลาม้าลายแล้วนำเข้าสู่พลาสมิด pGAPZ เพื่อสร้างพลาสมิดลูกผสม (pGAPZ:Δ6 pGAPZ: Δ6&Δ5 pGAPZ:E5 และ pGAPZ:E6) นอกจากนี้ยังได้สร้างพลาสมิดลูกผสมที่มีการแสดงออกของสองยีน คือ Δ6&Δ5 และ E6 (pGAPZ:Δ6&Δ5:E6) พลาสมิดลูกผสมทั้งหมดถูกถ่ายโอนเข้าสู่เซลล์แบคทีเรีย *Escherichia coli* แล้วสกัดออกมาเพื่อทำการบ่งชี้ลักษณะที่สำคัญของเอนไซม์ในกลุ่ม desaturase และ elongase จากผลการวิเคราะห์พบบริเวณสำคัญที่ถูกต้องตรงตามกลุ่มของเอนไซม์ desaturase และ elongase หลังจากนั้นจึงถ่ายโอน พลาสมิดลูกผสมทั้งหมดเข้าสู่เซลล์ยีสต์ *Pichia pastoris* เพื่อศึกษาการสะสมของกรดไขมันที่มีความไม่อิ่มตัวสูง โดยทำการเพาะเลี้ยง *P. pastoris* ลูกผสมเหล่านั้น สกัดกรดไขมัน เปลี่ยนกรดไขมันให้อยู่ในรูปเอสเทอร์ของกรดไขมัน (fatty acid methyl esters; FAMEs) และวิเคราะห์ FAMEs ที่ได้ด้วยแก๊สโครมาโทกราฟี ผลการทดลองชี้ให้เห็นว่า *P. pastoris* ลูกผสมที่มี pGAPZ:Δ6 และ pGAPZ:Δ6&Δ5 มีการสะสม gamma-linolenic acid (GLA, C18:3n-6) ซึ่งไม่พบในกลุ่มควบคุม นอกจากนี้ยังพบว่า *P. pastoris* ลูกผสมที่มี pGAPZ:Δ6&Δ5:E6 มีการสะสมของ EPA และ ARA ด้วย

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา _____

CHOTIKA GOSALAWIT : THE CONSTRUCTION OF *PICHIA PASTORIS* TO PRODUCE POLYUNSATURATED FATTY ACIDS (PUFAs). THESIS ADVISOR : ASSOC. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 79 PP.

PUFAs/DESATURASE/ELONGASE/*PICHIA PASTORIS*/pGAP plasmid

Polyunsaturated fatty acids (PUFAs) are generated by desaturase and elongase enzymes for example, Δ5 desaturase (Δ5), Δ6 desaturase (Δ6), Δ5 elongase (E5), and Δ6 elongase (E6). These enzymes can be found in all vertebrates including fish. In this study, cDNAs of Δ6 from Nile tilapia and Δ6&Δ5 bifunctional, E5, and E6 from zebrafish were amplified and cloned into pGAPZ to generate recombinant expression plasmids (pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E5, and pGAPZ:E6). Moreover, a co-express recombinant plasmid of Δ6&Δ5 and E6 (pGAPZ:Δ6&Δ5:E6) was constructed. All recombinant plasmids were transformed into *Escherichia coli*, and then extracted to identify the important characteristics of the desaturase and elongase enzyme families. The results indicated that the desaturases and elongases cloned contained important regions of their enzyme families. Thereafter, the recombinant plasmids were transformed into *Pichia pastoris*. To study the PUFAs accumulation, the recombinant *P. pastoris* were cultured, and fatty acids were extracted and methylated to form fatty acid methyl esters (FAMEs), which were determined by gas chromatography. The results indicated that recombinant *P. pastoris* containing pGAPZ:Δ6 and pGAPZ:Δ6&Δ5 accumulate gamma-linoleic acid (GLA, C18:3n-6) which is a fatty acid that is not present in the *P. pastoris* containing empty pGAPZ

plasmid. In addition, the EPA and ARA accumulation were detected in recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6

School of Biotechnology Student's Signature _____

Academic Year 2013 Advisor's Signature _____

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Chotika Gosalawit

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LIST OF ABBREVIATIONS

Δ4	=	Δ4 desaturase
Δ5	=	Δ5 desaturase
Δ6	=	Δ6 desaturase
Δ6&Δ5	=	Bifunctional Δ6&Δ5 desaturase
Δ8	=	Δ8 desaturase
Δ9	=	Δ9 desaturase
Δ12	=	Δ12 desaturase
Δ15	=	Δ15 desaturase
Δ17	=	Δ17 desaturase
ALA	=	Alpha-linolenic acid (C18:3n-3)
ARA	=	Arachidonic acid (C20:4n-6)
COX	=	Cyclooxygenase
DGLA	=	Di-homo gamma-linolenic acid (C20:3n-6)
DHA	=	Docosahexaenoic acid (C22:6n-3)
DPA	=	Docosatetraenoic acid (C22:5n-3)
DPAn-6	=	Docosapentaenoic acid (C22:5n-6)
DTA	=	Docosatetraenoic acid (C22:4n-6)
E5	=	Δ5 elongase
E6	=	Δ6 elongase
EDA	=	Eicosadienoic acid (C20:2n-6)
ELOVL	=	Elongation of very long chain fatty acid

LIST OF ABBREVIATIONS (Continued)

EPA	=	Eicosapentaenoic acid (C20:5n-3)
ERA	=	Eicosatrienoic acid (C20:3n-3)
ETA	=	Eicosatetraenoic acid (C20:4n-3)
FAMEs	=	Fatty acid methyl esters
FAS	=	Fatty acid synthase
GLA	=	Gamma-linolenic acid (C18:3n-6)
LA	=	Linoleic acid (C18:2n-6)
LOX	=	Lipoxygenase
OA	=	Oleic acid (C18:1n-9)
PGE	=	Prostaglandin
PUFAs	=	Polyunsaturated fatty acids
STA	=	Stearodonic acid (C18:4n-3)
THA	=	Tetracosahexaenoic acid (C24:6n-3)
TPA	=	Tetracosapentaenoic acid (C24:5n-3)

CHAPTER I

INTRODUCTION

1.1 Background

Polyunsaturated fatty acids (PUFAs) are long chain fatty acid that contained more than one double bonds in their backbones. PUFAs play important roles in human health such as reduced cardiovascular diseases, reduced rheumatoid arthritis (Justi et. al., 2003) including other inflammatory conditions, pulmonary disorder, and some neuropsychiatric disorder (Bourre, 2004). PUFAs can be classified into two groups, for example, omega-3 and omega-6 PUFAs. The omega-3 PUFAs contain long chain of carbon atoms with the first double bond at the third carbon position from the methyl end. The omega-6 PUFAs contain the first double bond at the sixth carbon position from the methyl end. Alpha-linolenic acid or ALA (C18:3n-3), eicosapentanoic acid or EPA (C20:5n-3), and docosahexanoic acid or DHA (C22:6n-3) are important forms of omega-3 PUFAs. Linoleic acid or LA (C18:2n-6), di-homo gamma-linolenic acid or DGLA (C20:3n-6) and arachidonic acid or ARA (C20:4n-6) are important forms of omega-6 PUFAs. The biosynthesis of PUFAs from LA to ARA and ALA to both EPA and DHA catalyzed by the Δ6 desaturase (Δ6) and Δ5 desaturase (Δ5) to add double bond at the Δ6 and Δ5 positions. In addition, elongases are enzymes that add two carbon atoms into the fatty acid chains. These enzymes can be found in various vertebrates including fish. Freshwater fish has been shown to have higher expression level of these enzymes than marine fish due to their diets.

However, all vertebrates lack Δ12 desaturase (Δ12) and Δ15 desaturase (Δ15) activities. Thus vertebrates are unable to synthesize the substrate LA and ALA (Hasting et al., 2001). Therefore, LA and ALA have to be obtained from their diets.

Pichia pastoris is not only an efficient host for expression of heterologous proteins but it is also easily grown to high cell density. This yeast also has been used in molecular research for production of PUFAs since it contains endogenous substrates LA and ALA. Li et al. (2009) has shown that expressing Δ6, Δ6 elongase (E6), and Δ5 in *P. pastoris* increased the production of stearodonic acid (STA, C18:4n-3), eicosatetraenoic acid (ETA, C20:4n-3) and EPA omega-3 PUFAs. The production of gamma-linolenic acid (GLA, C18:3n-6) from recombinant *P. pastoris* that expressed Δ6 has also been shown by Wan et al. (2009).

It has been reported that the expression of angiostatin under the gylceral-3-phosphate dehydrogenase (GAP) constitutive promoter of the pGAP plasmid showed higher level than in the AOX inducible system (Zhang et al., 2007). Also the expression of recombinant protein under the pGAP system is much easier than that of the inducible system. Therefore, the *P. pastoris* pGAP plasmid was the choice of this study for the production of PUFAs. In the future, the *P. pastoris* with high PUFAs could be used to increase the quality of human diets and animal feed.

In this work, several recombinant *P. pastoris* were constructed. Each type of recombinant yeast contained the gene that involved in PUFAs pathway (Δ6, bi-functional Δ6&Δ5 desaturase (Δ6&Δ5), Δ5 elongase (E5), and E6 genes). The pGAPZ plasmid was used for the recombinant plasmid construction (pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E5, and pGAPZ:E6). Furthermore, another recombinant yeast was constructed to co-express both the Δ6&Δ5 and the E6 (pGAPZ:Δ6&Δ5:E6) for

the production of EPA and ARA. Each type of recombinant *P. pastoris* was cultured and the fatty acid composition was measured by gas chromatography.

1.2 Objectives

1. To clone Δ6 cDNA from Nile tilapia into pGAPZ plasmid.
2. To clone Δ6&Δ5, E5, and E6 cDNAs from zebrafish into pGAPZ plasmid.
3. To clone both the Δ6&Δ5 and the E6 into pGAPZ plasmid.
4. To transform all recombinant plasmids and the pGAPZ empty plasmid (as control) into *P. pastoris*.
5. To determine the PUFAs level of the recombinant *P. pastoris* that contains each type of plasmid (pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E5, pGAPZ:E6 and pGAPZ:Δ6&Δ5:E6).

CHAPTER II

LITERATURE REVIEWS

2.1 Saturated fatty acid and unsaturated fatty acid

2.1.1 Definition and nomenclature

Fatty acids are hydrocarbon chain that contains methyl group at one end and a carboxyl group at the other end (Cockbain et al., 2012). Fatty acids are classified into two classes. There are saturated and unsaturated fatty acids. The saturated fatty acids are fatty acid that contained only single bond in their backbones. These fatty acids cannot absorb anymore hydrogen atom (Figure 2.1). The saturated fatty acids are classified into three groups: (i) short and medium chain saturated fatty acids (C4-C14) found in milk fats and in some vegetable oils; (ii) palmitic (C16) and stearic (C18) acids, the C16 palmitic acid is saturated fatty acid found in fish oils, milk and body fat of land animal, cotton seed oil and palm oil. The C18 stearic acid is the major fatty acid in ruminant animals, and cocoa butter. (iii) long-chain fatty acids are fatty acids that contain more than 18 carbon atoms, i.e. C20-C30 present in waxes (Gunstone, 1996). The unsaturated fatty acids are fatty acid chains that contained one or more than one double bond in their backbones. These fatty acids can absorb more hydrogen atom. The unsaturated fatty acids can be divided into two groups; (i) monounsaturated fatty acids and (ii) polyunsaturated fatty acids (PUFAs). The monounsaturated fatty acids are fatty acids that contain one double bond in their backbones, i.e. oleic acid omega-9 fatty acid (OA, C18:1n-9). The PUFAs are fatty

acids that contain more than one double bonds in their backbones, i.e. alpha-linolenic acid (ALA, C18:3n-3), eicosapentanoic acid (EPA, C20:5n-3) and docosahexanoic acid (DHA, C22:6n-3) which are omega-3 PUFAs and linoleic acid (LA, C18:2n-6), di-homo gamma-linolenic acid (DGLA, C20:3n-6) and arachidonic acid (ARA, C20:4n-6) which are omega-6 PUFAs. (Figure 2.1) (Varakamin, 2008; Gunstone, 1996).

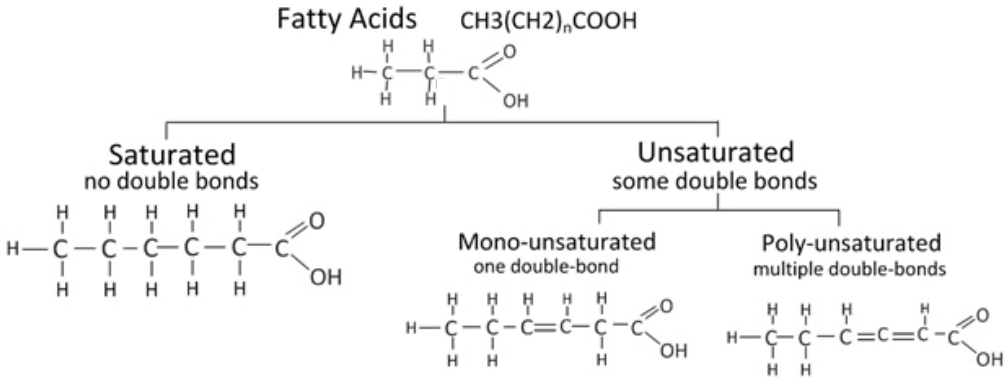

Figure 2.1 The structure of fatty acids: saturated fatty acid, monounsaturated fatty acid, and polyunsaturated fatty acid (http://click4biology.info/c4b/a/a1.htm).

In addition, PUFAs structure can be divided into two types: (i) *cis*-PUFAs and (ii) the *trans*-PUFAs. The *cis*-PUFAs contain hydrogen atoms on the same side of the C=C double bond resulted in bent of the fatty acid chain. These *cis*-PUFAs are normally found in cell membrane casing its fluidity, which benefit for transportation. Whereas, the *trans*-PUFAs contain hydrogen atoms on the opposite side of C=C double bond resulted in straight chain fatty acid (Figure 2.2) (Varakamin, 2008).

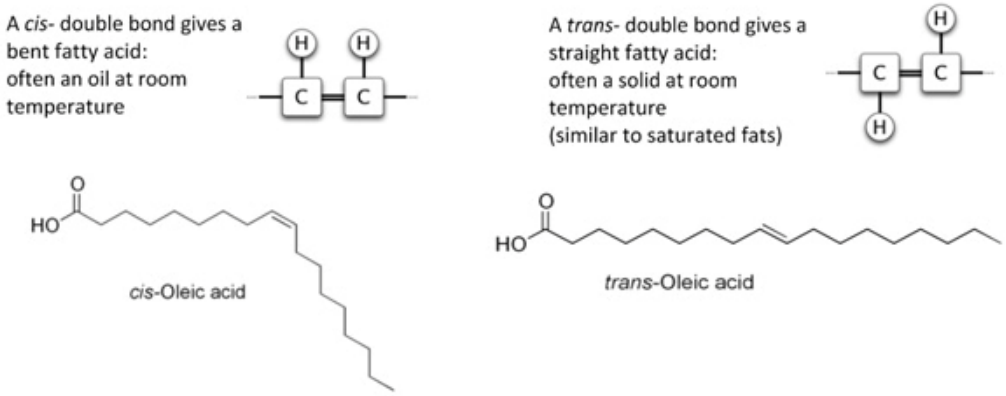

Figure 2.2 The structure of *cis*-PUFAs (left) and *trans*-PUFAs (right) (http:// click4biology.info/c4b/a/a1.htm).

The fatty acid nomenclature is classified base on the principle of fatty acids: (i) carbon chain length; (ii) number and first position of double bonds; and (iii) stereo configuration of the unsaturation. Davidson and Cantrill (1985) reviewed and divided systems for fatty acid nomenclature into four systems: (i) the trivial system; (ii) the systematic method; (iii) the structure system; and (iv) the 'n' and 'ω' system. The first three systems have shown several problems. For example, the trivial system elucidates only the name of the fatty acid but dose not show the structure of the fatty acid. The systematic method identify the number of carbon atom and the number of unsaturation counting from the carboxyl group, e.g. 5, 8, 11, 14-eicosatetraenoic acid. The relative compounds of different chain length and degree of unsaturation due to the elongation that occurred at the carboxyl group made it difficult to recognize. The structural system gives only the carbon and double bond number. Therefore, this system cannot separate between the fatty acids that have the same chain length and number of double bond but different position of double bonds. Therefore, the 'n' and 'ω' system is the simplest and widely used. Both 'n' and 'ω' showed the similarity

related to the position of first double bond from the methyl end. These systems can separate the stereo-isometric species (*cis*- and *trans*-), e.g. *cis*-C20:4n-6 and *cis*-C20:4ω6. Cx:yn-z: X is the number of carbon atom; y is the number of carbon-carbon double bond; and z is the position of the first carbon-carbon double bond counting from the methyl (n) end of the hydrocarbon chain (Cockbain et al., 2012; Calder and Grimble, 2003; Davidson and Cantrill, 1985).

2.1.2 Type of PUFAs

2.1.2.1 Omega-3 PUFAs Omega-3 fatty acids are long chain PUFAs with the first double bond at the 3rd carbon atom from the methyl end. The important forms of omega-3 PUFAs are ALA (C18:3n-3), EPA (20:5n-3) and DHA (22:6n-3) (Tocher, 2010) (Figure 2.3).

ALA is the substrate of omega-3 PUFAs pathway that can be bioconverted to EPA and DHA by desaturase and elongase activities. Both EPA and DHA have been detected at a high level in marine fish such as mackerel and salmon (Asif, 2011) but at a much lower level in plants and vegetable oils. ALA accumulates at a high level in green leaves and oil seeds such as rapeseed oil, soybean oil, flaxseed oil (also known as linseed), and walnut oil (Burdge and Calder, 2005).

2.1.2.2 Omega-6 PUFAs Omega-6 PUFAs contain the first double bond at the 6th carbon atom from the methyl end. The important forms of omega-6 PUFAs are LA (C18:2n-6), DGLA (C20:3n-6) and ARA (20:4n-6) (Figure 2.4). LA is the primary substrate of omega-6 PUFAs production. LA has been found in vegetable oils like soybean, canola oil, and nuts. Both DGLA and ARA could be produced from LA by desaturase and elongase activity (Olga and Napier, 2011).

2.1.3 Important of PUFAs

The PUFAs have revealed several benefits. Especially, *cis*-PUFAs (EPA and DHA) play important role in several functions. For infants, DHA and EPA are required for rapid growth of neural tissue related with cognitive function, visual development and a nutritional supply for development of pre- and postnatal.

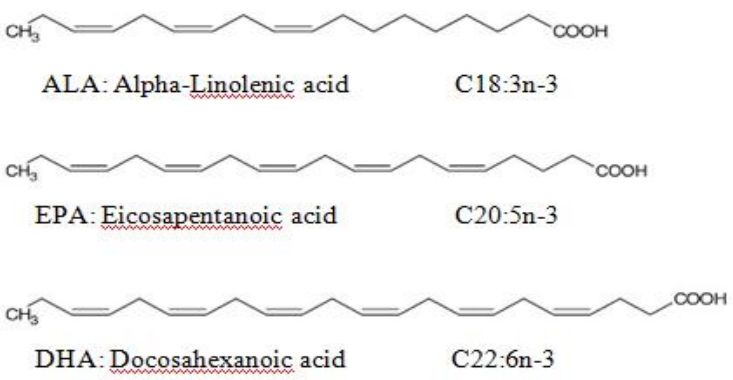

Figure 2.3 The important forms of omega-3 PUFAs; alpha-linolenic acid (ALA, C18:3n-3), eicosapentanoic acid (EPA, C20:5n-3), and Docosahexanoic acid (DHA, C22:6n-3) (Kashiwagi and Huang, 2012).

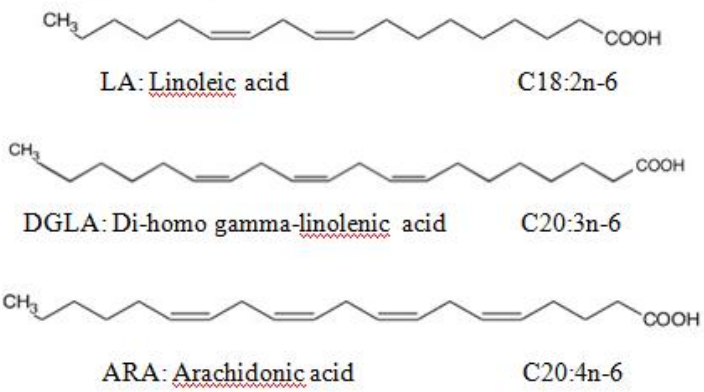

Figure 2.4 The important forms of omega-6 PUFAs; linoleic acid (LA, C18:2n-6), di-homo gamma-liolenic acid (DGLA, C20:3n-6), and arachidonic acid (ARA, C20:4n-6) (Kashiwagi and Huang, 2012).

Moreover, EPA influents the behavior and mood (Kidd, 2007). For adults, they are implicated that it can improve a wide range of clinical pathologies involving kidney, neural tissues (Brenna, 2002), reduce anxiety behavior for postpartum and decrease risk of suicide (Masood et al., 2005; Chen and Su, 2010; Uauy et al., 2001). In addition, previous study showed that used of DHA and EPA can prevent cardiovascular diseases (Cockbain et al., 2012), diabetes mellitus and colitis (Varakamin, 2008). In contrast, all of *trans*-PUFAs, saturated, *cis*-, and *trans*-monounsaturated fatty acid can stimulate the factors, which are responded to increase hypertension, atherosclerosis, and heart disease. Moreover, some omega-6 and omega-3 PUFAs are converted to eicosanoids for example; prostaglandins, prostacyclins, leukotrienes, and thromboxanes, which are important for biochemical and physiological, function of human body (Davidson and Cantrill, 1985). The eicosanoids are generated from 20 carbon atoms PUFAs. The EPA omega-3, DGLA, and ARA omega-6 PUFAs are eicosanoids precursors that can be converted to prostaglandin-E3 (PGE3), prostaglandin-E1 (PGE1), and prostaglandin-E2 (PGE2), respectively. The EPA, DGLA, and ARA can be converted to prostaglandins by cyclooxygenase (COX) (Tapiero et al., 2002). They all can be changed to leukotrienes (LT) by lipoxygenase (LOX) (Figure 2.5) (Calder and Grimble, 2002). ARA is an important precursor of eicosanoid synthesis. It is a major component of the cell membrane when compared with DGLA and EPA. The eicosanoids can activate the inflammatory and immune responses. The eicosanoids derived from EPA and ARA has opposite chemical property. For example, the PGE2 derived from ARA has a pro-inflammatory effect, e.g. induce fever, increase vascular permeability and vasodilation, and enhance pain. Whereas, the PGE3 derived from EPA has an anti-inflammatory activity, which is opposite of the PGE2. Previous studies reported that

the consumption of fish oil, which is rich of EPA, could reduce the production of eicosanoid from ARA by replacing ARA as a eicosanoids substrate (Calder, 2006; James et al., 2000). Thus, the EPA omega-3 PUFAs act as antagonistic. Normally, the suitable omega-6/omega-3 ratio is 1:1 or not over than 2:1. The consumption of omega-6/omega-3 ratio of human are from 20:1 to 50:1, resulting in an increase of eicosanoids derived from ARA. Therefore, the decrease consumption of ARA or increase consumption of EPA can balance between both of ARA-eicosanoids and EPA-eicosanoids (Varakamin, 2008).

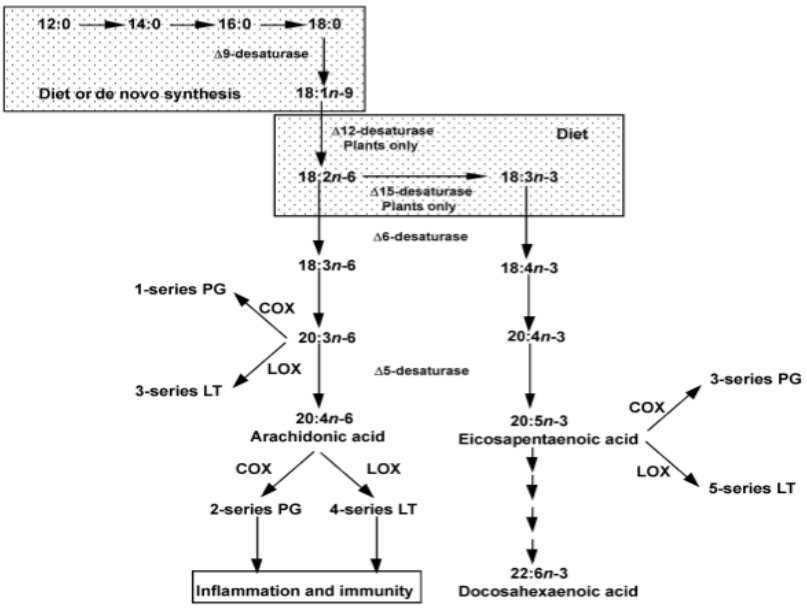

Figure 2.5 The eicosanoids production pathway (Calder and Grimble, 2002).

2.1.4 Omega-3 and omega-6 PUFAs biosynthesis

2.1.4.1 The fatty acid generation and elongation Enzymes for fatty acid biosynthesis are different between prokaryote and eukaryote but the reactions and mechanisms are similar. New fatty acids are synthesized by the activity of fatty acid synthase (FAS) in the cytosol. The first step of this process is condensation of

acetyl-CoA and malonyl-CoA to generated beta-ketoacyl-CoA. The second step, the beta-ketoacyl-CoA is reduced to beta-hydroxyacyl-CoA, which NADPH is required for this step. Thereafter, beta-hydroxyacyl-CoA is dehydrated to enoyl-CoA. The enoyl-CoA is reduced by enoyl-reductase in the fourth step to complete the elongation cycle and generate the elongated acyl-chain (Tehliverts et al., 2007; Jakobsson et al., 2006). The elongated acyl-chain or PUFAs, which are derived from diets, are further elongated in endoplasmic reticulum (ER) by three enzymes showed in figure 2.6 to generate longer chain fatty acids (Jakobsson et al., 2006).

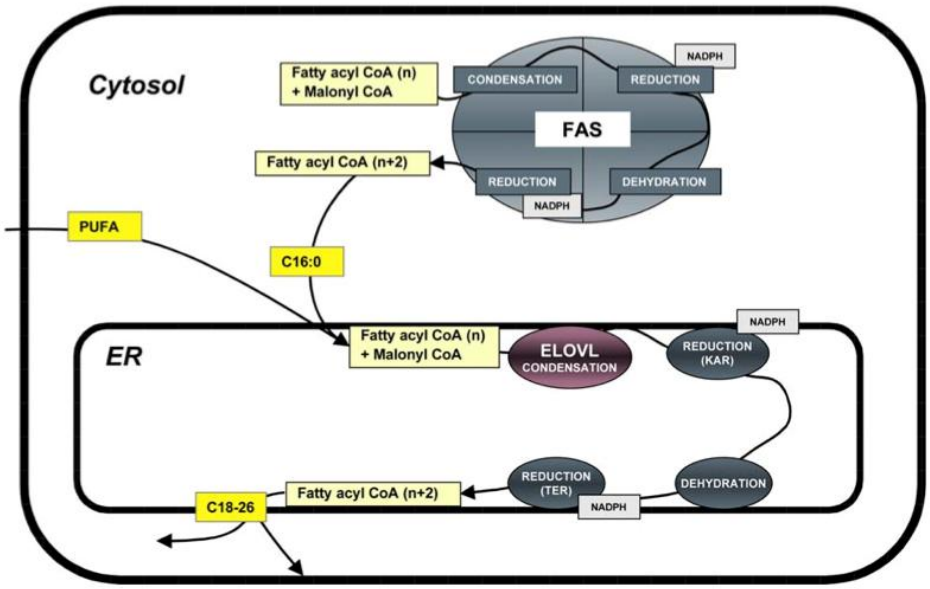

Figure 2.6 Fatty acid and long chain fatty acid synthesis; FAS: fatty acid synthase; ELOVL: elongation of very long chain fatty acid; KAR: 3-ketoacyl CoA reductase; and TER: trans-2,3-enoyl-CoA reductase (Jakobsson et al., 2006).

2.1.4.2 Enzymes involved in omega-3 and omega-6 PUFAs production

Desaturase – Fatty acid desaturases are enzymes that introduced double bond into fatty acid chains to increase the unsaturation of the fatty acid chain. These enzymes are found in all groups of organisms, e.g., bacteria, fungi, plants and animals (Los and Murata, 1998). Base on the insertion of double bonds, the desaturase enzymes are divided into two types; (i) front-end desaturase and (ii) methyl-end desaturase. The front-end desaturase (Δ4 desaturase (Δ4), Δ5 desaturase (Δ5), Δ6 desaturase (Δ6) and Δ8 desaturase (Δ8)) are enzymes that add a double bond between the pre-existing double bond and the carboxyl end of the fatty acid chain. The Δx desatuarse will add a double bond into the position x from the carboxyl end of the fatty acid chain. Whereas, the methyl-end desaturase (ω3 desaturase, (ω3)) inserts double bond between the pre-existing double bond and methyl-end. The ωy desaturase will add double bond at position y from the methyl end of fatty acid chain (Meesapyodsuk and Qiu, 2012). The activity of ω3 is conversion the substrate from omega-6 PUFAs to omega-3 PUFAs (Figure 2.9). For example, the ω3 can convert LA to ALA and ARA to EPA. The important regions of vertebrates fatty acid desaturases are (i) cytochrome *b5* domian that contains haem-binding motif fused to the N-terminal of the main desaturation domain; (ii) three histidine-rich boxes; and (iii) two transmembrane regions (Figure 2.7) (Meesapyodsuk and Qui, 2012; Vanger and Santigosa., 2011; Zheng et al., 2004; Hasting et al., 2001).

Membrane bound protein, Δ4 converts docosa-pentaenoic acid (DPA, 22:5n-3) to DHA. Furthermore, the Δ4 also converts docosatetraenoic acid (DTA, 22:4n-6) to docosapentaenoic acid (DPA, 22:5n-6). The Δ4 was recently identified from *Thraus tochytrium*, which is heterokont alga that produces high

level of DHA. This enzyme has presented the important region similarity to other desaturase but it is shown larger size (519 amino acids).

Delta 5 is also membrane bound protein that shows activity in the final step for production of EPA and ARA. The activity of Δ5 is conversion DGLA to ARA and Eicosatetraenoic acid (ETA, C20:4n-3) to EPA. In human, the expression of Δ5 is the greatest in adrenal gland, followed by liver, and brain. Generally, this enzyme is regulated by diets and expression level associated with various diseases (eye disorder, Alzheimer's disease, and diabetes). In fungal, the Δ5 contains important regions similar to other Δ5. But, the functional analysis found that fungal Δ5 can add double bond to only C16 and C18 fatty acids. Consequently, fungal Δ5 is not involved in PUFAs synthesis.

Delta 6 is a membrane bound protein that converts LA to GLA and ALA to STA. This step is the rate-limiting step of the PUFAs pathway. This enzyme has been identified from several organisms, e.g. human, rat, mouse, nematode, fungal, and plants. Interestingly, Δ6 of human and rat have contained dual function for desaturation of C18 (LA and ALA) and C24 (TPA) fatty acids (Pereira et al., 2003). The expression of this enzyme in human is the greatest in liver, followed by brain, heart, and lung. Moreover, the expression of Δ6 is regulated by dietary, age, and hormones. Fungal Δ6 contained an unusual histidine-rich motif (HKHHSH) downstream of the cytochrome *b5* region, which is absent in Δ6 of other organisms.

Delta 6&Δ5 is also a membrane bound protein that shows bifunctional activity of both Δ6 and Δ5 (Pereira et al., 2003). The Δ6&Δ5 has been found in several freshwater fish, e.g. trout, carp, and zebrafish. This enzyme is more active on Δ6 substrates than Δ5 substrates. In addition, it preferred to use omega-3 PUFAs over omega-6 PUFAs.

Delta 8 is also a membrane bound protein present in the alternative PUFAs pathway. This enzyme has been found in some eukaryote (*Euglena gracilis*), protozoa (*Tetrahymena*), and amoeba (*Acanthamoeba*). The Δ8 contains important regions similar to other desaturases. Δ8 elongates LA to Eicosadienoic acid (EDA, C20:2n-6) then desaturates to DGLA in the omega-6 PUFAs pathway. The Δ8 elongates ALA to Eicosatrienoic acid (ERA, C20:3n-3) then desaturates to ETA in the omega-3 PUFAs pathway (Figure 2.9) (Pereira et al., 2003).

Moreover, the fatty acid desaturases can also be classified into two types: (i) the acyl-ACP desaturase (the acyl-acyl carrier protein desaturase) and (ii) the integral membrane desaturase. The acyl-ACP desaturases are soluble desaturase found in plant plastid. The integral membrane desaturase is further divided into two group: (i) the acyl-lipid desaturase found in membrane of cyanobacterial thylakoid, plant endoplasmic reticulum (ER) and, plastid and (ii) the acyl-coenzyme A (CoA) desaturase found in ER membrane. All mammalian desaturases are acly-CoA desaturases that used cytochrome *b5* as electron donor (Shanklin et al., 2009; Nakamura and Nara, 2004; Los and Murata, 1998).

Elongase – Elongases are enzymes that added two carbon atoms into fatty acid chain to generate longer chain fatty acid. These enzymes are divided into two groups: (i) enzymes that involved in elongation of saturated and monounsaturated fatty acid and (ii) enzymes that involved in elongation of polyunsaturated fatty acid (Jakobsson et al., 2006). All of these elongase enzymes contain important regions indicates them being in the elongase family. There are KXXEXXDT, HXXHH, HXXMYXYY, TXXQXXQ motifs, and five or six transmembrane regions. Elongase enzymes have been studied from several

organisms, e.g. human, mouse, and yeast. Yeast cells exhibit three types of elongase. There are ELO1, ELO2, and ELO3. They have specific elongation activity to different length of long chain fatty acid. The ELO1 prefer to elongate C14 to C16. The ELO2 prefer to elongate saturated and monounsaturated fatty acid up to C24. The ELO3 prefer to extended saturated and monounsaturated fatty acid from C24 to C26 (Matsuzaka et al., 2002; Cook and McMaster, 2002). In zebrafish, the E5&E6 elongase has been shown to have a multifunction elongase activity similar to the Δ6&Δ5 that is also a multifunction Δ6 and Δ5 desaturase (Vagner and Santigosa, 2011; Agaba et al., 2004).

2.1.5 Omega-3 and omega-6 PUFAs pathways

Both omega-3 (EPA and DHA) and omega-6 (DGLA and ARA) PUFAs can be produce by several desaturase and elongase enzymes such as Δ6, Δ5, Δ4 desaturase, Δ5 elongase (E5), and Δ6 elongase (E6). The process start with Δ6 desaturates ALA (18:3n-3) to STA (18:4n-3) as well as LA (18:2n-6) to GLA (18:3n-6). Thereafter, the E6 elongates STA to ETA and GLA to DGLA. Both ETA and DGLA are the substrate for the Δ5 to generate EPA (20:5n-3) and ARA (20:4n-6), respectively.

Both EPA and ARA elongate to DPA and DTA, respectively. The production of DHA (22:6n-3) occurred in two pathways. First, the DPA elongates to tetracosapentaenoic acid (TPA, 24:5n-3) then Δ6 converts TPA to tetracosahexaenoic acid (THA, 24:6n-3), which further alters to DHA in peroxisome by β-oxidation process. Another pathway is the activity of Δ4 converts DPA to DHA as well as DTA to DPA (Figure 2.9) (Zheng et al., 2004; Pereira et al., 2003). Unfortunately, all

vertebrates lack Δ12 desaturase (Δ12) and Δ15 desaturase (Δ15) activities for the conversion of oleic acid (OA, 18:1n-9) to LA and ALA. Therefore, vertebrates cannot produce LA and ALA. They have to get LA and ALA from their diets (Harris et al., 2009; Hasting, 2001).

Generally, PUFAs accumulate higher in marine fish than freshwater fish due to feed and feeding behavior of fish. For freshwater fish, the feeding behaviors are herbivorous and omnivorous. Most freshwater fish feeds (phytoplankton, periphyton and detritus) contain LA and ALA with low level of EPA and DHA.

Therefore, freshwater fish has to retain the ability of the desaturase and elongase enzymes for PUFAs production. In contrast, in marine fish, the feeding behaviors are herbivorous and carnivorous. Most marine fish feeds (phytoplankton, zooplankton and larva fishes) contain high level of PUFAs. Therefore, marine fish does not need to retain the enzymes ability for the production of PUFAs.

The PUFAs pathway of lower eukaryotes (fungi, algae and protozoa), contain Δ9 desaturase (Δ9), Δ12, and Δ15. Lower eukaryotes also have Δ8 desaturase (Δ8) which is an alternative pathway for generation of EPA (C20:4n-3). Another enzyme, Δ4 can add double bond at the forth carbon atom of DPA (22:5n-3) to produce DHA (Vanegas-Caleron et al., 2010; Pereira et al., 2003). Li et al. (2010) suggested that the expression of Δ4 is an alternative pathway for the generation of DHA in vertebrate (*Siganus canaliculatus*). Some fungi can add double bond by the activity of Δ17 desaturase (Δ17) for generation of both ETA and EPA (Figure 2.9) (Martin et al., 2007).

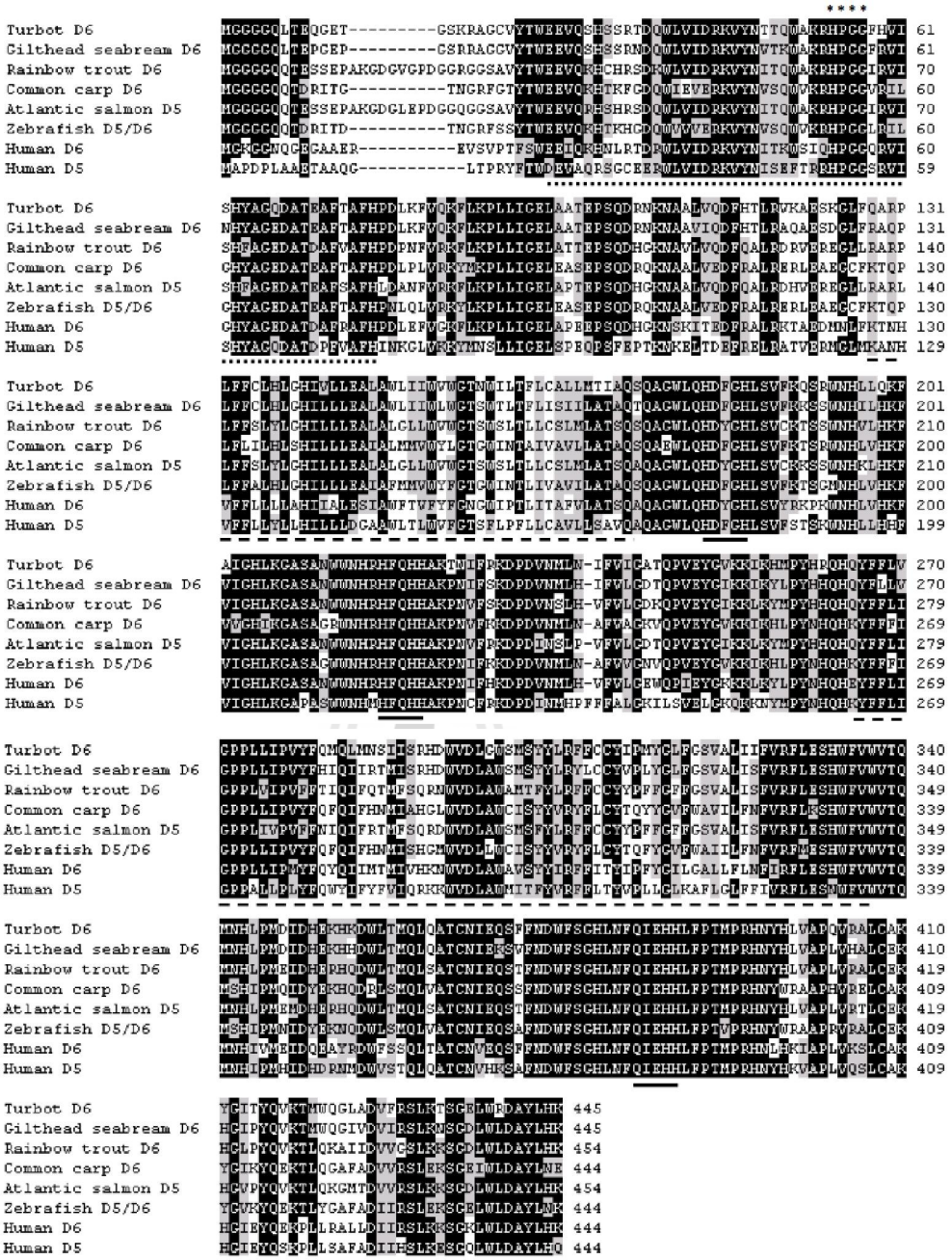

Figure 2.7 Comparison of fatty acid desaturase from several fish (carp, rainbow trout, sea bream, turbot, zebrafish, salmon and, human). Dotted line: the cytochrome b5, asterisks above: haem-binding motif, dashed line: two transmembrane regions, and solid lines: three histidine boxes (Zheng et al., 2004).

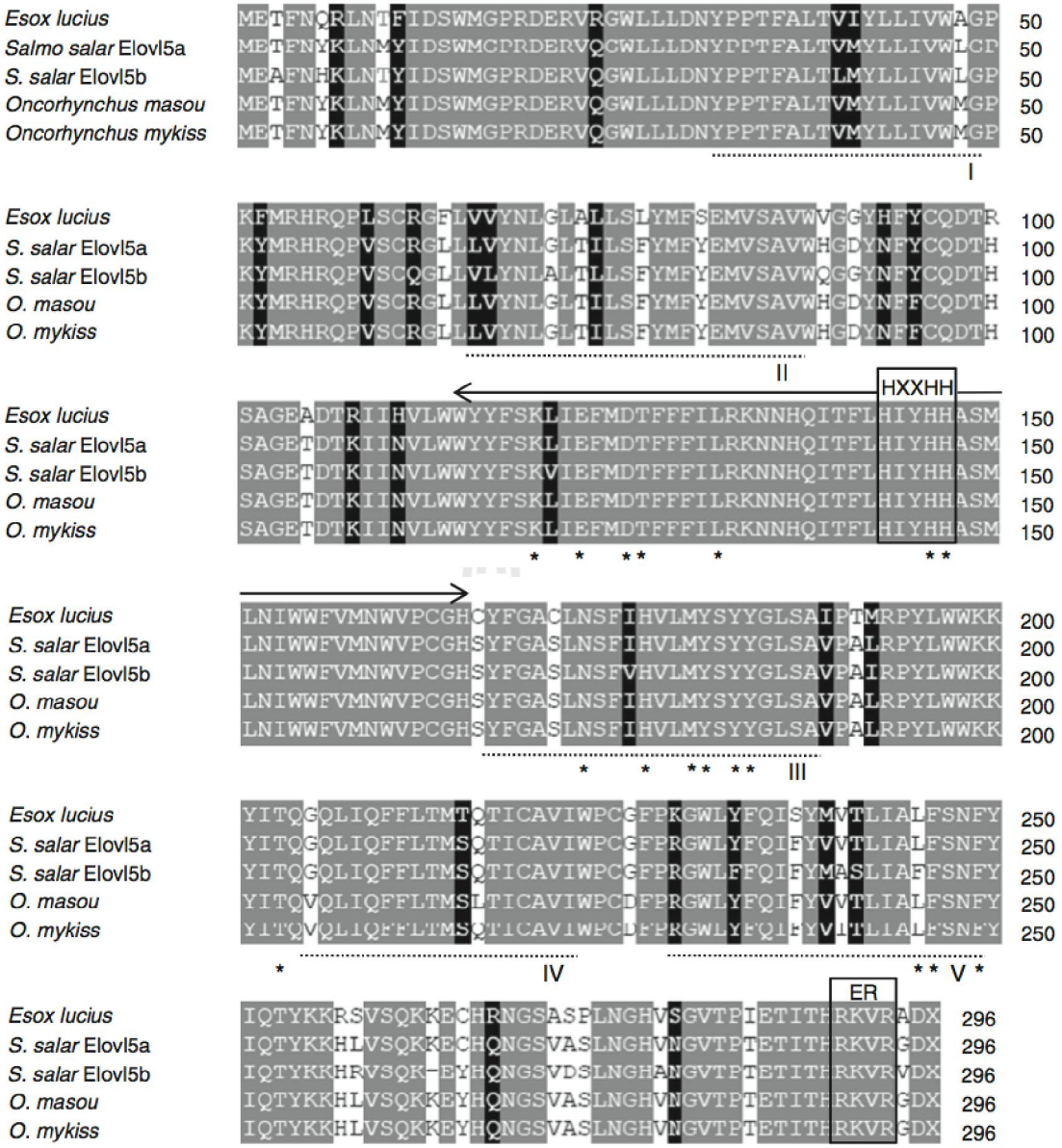

Figure 2.8 Comparison of fatty acid elongase from several fish (northern pike, Atlantic salmon, masu salmon and, trout). The single HXXHH histidine box, endoplasmic reticulum retention signal (ER), and five transmembrane regions is dot line (Carmona-Antoñanzas et al., 2013).

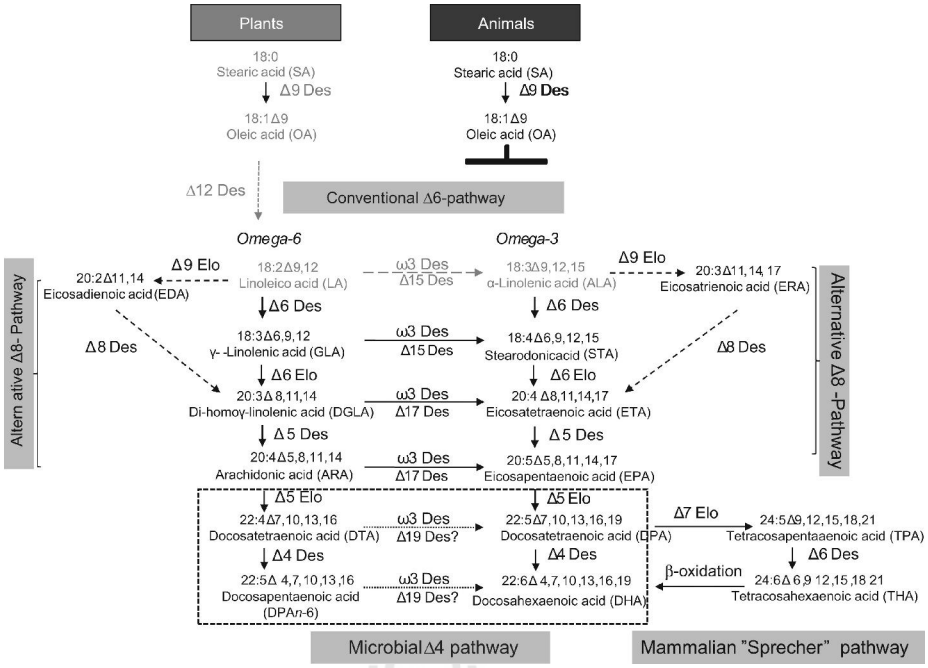

Figure 2.9 PUFAs biosynthesis pathway (Olga and Napier, 2011).

2.2 Yeast

2.2.1 The structure of yeast cells

Yeast is a eukaryotic cell classified in the kingdom fungi. Yeast exhibits several shapes. There are round, ellipsoidal, oval, apiculate, triangular, elongated, and filamentous. Yeast cell consists of several components, e.g. cell wall, periplasm space, plasma membrane, cytosol, nucleus, mitochondrion, endoplasmic reticulum, golgi apparatus, secretory vesicles, vacuole, and peroxisome. The cytoplasm is surrounded by plasma membrane, periplasmic space (cell-wall associated region external to plasma membrane), and cell wall. The cell wall consists of four classes of macromolecules, i.e. highly glycosylated gylcoproteins, two types of beta-glucans, and actin (Feldmann, 2010). The structure of plasma membrane is a lipid bilayer with protein inserted into this layer. All of the yeast cell components are similar to higher

eukaryote, which is an ideal model for eukaryotic cell biology. The reproduction of yeast is sexual and asexual. Most yeast reproduces by asexual budding. A small bud is a daughter cell that forms on the parent cell. The nucleus of parent cell doubled and separated then transferred to daughter cell. The small buds attached and grow until separated from the parent cell.

2.2.2 *Pichia pastoris*

2.2.2.1 History and taxonomy The methylotrophic yeast (*Pichia*), which can use methanol as a carbon source and energy, was discovered around 40 years ago by Koichi Ogata. Since, the cost of methanol is inexpensive. The methylotrophic yeasts were interested to generate yeast biomass for animal feed. During the 1970s, Phillips Petroleum Company had developed medias and methods for growing *P. pastoris* using methanol in continuous culture at high cell densities. Unfortunately, the cost of methanol had increased higher than the cost of soybeans, which are the major alternative source of animal feed protein. Thereafter, in the early 1980s, *P. pastoris* was developed as a heterologous gene expression system by Phillips Petroleum and the Salk Institute Biotechnology/Industrial Associate Inc. (SIBIA), a biotechnology company located in La Jolla, CA, USA. The developed expression system included AOX1 promoter, plasmids, strains and methods for molecular genetic manipulation. In 1993, Phillips Petroleum sold the patent of *P. pastoris* expression system to Research Corporation Technologies (RCT) who is currently the patent holder. In addition, Phillips Petroleum licensed Invitrogen to sell components of the system to researchers worldwide. (Macauley-Patrick, et al., 2005; Cereghino and cregg, 2000; Cregg et al., 2000).

The general taxonomy of *P. pastoris* is as follows (http:// www.platform-fefana.org/Website/DOCS/07-03-05_pp_qps.pdf)

Kingdom: Fungi

Phylum: Ascomycota

Class: Saccharomycetes

Order: Saccharomycetales

Family: Saccharomycetaceae

Genus: *Pichia*

Species: *Pichia pastoris*

2.2.2.2 Benefits of *P. pastoris* *P. pastoris* is a eukaryotic single cell microorganism easy to manipulate and culture. *P. pastoris* has been employed to use as host system for the production of heterologous proteins (Waterham et al., 1997). Unlike the bacteria system (*Escherichia coli*), this yeast shows more similar activity to higher eukaryote, e.g. posttranslation modification (proteolytic processing, protein folding, disulfide bond formation, and glycosylation). Therefore, the inactive protein in bacteria expression system can most of the time be active in *P. pastoris* expression system (Cregg et al., 2000). Moreover, *P. pastoris* has shown to faster growing and less expensive for using when compared to mammalian expression system (Balamurugan et al., 2007). In addition, *P. pastoris* has been used as a model system to observe molecular mechanism. For example: (i) the import and assembly of peroxisome; (ii) the selective autophagic degration of peroxisome; and (iii) the organization and function of secretory pathway in eukaryotes (Cregg et al., 2000).

Since, the *P. pastoris* exhibited several advantages when compared with other eukaryotic and prokaryotic systems, e.g.

(i) Rapidly growth rate and able to grow to high cell density fermentation (Romanos, 1995).

(ii) High level of recombinant protein production in a protein-free medium.

(iii) Elimination of endotoxin and bacterial contamination.

(iv) Safety for human.

(v) Contains several posttranslation modifications (glycolsylation, methylation, acylation, proteolytic adjustment).

(vi) Ability to purify secreted protein from growth medium without harvesting the yeast cells (Li et al., 2007).

(vii) The cost for culturing is lower than mammalian expression system and also gives higher recombinant protein expression level (Balamurugan et al., 2007; http://www. pichia.com/pichia_system.pdf).

(viii) The simplicity of techniques needed for manipulation of the cells (Cereghino and Cregg, 2000).

(ix) Expressed all of the Δ9, Δ12 and Δ15 desaturase activity. Therefore, it should be easy to construct the recombinant *P. pastoris* containing high level of PUFAs. Li et al. (2009) showed that the insertion of Δ6, E6 and Δ5 could generate EPA and DHA in *P. pastoris* cells.

Therefore, *P. pastoris* is an important host organism for the production of recombinant proteins (Krainer et al., 2012) and also have the generally regarded as safe (GRAS) status (Noronha et al., 2002).

2.2.2.3 Expression plasmid and alternative promoter for expression in *P. pastoris* To construct the expression plasmid in *P. pastoris* three steps are needed: (i) the construction of plasmid that contained interesting gene; (ii) determination of potential expression strains for foreign gene product; and (iii) transformation of expression plasmid into *P. pastoris* genome. The components of all *P. pastoris* expression plasmid are: (i) promoter sequence (most often the AOX1 promoter); (ii) transcription termination sequence for polyadenyllation of mRNAs; (iii) multiple cloning sites for insertion of foreign gene; (iv) origin of replication for maintenance of plasmid in *E. coli*; and (v) selectable maker for *E. coli* and *P. pastoris* (Li et al., 2007).

In *P. pastoris*, the *AOX1* and *AOX2* genes encode alcohol oxidase. The AOX1 is a dominant alcohol oxidase activity in the cell. The expression of *AOX1* gene is highly regulated and induced by methanol to high level. Although the expression of AOX1 promoter has been used for expression of many foreign proteins successfully, however, several problems are still of concerned. The used of methanol as inducer is not suitable for expression of product used as food or feed. Due to its petroleum base related compound methane. When high amount of methanol are store, fire hazard is of concerned. Some other promoters for example GAP, FLD1, PEX8, and YPT7 have been developed. The glyceroaldehyde-3-phosphate dehydrogenase (GAP) promoter has been shown to be a strong constitutive promoter with high expression when glucose was used as carbon source. This promoter has shown several advantages, e.g. not required methanol for induction, convenient for growing and protein expression. However, GAP promoter is not suitable for production of protein that is toxic for the cell since it is constitutively express. The formaldehyde dehydrogenase (FLD1) is an inducible promoter induced

by either methanol as a carbon source with ammonium sulfate as a nitrogen source, or methylamine as a nitrogen source with glucose as a carbon source. Moreover, FLD1 promoter is repressed in medium with glucose and ammonium sulfate. The peroxin (PEX8) is a gene that encoded peroxisomal matrix protein. This protein is important for peroxisome biogenesis. This promoter expressed at a low but significant level on glucose and can produce small level of recombinant protein when shift to methanol. The yeast protein 1 (YPT1) encoded a GTPase involved in secretion. The YPT1 promoter has shown a low constitutive expression when expressed in media containing glucose, methanol, or manitol as a carbon source (Li et al., 2007; Cereghino and Cregg, 2000; Cregg et al., 2000).

2.2.2.4 Strains of *P. pastoris* *P. pastoris* host strains can be divided into three groups vary with their ability to utilize methanol for recombinant protein production. First group is the most strains that grow on methanol at the wild type rate (Mut^+, methanol utilization plus phenotype). For example, the most commonly used host GS115 (*his4*$^-$) contains both *AOX1 and AOX2* genes, therefore can be grown on methanol at wild type rate. Moreover, this GS115 strain has been modified to be defective in histidine dehydrogenase gene (*his4*) that cannot grow on non-histidine containing media (Daly and Hearn, 2005). Another two groups are Mut^s (methanol utilization slow phenotype) and Mut^- (methanol untilization minus phenotype). Both Mut^s and Mut^- varied with utilization of methanol due to deletion of one or both *AOX* genes. Mut^s grow on methanol at the slow late due to deletion of *AOX1* gene. For example, KM71 (*his4*$^-$ *arg4*$^-$ *aox1Δ::ARG4*), the *AOX1* gene is deleted and replaced by *ARG4* gene of *S. cerevisiae.* Mut^- cannot grow on methanol due to deletion both *AOX1* and *AOX2* genes. For example, MC100-3 (*his4*$^-$ *arg4*$^-$ *aox1Δ::SARG4 aox2Δ::Phis4)*

(Li et al., 2007). Moreover, some secretion of foreign proteins can be degraded by proteases of *P. pastoris.* To overcome this problem, the host strain that defected in those proteases can reduce the degradation of foreign proteins. For example, SMD1163 (*his4*$^{-}$ *pep4*$^{-}$ *prb1*$^{-}$), SMD1165 (*his4*$^{-}$ *prb1*$^{-}$), and SMD1168 (*his4*$^{-}$ *pep4*$^{-}$). All of these strains are Mut^{+} that showed protease-deficient phenotype, which is suitable for expression of foreign proteins. The *PEP4* gene encodes proteinase A that required for activation of other vascular proteases such as carboxypeptidase Y and proteinase B. Therefore, the mutation of *PEP4* gene can decrease or eliminate the proteinase A, carboxypeptidase Y activity and reduction of proteinase B activity. The *PRB1* gene encodes proteinase B, the mutation of *PRB1* gene can eliminate the proteinase B activity (Cregg et al., 2000; Cereghino and Cregg., 2000).

2.3 pGAPZ plasmid

2.3.1 The components and map of pGAPZ plasmid

pGAPZ contains pUC origin of replication for *E. coli*, Zeocin resistant gene that worked as antibiotic resistant marker in both *E. coil* and *P. pastoris*. The pGAPZ plasmid also contains the glyceraldehyde-3-phophate dehydrogenase (GAP) promoter to drive foreign protein expression. The GAP promoter is a constitutive expressed promoter that has been shown to transcribe recombinant proteins to high level in *P. pastoris.* Behind the pGAP promoter are the multiple cloning sites for easy and convenient cloning of interested gene. The interested gene can be designed to fuse with myc epitope and 6xHis tag followed by stop codon (Figure 2.10). This plasmid contains the AOX transcription termination for performing efficient 3' mRNA and polyadenylation processing. The pGAPZ plasmid can generate the

multiple copies of expression cassette gene by *Bam* HI and *Bgl* II restriction site. Since, both *Bam* HI and *Bgl* II have contained complementary site that can ligate both of them together. Moreover, the ligated *Bam* HI and *Bgl* II will present the new site that cannot digest with both enzymes.

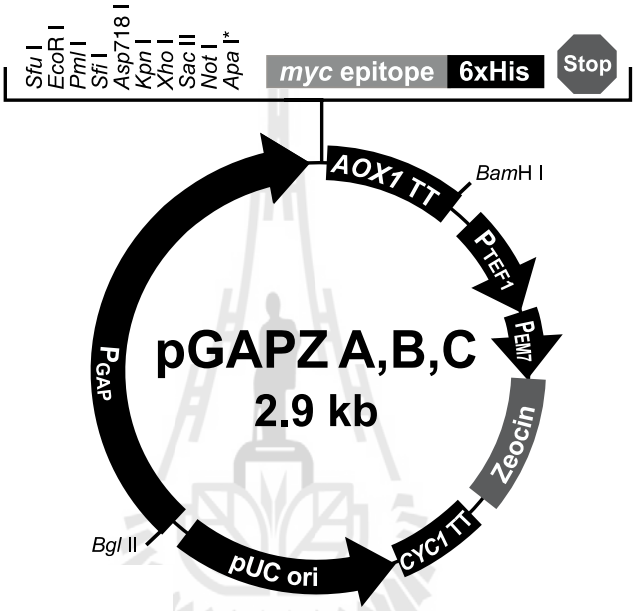

Figure 2.10 Map of pGAPZ plasmid.

2.3.2 Integration into *P. pastoris* genome of pGAPZ plasmid

Normally, the *P. pastoris* has no stable episomal plasmid for expression. Consequently, the linearized recombinant plasmid has been generated for stability of heterologous protein expression by homologous recombinant between recombinant plasmid and *P. pastoris* genome (Li et al., 2007). Several reasons for chromosomal integration were considered. For example: (i) the episomal plasmid tend to have low copy number in host cell that effected the amount of products; (ii) the large size of plasmid effected the replication process; (iii) the transformation with episomal

plasmid required antibiotic resistant marker for maintaining the transformed cells (Daly and Hearn, 2005). The method for integration into *P. pastoris* genome is linearized the recombinant plasmids at specific site. Thereafter, the linearized recombinant plasmids will transform into *P. pastoris.* The recombinant plasmids with free termini stimulate the homologous recombination by single gene insertion (Figure 2.11) or multiple gene insertion (Figure 2.12). The multiple gene insertion events can occur spontaneously with low, but detectable frequency (1-10% of selected Zeocin transformants) (Invitrogen).

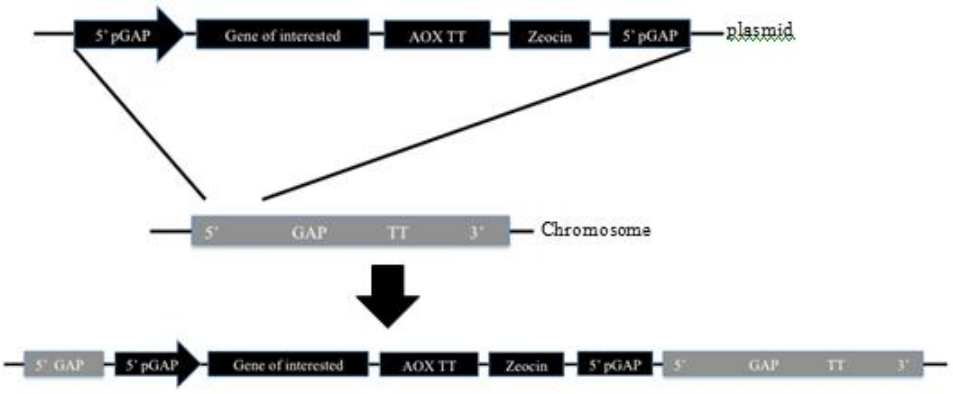

Figure 2.11 Integration into host genome by single gene insertion.

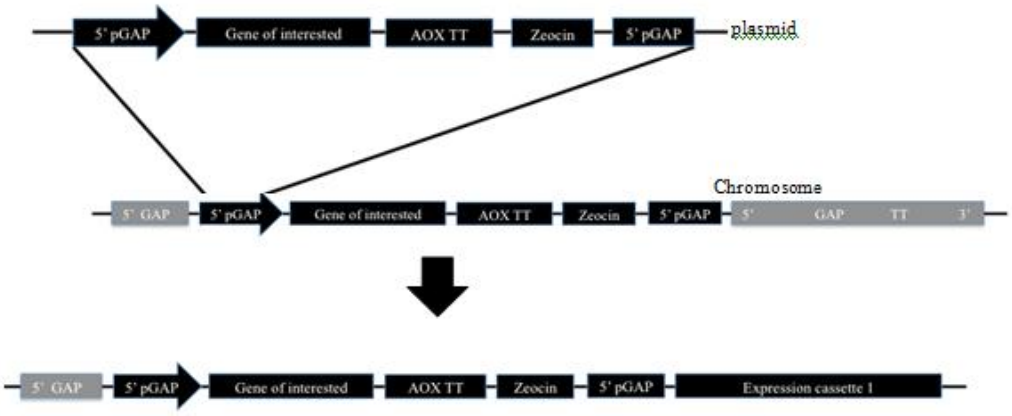

Figure 2.12 Integration into host genome by multiple gene insertion.

2.4 Nile tilapia (*Oreochromis niloticus*)

2.4.1 History and taxonomy

Tilapia is a freshwater fish in the family Cichlidae, which is one of four families (Cichlidae, Embiotocidae, Pomacentridae and Labridae) of suborder Labroidei. The Cichlidae family consists of three important genus; *Oreochromis, Sarotherodon* and *Tilapia* that divided by reproductive behaviors. It is the most expansive cultured fish of world (Ferraz De Arruda et al., 2006). Tilapia is a word derived from the American Bushman word meaning to fish and a part of cichlid fish that is a native to Africa. They are native to Africa, but introduced into many tropical, subtropical region of the world. Africa is the most important producer of the global production of tilapia by capturing fisheries around 70% of the world, follow by Asia 18%, North America 9% and South America 3%. For the production of tilapia by farming, only 5 countries are the major important producer (China 50%, Egypt 12%, the Philippines 9%, Indonesia 8% and Thailand 7%) (El-Sayed, 2006). More than 90% of the culturing tilapia is Nile tilapia (*O. niloticus)*. Lower than 10% are other species such as blue tilapia (*O. aureus),* mozambique tilapia (*O. mossambicus)* and the zanzibar tilapia (*O. urolepis hornorum)* (Popma and Masser, 1999).

On 25 March 1965, fifty Nile tilapias were offered to His Majesty the King Bhumibhol by the Emperor Akihito, who is the Crown Prince of Japan at that time. His Majesty the King Bhumibhol cultured the Nile tilapias in a pond size of around 10 square meters in Chitlada palace. After five months, there were a lot of Nile tilapia fry in the pond. Thereafter, His Majesty the King Bhumibhol ordered to dig six new ponds with the size around 70 square meters and transferred the fish from the old pond to the new ponds by himself on 1 September 1965. Consequently, His Majesty the King Bhumibhol asked

technical staff from Department of Fisheries to monitor the fish every month. The Nile tilapia has shown rapid growth, easy culturing, good taste, and producing lots of fry. Consequently, His Majesty the King Bhumibhol distributed the Nile tilapia to his people. On 17 March 1966, His Majesty the King Bhumibhol designated the name of Nile tilapia as a "Pla-Nil". Subsequently, The Pla-Nil was offered to Department of Fisheries for culturing, breeding, and dispensed to the Thai people (www.fisheries.go.th).

The general taxonomy of Nile tilapia (*Oreochromis niloticus*) is as follows (Nelson, 2006)

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Periformes

Family: Cichlidae

Genus: *Oreochromis*

Species: *Oreochromis niloticus*

2.4.2 Habitat and environmental requirement

The physical characteristics of Nile tilapia are shaped like sunfish but can be identified by interrupted lateral lines, which are the characteristic of the Cichlid family of fishes. They are laterally compressed with deep-bodied and long dorsal fins. Nile tilapia shows both spine and soft ray. The dorsal fins, 16-17 spines continue with 11-15 soft rays and also found in pelvic fins and anal fins. Caudal fin shows truncated style (Figure 2.13) (http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en#tcNA0078). Nile tilapia male excavates a nest in the pond bottom (generally in

water shallower than 3 feet) and mates with several females. After short mating the female spawns in the nest then male fertilizes the egg, holds and incubates the eggs in the female mouth (buccal cavity) until they hatched. Fry remains in the female's mouth through yolk sac food source and often lives in the mother's mouth for several days before they begin to feed (Popma and Masser, 1999). The Nile tilapia can tolerate to wide range of environment conditions. For example: physical parameter (temperature, photoperiod, depth, current velocity, and turbidity); chemical parameters (salimity, pH, dissolved oxygen, minimal, and gas content); and biological parameters (competition, food availability, and productivity). Nile tilapia is herbivorous and omnivorous. The period from juve Nile to adult consists of variety of food, e.g. phytoplankton, zooplankton, periphyton and detritus of plant origin. The trend consumption and culturing of Nile tilapia have increased because of many positive points, for example; Nile tilapia can reproduce in short generation time, fast growth, tolerance to wide range of environmental conditions such as poor water quality, ranging of temperature from 8 to 42 degree Celsius, tolerance to brackish water up to 36 parts per thousand, low dissolved oxygen concentration down to 0.1 mg/L but optimum growth is more than 3 mg/L, pH between 3.7 to 11, ammonia up to 7.1 mg/L and wide range of natural food organisms (Mjoun et al., 2010; Popma and Masser, 1999). Moreover, the Nile tilapia has shown to be a rich source of protein, phosphorus, potassium, selenium, niacin, vitamin B-12, carbohydrates, sodium (Mjoun and Rosentrater, 2010) and some omega-3 PUFAs (EPA and DHA) (Du et al., 2012).

Figure 2.13 Nile tilapia (*Oreochromis niloticus*).

2.5 Zebrafish (*Danio rerio*)

2.5.1 History and taxonomy

Zebrafish (*Danio rerio*) is a small freshwater fish originated in South Asia, mainly India, Bangladesh, Nepal, Myanmar, and Pakistan (Lawrence, 2007). This fish is often found in shallow, slow-moving water near the streams. The zebrafish is often used for studying genetics and development, human disease, and screening of therapeutic drug. Zebrafish are small, rapid development, short generation time and large numbers of eggs production. Therefore, they have been used as a laboratory animal model (Lawrence, 2007; Briggs, 2002).

The general taxonomy of zebrafish (*Danio rerio*) is as follows (http://eol.org/pages/204011/overview)

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Danio*

Species: *Danio rerio*

2.5.2 Habitat and environmental requirement

The shape of zebrafish is fusiform and laterally compressed with the mouth direct upward (Figure 2.14). Male are torpedo-like shape. Moreover, male zebrafish expresses gold stripes between blue stripes. Female zebrafish are larger than male and exhibits silver stripes instead of gold. The zebrafish can grow to 6.4 cm with a lifespan of around two to three years. The season for zebrafish breeding is during the monsoons (April to August). This fish normally breed in vegetated pools. They have non-adhesive eggs. The hatching depends on water temperature. They hatched between 48 and 72 hours after fertilization. Zebrafish is omnivores. The foods of zebrafish are zooplankton, aquatic insects, arachnids and mosquito larvae (Engeszer et al., 2007). The optimal conditions that generally recommended for zebrafish are tolerance range of 6.7-41.7°C but 28.5°C is optimal temperature, pH range from 7-8, salinity range from 0.25-0.75 ppt, and dissolved oxygen around 7.8 mg/L at 28.0°C (Lawrence, 2007).

Figure 2.14 Zebrafish (*Danio rerio*) (http://animaldiversity.ummz.umich.edu/accounts/Danio_rerio/.

CHAPTER III

MATERIALS AND METHODS

3.1 Construction of expression plasmid and recombinant *Pichia pastoris*

3.1.1 Primer design (Δ6 desaturase, bifunctional Δ6&Δ5 desaturase, Δ5 elongase, and Δ6 elongase genes)

The Δ6 desaturase (Δ6) (XM_003440470) of *Oreochromis niloticus* (Nile tilapia) predicted to be Δ6 gene. The bi-functional Δ6&Δ5 desaturase (Δ6&Δ5) (NM_131645.2), Δ5 elongase (E5) (NM_200453.1), and Δ6 elongase (E6) (NM_199532.1) of *Danio rerio* (zebrafish) predicted to be Δ6&Δ5, E5, and E6 genes, respectively. Each of the nucleotide sequence was used for primer designed to amplify Δ6, Δ6&Δ5, E5, and E6 genes.

3.1.2 RNA extraction (Nile tilapia and zebrafish), cDNA synthesis and PCR

Nile tilapia and zebrafish were shocked on ice and liver tissues were removed and dissected on ice. The excised livers were homogenized by mortar and pestle in liquid nitrogen. The total RNA was extracted by RNeasy Mini kit (QIAGEN) then converted to cDNA by SuperScript® III Reverse transcriptase (Invitrogen). The 4 genes (Δ6, Δ6&Δ5, E5, and E6 genes) were amplified by nested PCR method using specific primers shown in table 3.1. *Eco* RI and *Xho* I restriction sites (underline) were introduced to the inner forward and reverse primers of Δ6, Δ6&Δ5, and E5, respectively. The restriction sites *Pml* I and *Xho* I (underline) were

added to the inner forward and reverse primers of the E6, respectively (Table 3.1). The PCR amplifications of the outer primers of 25 µl PCR mixture contained 1 µl of cDNA template, 1X Go-taq buffer (Promega), 2.0 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer and 2.5 U GoTaq polymerase (Promega). PCR condition consists of incubation at 95°C for 5 minutes and then 35 repetitive cycles of denaturing step at 95°C for 30 seconds, annealing step at 52-66°C (depended on primers in table 3.1) for 30 seconds, and extension step at 72°C for 1 minute. The extension step, 72°C for 1.30 minutes was used for amplification of genes that were more than or equal to 1,000 bp. The final step was performed at 72°C for 5 minutes for the last extension. The amplification with the inner primers, the 1 µl of each PCR product from the outer primers amplification was used as a template. The PCR condition of the inner primers was similar to the outer primer amplification. The PCR products were separated on 1% agarose gel then detected under UV.

3.1.3 Ligation into pGAPZ plasmid

Escherichia coli (DH5α) competent cells were prepared by culturing single colony of *E. coli* in 5 ml low salt LB broth at 37°C with 200 rpm shaking overnight. Thereafter, 1 ml of overnight cultured was transferred to new 100 ml low salt LB broth. The cells were incubated at 37°C with 200 rpm shaking for 3 hour or OD_{600} equal to 0.3-0.6. The *E. coli* cells were incubated on ice for 30 minutes then collected by centrifugation at 4,000 rpm 4°C for 5 minutes. The supernatant was removed and cells were washed with 100 ml ice-cold 10% glycerol then resuspended on ice. The *E. coli* cells were centrifuged again at 4,000 rpm 4°C for 5 minutes then the supernatant was discarded. Ten ml ice-cold 10% glycerol were added then resuspended on ice and centrifuged at 4,000 rpm at 4°C for 5 minutes. The

supernatant was removed then 2 ml of GYT (10% glycerol, 0.125% (w/v) yeast extract, and 0.25% (w/v) tryptone) were added. The 100 µl *E. coli* competent cells were aliquot into PCR tube and put in liquid nitrogen before stored at -80°C until use.

pGAPZ plasmid was used as expression plasmid. The pGAPZ plasmid was digested with *Eco* RI (or *Pml* I for ligation with E6 gene) and *Xho* I. The total volume of digestion was 30 µl consists of 1X buffer (depended on enzyme in table 3.2), 10 U for each *Eco* RI (or *Pml* I) and *Xho* I and 1 µg of pGAPZ plasmid. The digested pGAPZ plasmid was separated on 1% agarose gel. The 1% agarose gel that contained digested pGAPZ plasmid was cut and purified by Gel extraction Kit (Invitrogen).

The Δ6, Δ6&Δ5, E5, and E6 genes were digested at 37°C overnight. The total volume of the reaction was 30 µl consists of 1X buffer (Table 3.2), 10 U of *Eco* RI or *Pml* I and *Xho* I and 1 µg of each gene from the nested PCR reaction. The digested fragments were separated on 1% agarose gel. The 1% agarose gel that contained digested genes were cut and purified by Gel extraction kit (Invitrogen).

For generation of recombinant plasmid pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E5, and pGAPZ:E6, the digested pGAPZ plasmid and the digested genes were ligated together. The ligation, the total volume was 10 µl consisted of 200 U T4 DNA ligase (NEB), 1X T4 DNA ligation buffer, 100 ng of digested pGAPZ plasmid and 300 ng of digested genes.

Table 3.1 Primer name, sequence of forward and reverse primers and annealing temperature (Ta).

Primer name	Forward*	Reverse*	Annealing Temp. (^{o}C)
pGAPZ plasmid			
pGAPZ	GTCCCTATTTCAATCAATTGAA	AAGTGCCCAACTTGAACTGAGG	47
pGAPZ(M) plasmid			
pGAPZ(M)	TCTTGGTGTCCTCGTCCAA	AAGTGCCCAACTTGAACTGAGG	51
Δ6			
Outer primers	GTGCATTCAGACCAGAGGCAGC	CATTCCTTTTACACCAGTGGAGG	55
Inner primers	CCGAATTCATGGGAGGTGGAAGCCAGC	CCCTCGAGGATTTATGGAGATATGCATCC	61
Δ6&Δ5			
Outer primers	AGCAGCACTGTTCAGAGATC	CTATGTAGGGAGAAGTGATGC	52
Inner primers	CCGAATTCATGGGTGGCGGAGGACAGCA	CCCTCGAGGATTTGTTGAGATACGCATCCAGCC	66
E5			
Outer primers	CTCGCACACTTCTAAAGACT	GCGTATCAATAATGGATGAAC	49
Inner primers	CCGAATTCATGGAGACGTTTAGTCACAG	CCCTCGAGGAATCTGCTCGTGCTTTTCT	60
E6			
Outer primers	AGCGTTCAGAGGACCACCAG	TTCCTCCCAACTTCTCCTAGTG	55
Inner primers	CCCACGTGATGTCGGTGCTGGCTTTGCA	CCCTCGAGGATTGGCTTTTCTTGGCTGCG	65

*: The primer sequences were arranged from 5'- to 3'- end; Underline: restriction site.

Table 3.2 Restriction enzymes used in the experiment.

Restriction enzyme	Restriction site	Buffer	Company
***Eco* RI**	G↓AATTC	Buffer R	Fermentas Ltd.
***Pml* I**	CAC↓GTG	Buffer tango	Fermentas Ltd.
***Xho* I**	C↓TCGAG	Buffer R	Fermentas Ltd.
***Avr* II**	C↓CTAGG	CutSmart buffer	New England Biolabs
***Bam* HI**	G↓GATCC	Buffer tango	Fermentas Ltd.
***Bgl* II**	A↓GATCT	Buffer D	Promega

All enzymes were digested and incubated at 37°C overnight

The mixtures were incubated at 16°C overnight, according to manufacture's protocol. The ligation mixtures were transformed into *E. coli* competent cells by electroporation at 1,800 volts then incubated with 1 ml low salt LB broth at 37°C for 60 minutes. The transformed cells were spread on low salt LB plate that contained 50 μg/ml Zeocin then incubated in an upside down direction at 37°C overnight. The transformed cells were checked for each recombinant plasmid by colony PCR technique. The colony PCR technique, each single recombinant colony was resuspended in 5 μl DI water then boiled at 100°C for 5 minutes. Two microliter of the boiled cell was used as template in the PCR reaction. The PCR condition was similar to the inner primers amplification. Each type of recombinant plasmid was extracted and the target gene was confirmed by digestion then sent for sequencing. The DNA sequencing was performed by Macrogen (Korea)

(http://dna.macrogen.com/eng/) using pGAPZ forward and reverse primers (Table 3.1). Resulting amino acid sequences were used in BLAST program (blast.ncbi.nlm.nih.gov) for amino acid comparison with the database in GenBank. The alignment was created by ClustalW2-multiple sequence alignment tool (http://www.ebi.ac.uk/ Tools/msa/clustalw2/).

The recombinant plasmid pGAPZ:Δ6&Δ5:E6 was constructed from the 2 recombinant plasmids; (i) pGAPZ:Δ6&Δ5 and (ii) the mutant pGAPZ:E6 (pGAPZ(M):E6). Since, Transformation into *P. pastoris* has to digest with *Avr* II to generate linear form of plasmid. For that reason, the mutation of GAP promoter was produced. The construction of pGAPZ(M), the pGAPZ plasmid was digested with *Avr* II to generate sticky end. Thereafter, PCR reaction was done for adding of nucleotide to produce blunt end that altered the *Avr* II restriction site. The pGAPZ(M) was transformed into *E. coli* competent cells for multiplication of this plasmid then extracted and confirmed the mutation by negative digestion with *Avr* II. The pGAPZ(M) was sequenced by Macrogen (Korea) (http://dna.macro gen.com/eng/) using pGAPZ(M) plasmid forward and reverse primers (Table 3.1). Afterward, the E6 gene was cloned into pGAPZ(M) plasmid for construction of pGAPZ(M):E6.

To construct the recombinant plasmid pGAPZ:Δ6&Δ5:E6, the pGAPZ: Δ6&Δ5 was digested with *Bgl* II to generate linear form. The pGAPZ(M):E6 was digested by *Bam* HI and *Bgl* II for generation of the DNA fragment. Thereafter, both of them were ligated together then transformed into *E. coli* competent cells. The digestion, ligation, and transformation conditions were similar to previously report.

3.1.4 Transformation into *P. pastoris*

The *P. pastoris* (SMD1168H) competent cells were prepared by culturing single colony of *P. pastoris* in 5 ml YPD broth at 30°C with 200 rpm shaking overnight. The 1% overnight cultured was inoculated to new 50 ml YPD broth. The *P. pastoris* cells were incubated at 30°C with 200 rpm shaking for 5 hours or till OD_{600} around 1.3. The cells were incubated on ice for 30 minutes then centrifuged at 2,000 rpm 4°C for 5 minutes. The supernatant was removed then 20 ml of ice-cold DI water was added and then centrifuged. The cells were resuspended twice with 5 ml and 1 ml ice-cold DI water, respectively. One ml of ice-cold 1M sorbitol was added and resuspended then centrifuged. The supernatant was removed. Afterward, 400 µl of 1M sorbitol was added to the cells then resuspended. Then 100 µl of *P. pastoris* competent cells was aliquot into PCR tube for further transformation.

Transformation of recombinant pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E5, pGAPZ:E6, pGAPZ:Δ6&Δ5:E6, and empty pGAPZ plasmid as a control were done. All of the recombinant plasmids and empty pGAPZ plasmid were digested with *Avr* II to generate linear form of plasmids and 500 ng of plasmids were used for transformation. The electroporation condition was done at 1,500 volts then incubated with 1 ml 1M sorbitol at 30°C for 1 hour without shaking. The incubated cells were filled with 1 ml of YPD broth then incubated at 30°C with 200 rpm shaking for 1 hour. Each recombinant *P. pastoris* was spread on YPD plate that contained 100 µg/ml Zeocin. The plates were incubated at 30°C for 2 days in an upside down direction. Each type of recombinant *P. pastoris* was checked for the target gene by colony PCR technique. The colony PCR technique for recombinant *P. pastoris*, single colony was inoculated into 100 µl of solution containing 200 mM LiOAC and 1%

SDS. The cells were vortex and incubated at 65°C for 10 minutes. Three hundreds microliters of absolute ethanol was added and vortex. The samples were centrifuged at 14,000 rpm for 3 minutes then removed the supernatant. The pellet was resuspended in 70% ethanol then vortex and centrifuged. The supernatant was removed and the pellet was dried. The pellet was resuspended in 30 μl TE buffer. Two μl of sample was used as PCR template.

All of recombinant *P. pastoris* types were restreak on YPD plate containing difference concentration of Zeocin (100, 200, 300, 400, and 500 μg/ml) for prediction of high copy number that integrated into genome of *P. pastoris*. The recombinant *P. pastoris* that can grow on YPD plate containing high concentration Zeocin (500 μg/ml) was used for cultured then observed the fatty acid compositions.

3.2 Polyunsaturated fatty acid analysis of recombinant *P. pastoris*

3.2.1 *P. pastoris* culturing, fatty acid extraction and conversion to fatty acid methyl esters (FAMEs)

Each type of recombinant *P. pastoris* (*P. pastoris* containing empty pGAPZ, pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E5, pGAPZ:E6, and pGAPZ:Δ6 &Δ5:E6) including wild type *P. pastoris* (SMD1168H) were inoculated in 5 ml YPD then incubated at 30°C with 200 rpm shaking overnight. Then 1% overnight cultured was inoculated into new YPD broth then incubated at 30°C with 200 rpm shaking 4 days in incubator shaker. At day 5, all *P. pastoris* (SMD1168H) and the recombinant *P. pastoris* were collected by centrifugation at 4,000 rpm for 20 minutes then stored at -80°C until use for omega-3 and omega-6 PUFAs determination.

For the fatty acid extraction, 90 ml of chloroform:methanol (2:1, v/v) was added to 30 grams of wet cells then break in blender. The samples were filtered by

filter paper (Ø70 mm). The filtered solution of samples were added with 30 ml chloroform, 30 ml DI water, and 50 ml 0.58% NaCl then wait until separated into 2 layers. The lower phase was collected and the water residue was removed by addition of Na_2SO_4. The samples were filtered by filter paper (Ø125 mm) then evaporated under stream of oxygen-free nitrogen (Folch et al., 1957).

For the FAMEs conversion, 1.5 ml of 0.5M methanolic NaOH was added to 25 mg of samples then heated at 80-100°C for 2 minutes while shaking. The 1 ml of internal standard C17 fatty acid in methanol and 2 ml BF_3 were added to the samples then heated for 30 minutes. The samples were cool down to room temperature then 1 ml of iso-octane was added for phase separation. Saturated NaCl solution 5 ml was added and the upper phase was collected. Two ml of iso-octane was added to the samples then the upper phase was collected again. All collected upper phase was dried with N_2 gas until only 1 ml left then transferred into vial (Ichihara and Fukubayashi, 2010). The FAMEs were measured by gas chromatography (GC) that no repeated.

3.2.2 PUFAs analysis by gas chromatography (GC)

One µl FAMEs of wild type *P. pastoris* (SMD1168H), *P. pastoris* with pGAP empty plasmid and each recombinant *P. pastoris* were carried out on an Agilent Technologies 7890A GC system equipped with the SP-2560 column (Supelco) (100 m x 250 µm i.d., thickness of 0.2 µm). Helium was used as a carrier gas. The program for analysis of FAMEs was modified from work of Mart´ınez-Monteagudo et al (2012) and KaršuLíNoVá et al (2007). The GC oven was initiated held at 70°C for 4 minutes then increased to 175°C for 27 minutes then heated to 215°C for 17 minutes, and then heated to 240°C for 10 minutes. The Supelco® 37

Component FAME Mix (47885-U) was used as standard. The target fatty acid composition of each recombinant *P. pastoris* was compared with the standard by similarity of retention time.

The percent total fat was calculated by [weight of fat after dried (g) / starter cells (g)] x 100. The percent of each fatty acid was performed by [amount of each FAMEs (µg/µl) / amount of total FAMEs (µg/µl)] x 100. The conversion efficiency was calculated by [amount of product (µg/µl) / (amount of product (µg/µl) + amount of substrate (µg/µl))] x 100.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Construction of expression plasmid and recombinant *P. pastoris*

The Nile tilapia Δ6 desaturase (Δ6) and the zebrafish bifunctional Δ6&Δ5 desaturase (Δ6&Δ5), Δ5 elongase (E5), and Δ6 elongase (E6) genes were amplified from total RNA by nested RT-PCR technique. Each of them was cloned into pGAPZ plasmid and transformed into *E. coli*. The transformed cells were confirmed by colony PCR technique. The plasmids were extracted and sequenced. Resulting sequence analysis indicated that the cloned Δ6 contained 1,335 bp coded for 445 amino acids. The amino acid sequence BLAST showed 99% identity with Nile tilapia (AGV52807.1), 82% with barramundi perch (ACY25091.2), 81% with both mandarin fish (ACH53604.1) and cobia (ACJ65149.1), 80% with the southern bluefin tuna (ADG62353.1), orange-spotted grouper (ACJ26848.1), and European seabass (ACD10793.1). The 1,332 bp Δ6&Δ5 coded for 444 amino acids with 99% identity with zebrafish (NP_571720.2), 91% with rohu (ABV01368.2), 89% with common carp, and 71% with Nile tilapia (XP_005470690.1).

The amino acid alignment comparing between each of the Δ6 and Δ6&Δ5 with other desaturases showed that both of our Δ6 and Δ6&Δ5 consisted of N-terminal cytochrome *b5* containing haem-binding motif (H-P-G-G), three histidine boxes and two trans-membrane regions similar to desaturases of other organisms (Figure 4.1-4.2). (Los and Murata, 1998).

The 873 bp E5 coded for 291 amino acids. The E5 amino acid sequence BLAST showed 100% identity with E5 of zebrafish (NP_956747.1), 91% with common carp (AER39745.1), 89% with grass carp (ADU04500.1), and 80% with Japanese eel (ACI32414.1). The 798 bp E6 coded for 266 amino acids. The E6 amino acid sequence BLAST showed 99% similarity with E6 of zebrafish (NP_955826.1), 93% with zebra mbuna (XP_004553671.1), Nile tilapia (XP_003443447.1), and fugu rubripes (XP_003970691.1), and 91% with channel catfish (NP_001187477.1).

The amino acid sequence alignment compared between each of the E5 and E6 with other E5 and E6 from other fishes showed that the E5 contained multiple putative trans-membrane regions, which are found in member of elongase family. A single HXXHH histidine box was also found between the 2nd and the 3rd trans-membrane regions (Figure 4.3). The E6 contained multiple putative trans-membrane regions and a single HXXHH that located between the 3rd and the 4th trans-membrane regions (Figure 4.4) (Carmona-Antoñanzas et al., 2013; Matsuzaka et al., 2002).

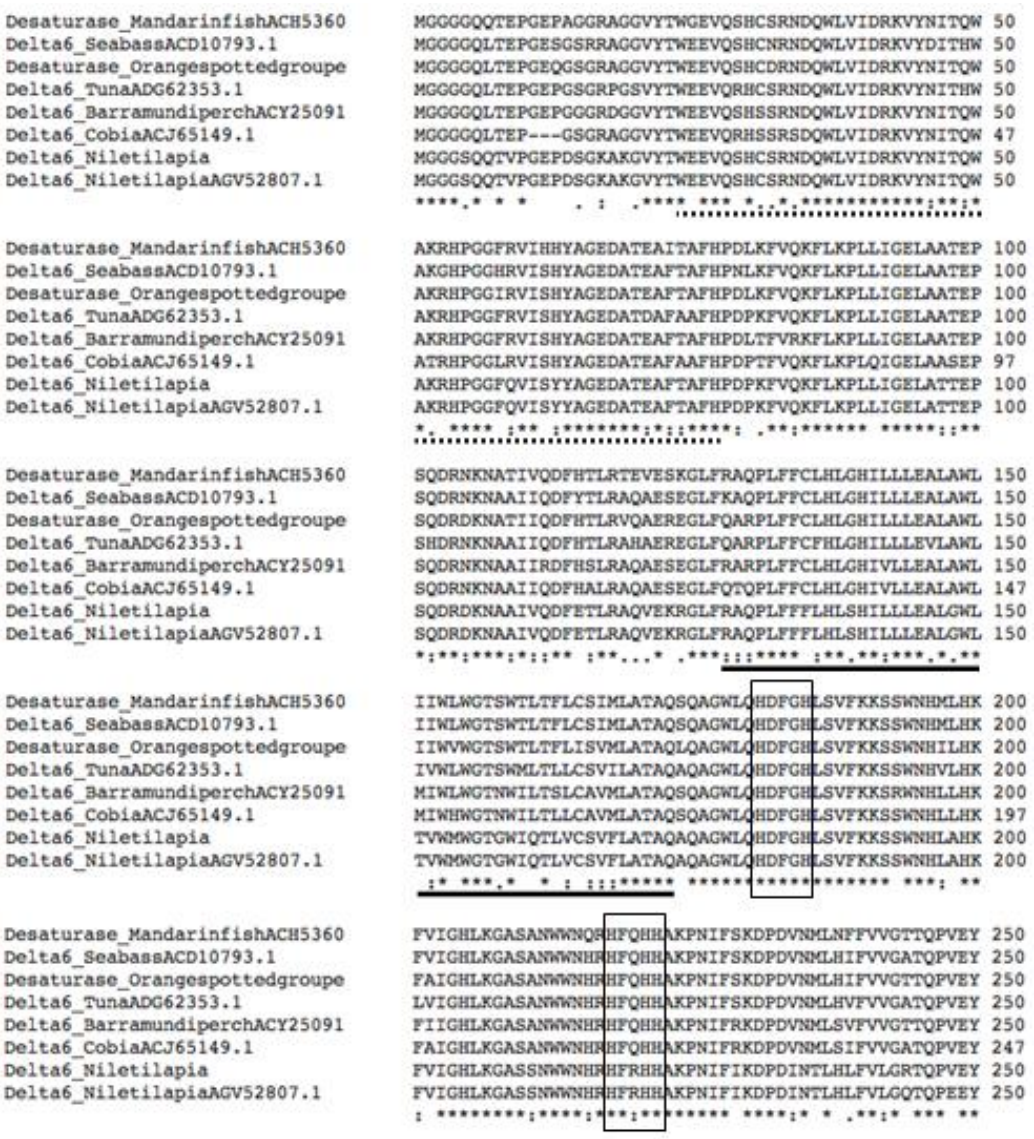

Figure 4.1 Alignment between the cloned Δ6 with Δ6 from other organisms. Dotted line: N-terminal cytochrome *b5* region; Solid lines: trans-membrane regions; Black frames: three histidine boxes; "*" indicates identical amino acid; ":" conserved substitutions and "."semi-conserved substitutions.

Figure 4.1 (Continued) Alignment between the cloned Δ6 with Δ6 from other organisms. Dotted line: N-terminal cytochrome *b5* region; Solid lines: trans-membrane regions; Black frames: three histidine boxes; "*" indicates identical amino acid; ":" conserved substitutions and "."semi-conserved substitutions.

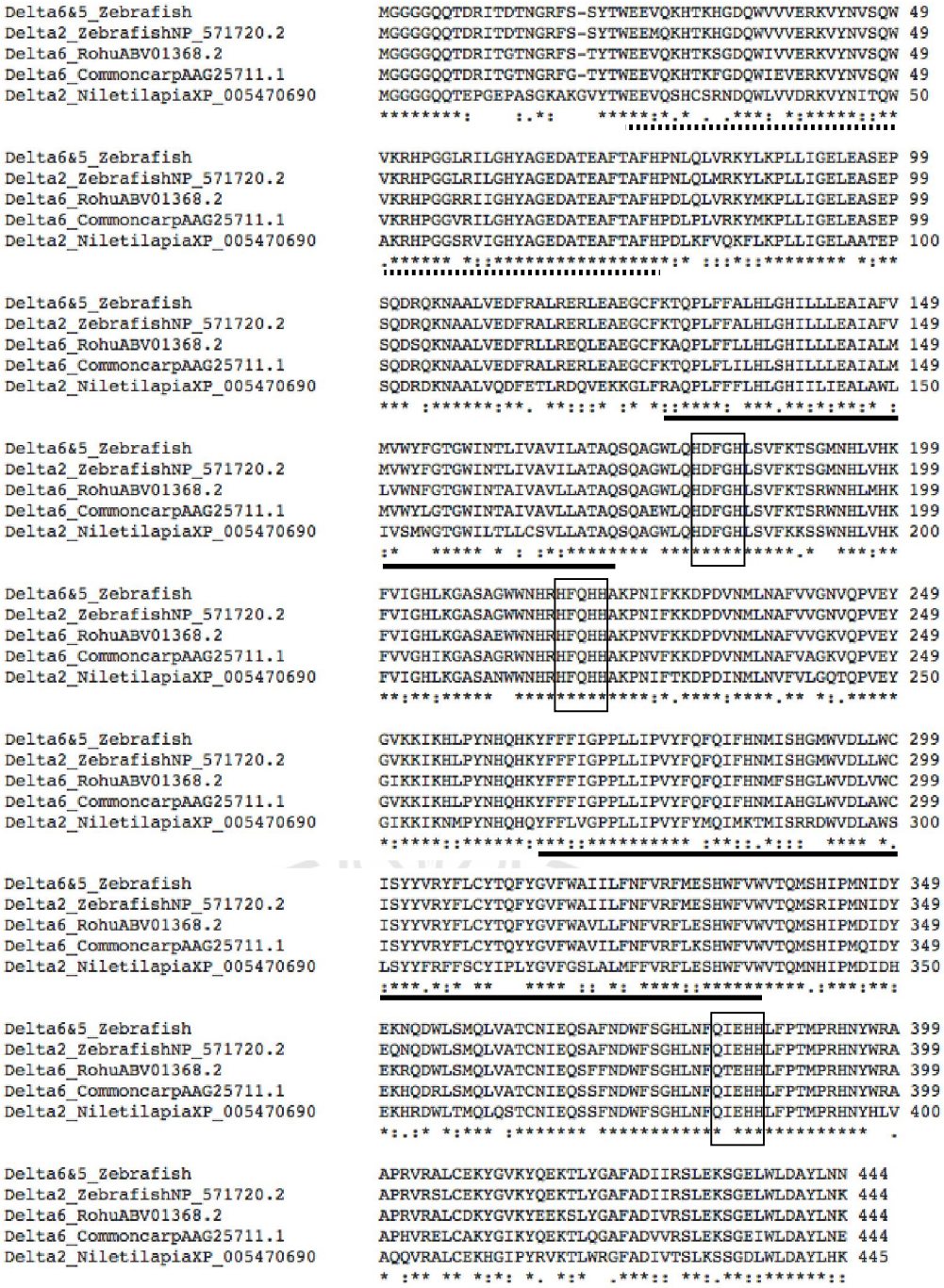

Figure 4.2 Alignment between the cloned Δ6&Δ5 with desaturases from other organisms. Dotted line: N-terminal cytochrome *b5* region; Solid lines: trans-membrane regions; Black frames: three histidine boxes; "*" indicates identical amino acid, ":" conserved substitutions and "."semi-conserved substitutions.

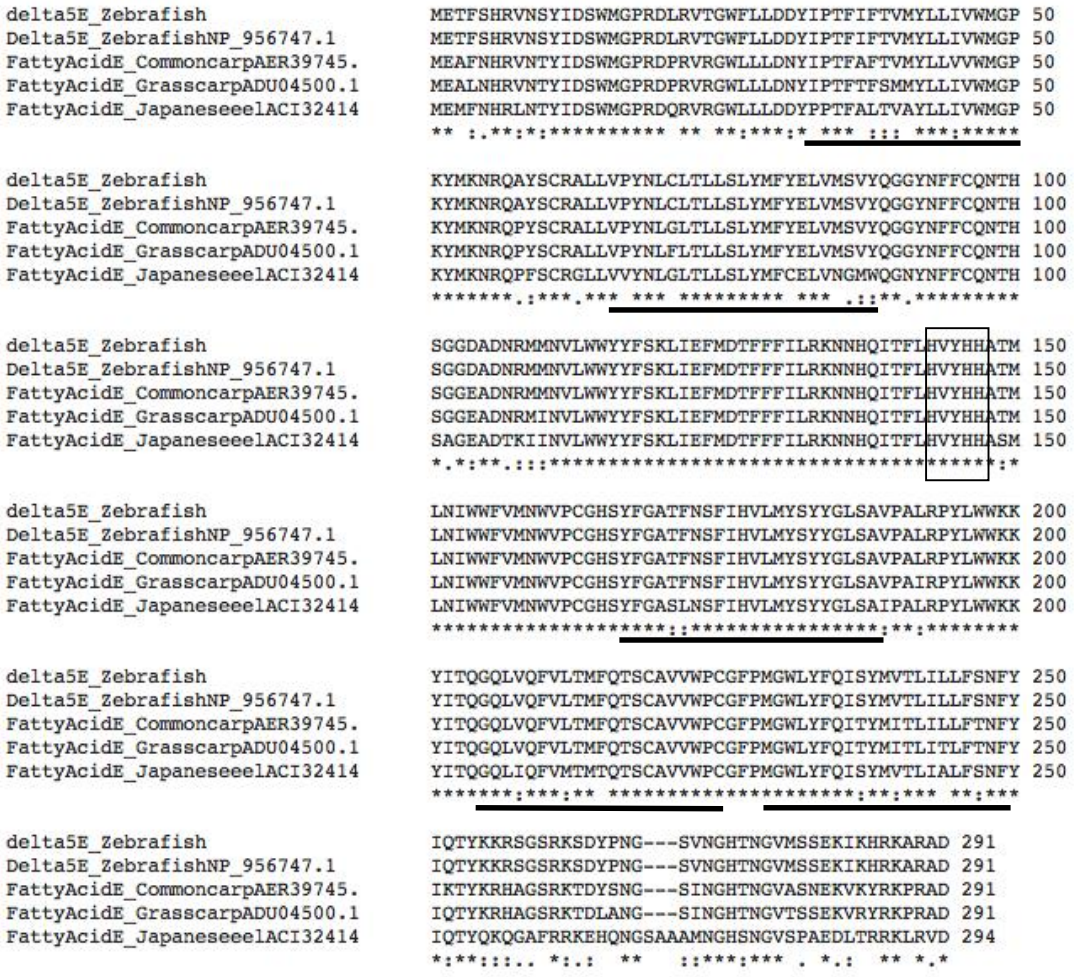

Figure 4.3 Alignment between the cloned E5 with elongases from other organisms. Solid lines: trans-membrane regions; Black frame: histidine box; "*" indicates identical amino acid; ":" conserved substitutions and "." semi-conserved substitutions.

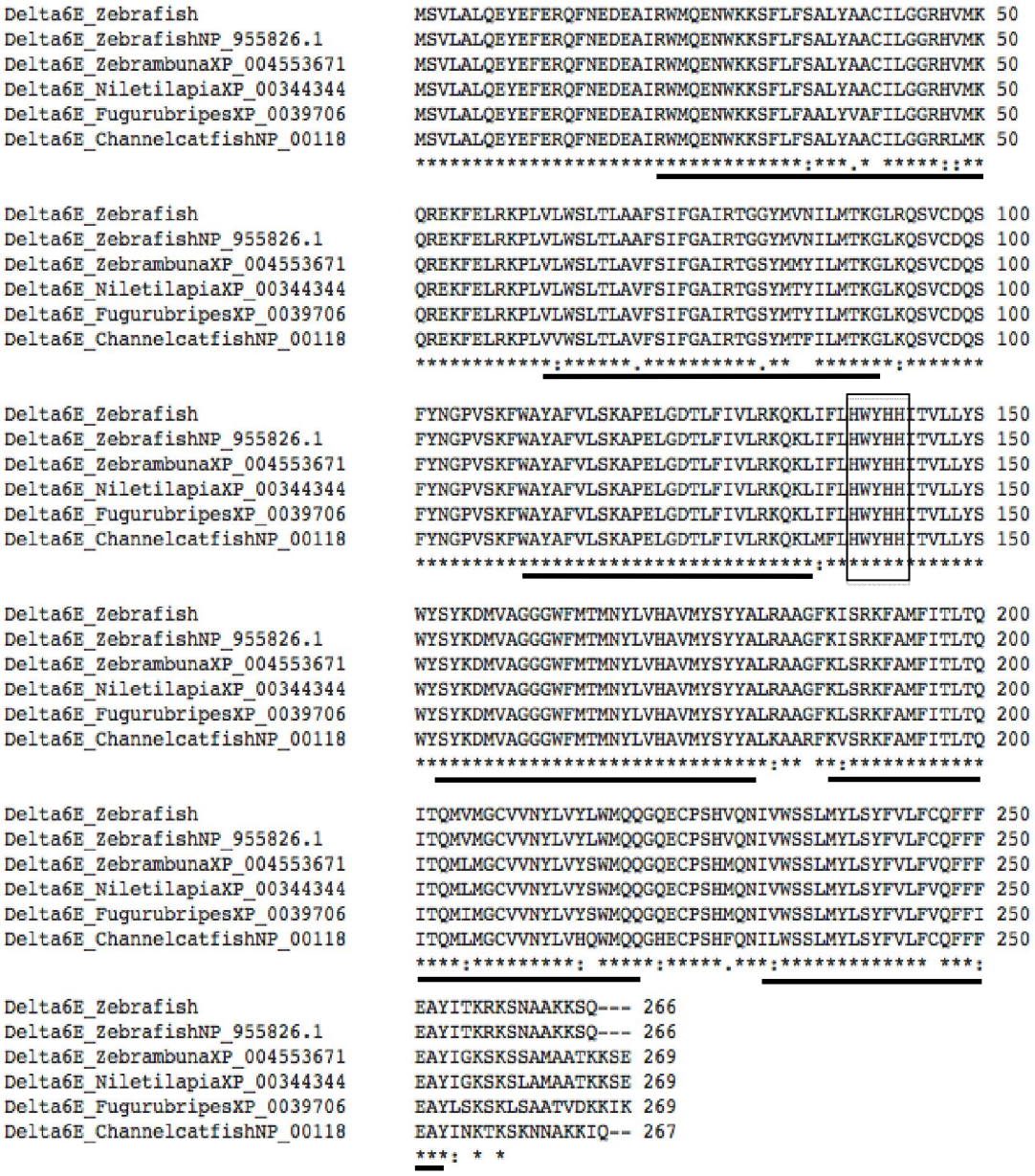

Figure 4.4 Alignment between the cloned E6 with E6 from other organisms. Solid lines: trans-membrane regions; Black frame: histidine box; "*" indicates identical amino acid; ":" conserved substitutions and "."semi-conserved substitutions.

Each recombinant plasmid (pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E5, pGAPZ:E6, and pGAPZ:Δ6&Δ5:E6) including empty pGAPZ plasmid were linearized by *Avr* II then transformed into *P. pastoris* (SMD1168H) by electroporation technique. Each recombinant *P. pastoris* was confirmed by colony PCR technique using pGAPZ plasmid and specific gene primers. Each recombinant *P. pastoris* was shown to contain the expected band size (Figure 4.5-4.10).

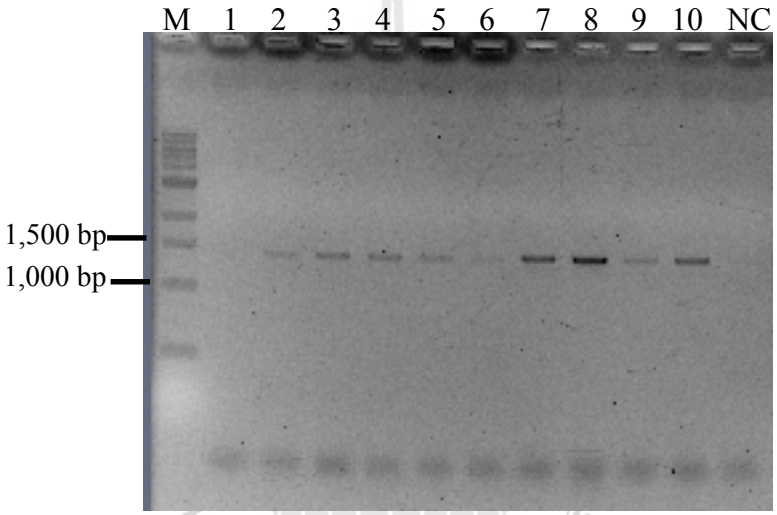

Figure 4.5 Colony PCR of recombinant *P. pastoris* containing pGAPZ:Δ6 using pGAPZ plasmid forward and Δ6 reverse primers. Lane M: 1 kb marker (NEB); Lane 1-10: clone 1-10; Lane NC: negative control.

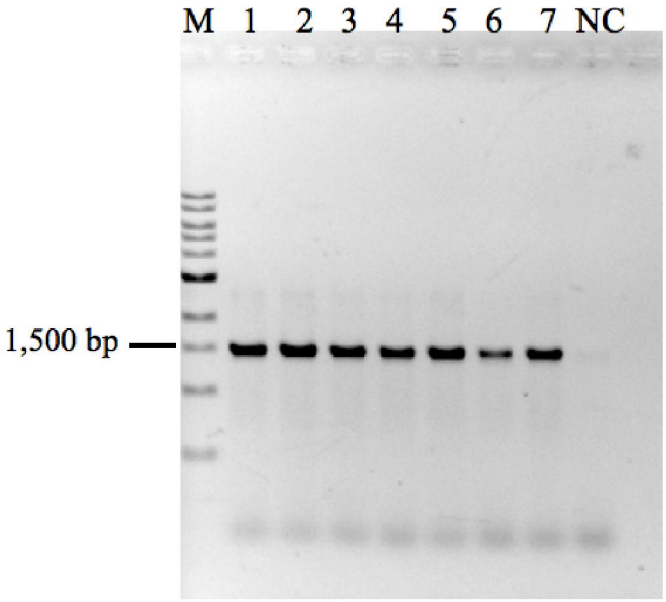

Figure 4.6 Colony PCR of recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5 using Δ6&Δ5 forward and pGAPZ plasmid reverse primers. Lane M: 1 kb marker (NEB); Lane 1-7: clone 1-7; Lane NC: negative control.

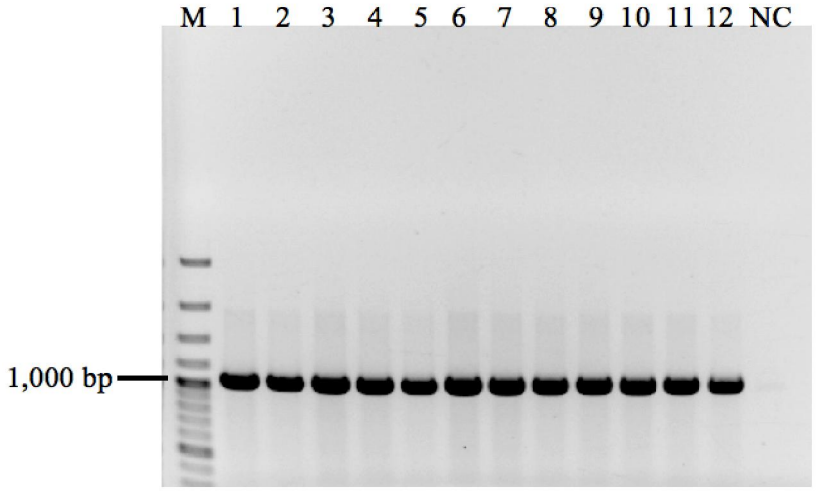

Figure 4.7 Colony PCR of recombinant *P. pastoris* containing pGAPZ:E5 using E5 forward and pGAPZ plasmid reverse primers. Lane M: 100 bp marker (Fermentas); Lane 1-12: clone 1-12; Lane NC: negative control.

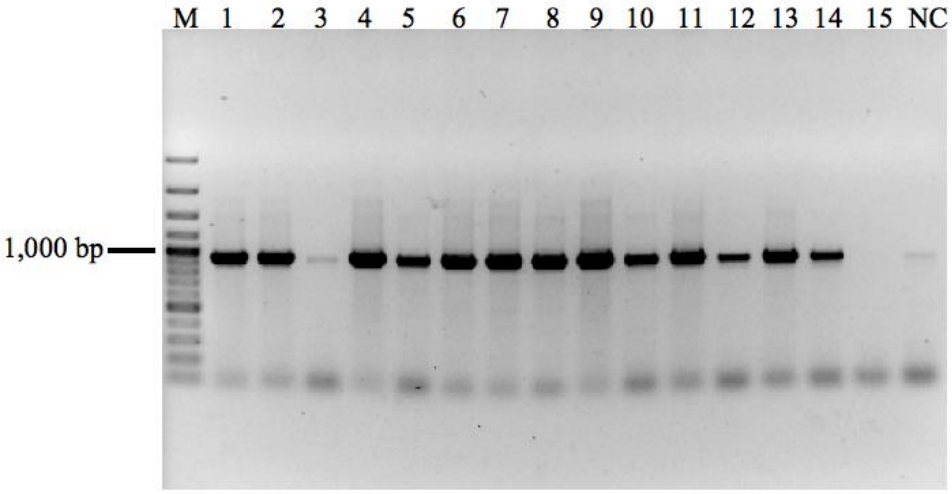

Figure 4.8 Colony PCR of recombinant *P. pastoris* containing pGAPZ:E6 using E6 forward and pGAPZ plasmid reverse primers. Lane M: 100 bp marker (Fermentas); Lane 1-15: clone 1-15; Lane NC: negative control.

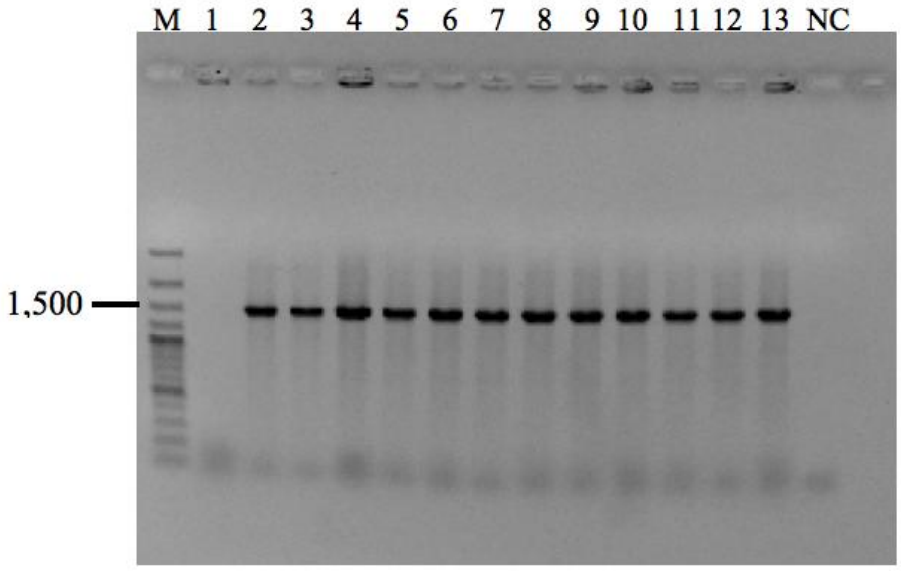

Figure 4.9 Colony PCR of recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6 using Δ6&Δ5 forward and pGAPZ plasmid reverse primers. Lane M: 100 bp marker (Fermentas); Lane 1-13: clone 1-13; Lane NC: negative control.

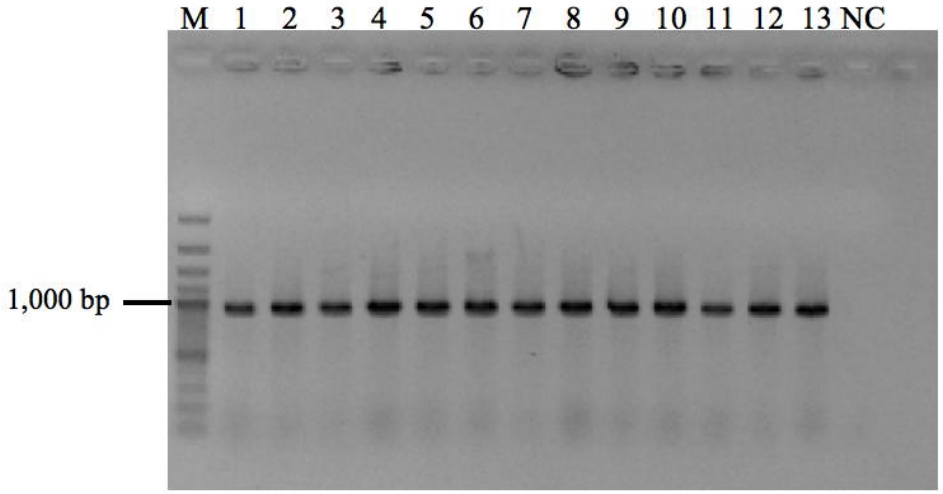

Figure 4.10 Colony PCR of recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6 using E6 forward and pGAPZ plasmid reverse primers. Lane M: 100 bp marker (Fermentas); Lane 1-13: clone 1-13; Lane NC: negative control.

Since, the pGAPZ plasmid contains *Sh ble* (*streptoalloteichus hindustanus* bleomycin) gene, which confirm the resistance to Zeocin. The tolerance to high concentration of Zeocin antibiotic has shown to predict the high copy number of recombinant plasmid integrated into chromosome of *P. pastoris*. The review of Daly and Hearn (2004) showed that transformed *P. pastoris* resistant to 100, 500, 1,000, and 2,000 μg/mL of Zeocin contained 1, 2, 3, and 4 copies of recombinant pGAPZ plasmid. In addition, Sarramegna et al. (2002) study found that recombinant *P. pastoris* resistant to 1,000 μg/mL Zeocin antibiotic contained 15–25 copy numbers of recombinant pPICZ plasmid. The events of multiple recombinant plasmid insertion are occurred spontaneously in cell at a low but detectable frequency (1-10% of selected Zeocin transformants) (Sarramegna et al., 2002; Haggins and Cregg, 1998; www.invitrogen.com). The factors for higher copy number insertion of AOX promoter (pPICZ plasmid) than GAP promoter (pGAPZ plasmid) might be diverse

site for homologous recombination. For example, the insertion at AOX I has occurred at homologous sequence between yeast genome and any of the three AOX I regions in the plasmid: (i) AOX I promoter; (ii) the AOX I transcription termination regions (TT), or (iii) sequence even further downstream of AOX I (3' AOX I) (Balamurugan et al., 2007). Therefore, transformant yeasts needed to be screen to find "jack-pot" clones. In this work, each recombinant *P. pastoris* type was restreaked on YPD plate containing different concentrations of Zeocin (100, 200, 300, 400, and 500 μg/ml). When restreak on YPD containing 500 μg/ml Zeocin, not every clone of the recombinant *P. pastoris* were able to grow (Table 4.1). The reasons might be come from size, concentration of linearized transformant and the process for preparation of the competent yeast cell. However, in this work the numbers of plasmid copy were not determined. But one of each recombinant *P. pastoris* type that can grow in high (500 μg/ml) Zeocin was selected and fatty acid compositions were determined.

Table 4.1 Number of recombinant *P. pastoris* clone on YPD with 100 and 500 μg/ml of Zeocin.

Type of recombinant pGAPZ plasmid	**Number of clone on different concentration of Zeocin**	
	100 μg/ml	**500 μg/ml (%)**
pGAPZ:Δ6	10	2 (20%)
pGAPZ:Δ6&Δ5	7	2 (29%)
pGAPZ:E5	12	4 (33%)
pGAPZ:E6	14	2 (14%)
pGAPZ:Δ6&Δ5:E6	13	4 (31%)

4.2 Polyunsaturated fatty acid analysis by gas chromatography (GC)

In this work, the function of Δ6, Δ6&Δ5, E5, and E6 were determined. The wild type *P. pastoris* (SMD1168H) and each recombinant *P. pastoris* were cultured in YPD broth for 4 days. At day 5, total fat were extracted and methylated to FAMEs then the fatty acid compositions were measured by GC.

In general, long chain fatty acids such as palmitic acid (PA, C16), palmitoleic acid (POA, C16:1), margaric acid (MA, C17), ginkgolic acid (GA, C17:1), stearic acid (SA, C18), oleic acid (OA, C18:1), linoleic acid (LA, C18:2n-6), and alpha-linolenic acid (ALA, C18:3n-3) are endogenous fatty acid in *P. pastoris* (Li et al., 2009; Zhang et al., 2005). The long chain fatty acids (PA, POA, MA, GA, SA, OA, LA, and ALA) were detected in *P. pastoris* (SMD1168H) and recombinant *P. pastoris* containing empty pGAPZ plasmid, which were used as control group in this work (Figure 4.12-4.13).

Both LA and ALA (No. 1 and 2 in figure 4.11) are the substrates in the omega-6 and the omega-3 PUFAs pathways (conventional Δ6 pathway), which can be converted to other PUFAs in their pathway by desaturases and elongases activities. The Δ6 has been shown to act in the first step, which is the rate-limiting step in the biosynthesis pathway of PUFAs (Baylin et al., 2007; Zheng et al., 2004). Furthermore, other desaturase (Δ5) and elongase (E5 and E6) are also presented in this pathway (Figure 4.11) (Zheng et al., 2004). Therefore, the function of Δ5, Δ6, E5, and E6 were determined.

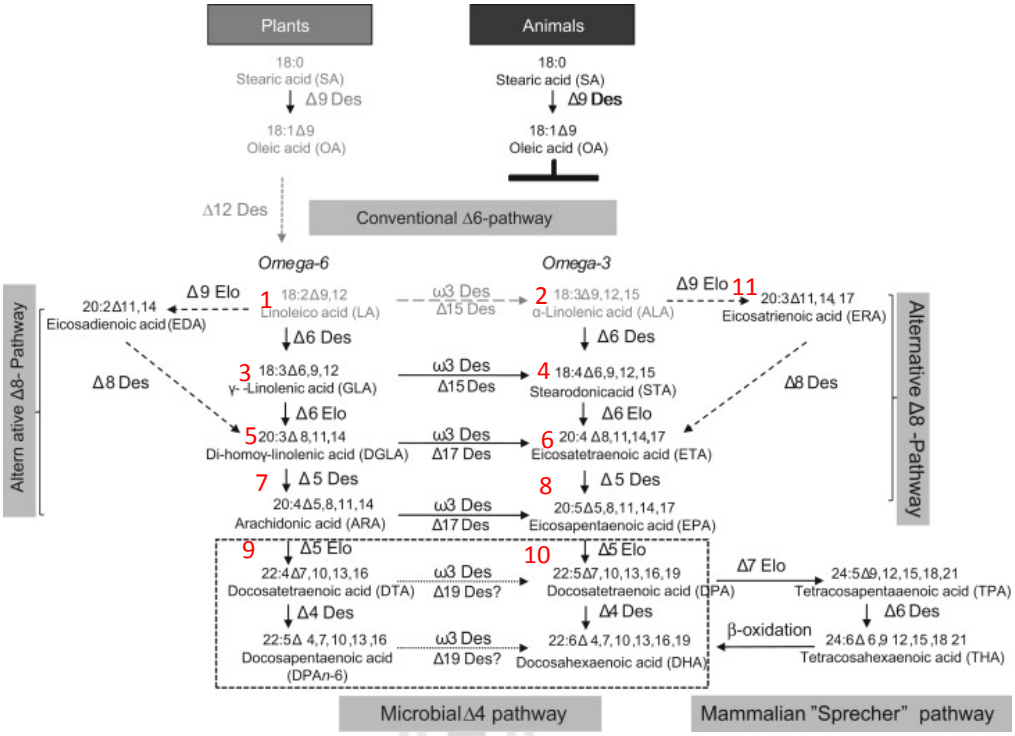

Figure 4.11 PUFAs biosynthesis pathway.

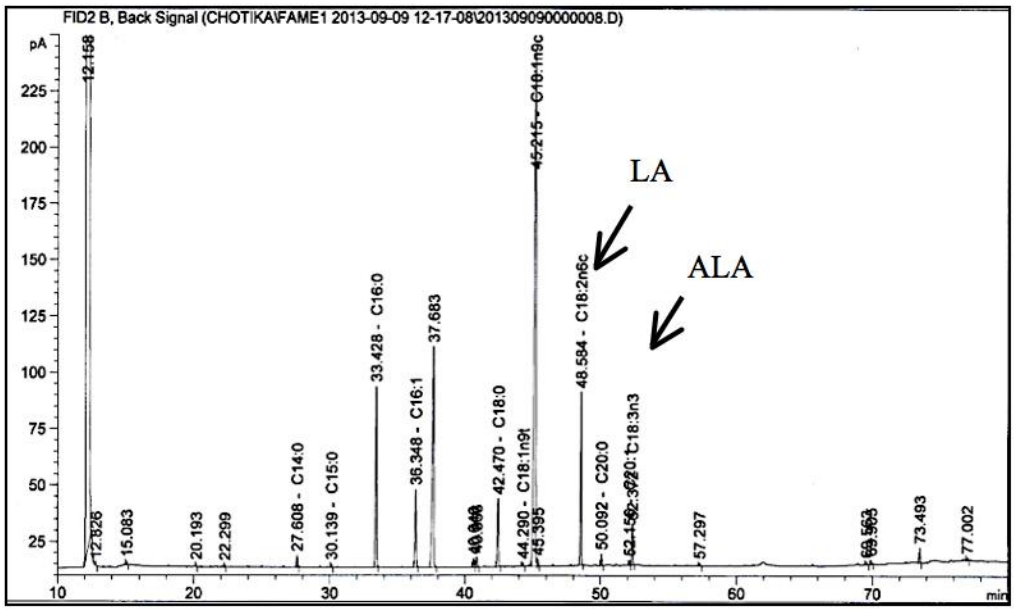

Figure 4.12 Identification of fatty acid profile of *P. pastoris* (SMD1168H). LA is Linoleic acid (LA, C18:2n-6) and ALA is alpha-linolenic acid (ALA, C18:3n-3).

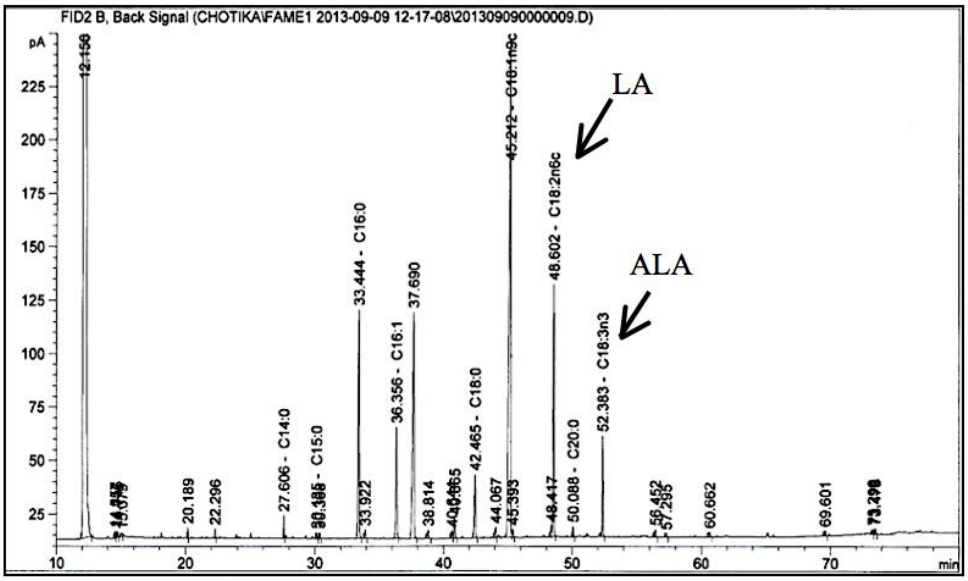

Figure 4.13 Identification of fatty acid profile of *P. pastoris* containing empty pGAPZ plasmid. LA is Linoleic acid (LA, C18:2n-6) and ALA is alpha-linolenic acid (ALA, C18:3n-3).

Recombinant *P. pastoris* containing pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E5, and pGAPZ:E6 were constructed and cultured in YPD for four days and then the fatty acid compositions were determined. The GC analysis results indicated that recombinant *P. pastoris* with pGAPZ:Δ6 and pGAPZ:Δ6&Δ5 contained the LA and ALA (No. 1 and 2 in figure 4.11). In addition, both of them present a new peak of gamma-linolenic acid omega-6 PUFAs (GLA, C18:3n-6) (No. 3 in figure 4.11), which is the product from Δ6 activity. Whereas, the stearodonic acid omega-3 PUFAs (STA, C18:4n-3) (No. 4 in figure 4.11) could not be observed due to the limitation of the FAMEs standard that used at The Center for Scientific and Technology Equipment of SUT (Figure 4.14-4.15). The recombinant *P. pastoris* containing pGAPZ:Δ6, and pGAPZ:Δ6&Δ5 had 10.6 and 8.9% conversion from LA to GLA, respectively.

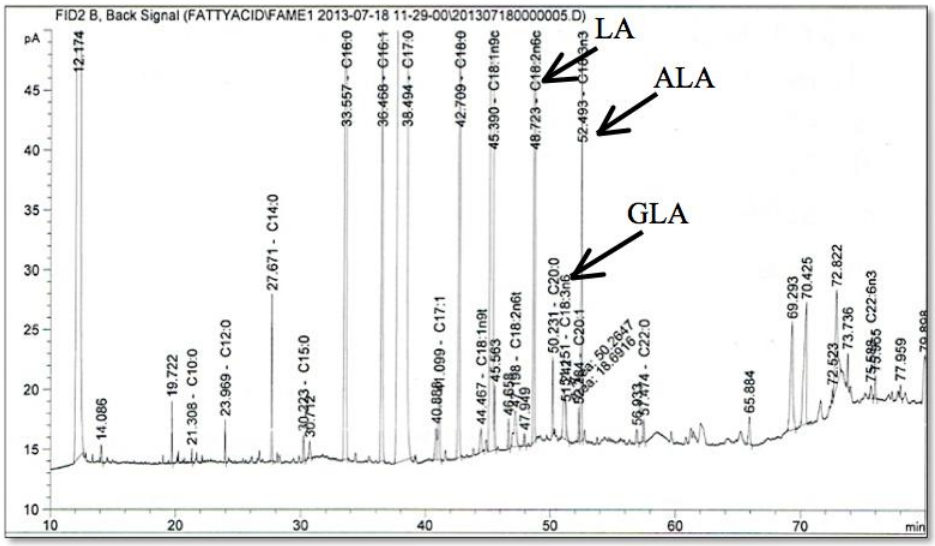

Figure 4.14 Identification of fatty acid profile of *P. pastoris* containing pGAPZ:Δ6. LA is Linoleic acid (LA, C18:2n-6), ALA is alpha-linolenic acid (ALA, C18:3n-3), and GLA is gamma-linolenic acid (GLA, C18:3n-6).

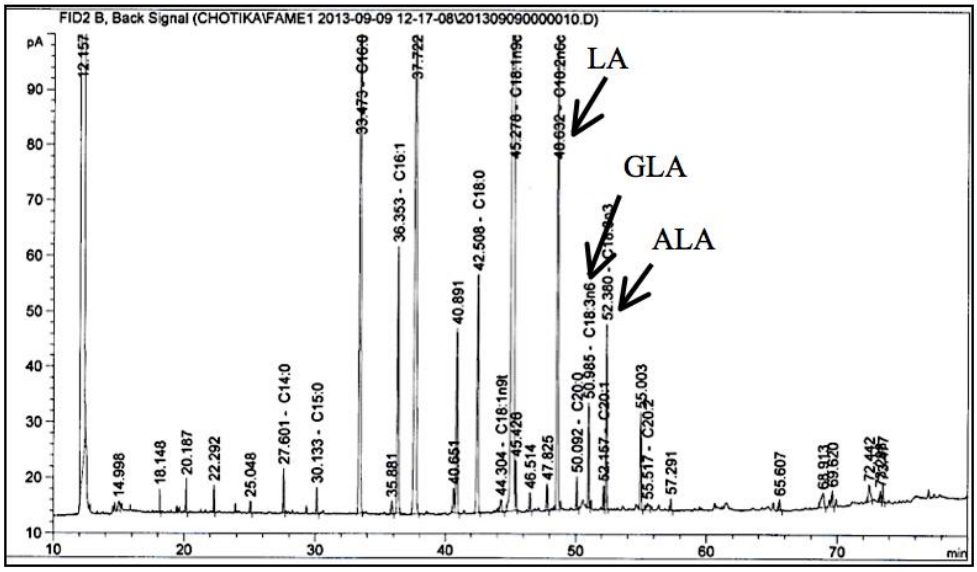

Figure 4.15 Identification of fatty acid profile of *P. pastoris* containing pGAPZ:Δ6&Δ5. LA is Linoleic acid (LA, C18:2n-6), ALA is alpha-linolenic acid (ALA, C18:3n-3), and GLA is gamma-linolenic acid (GLA, C18:3n-6)

Several studies have shown that GLA and STA can be produced from LA and ALA, respectively in recombinant *P. pastoris.* Zhang et al. (2005) shown that *Rhizopus arrhizus* Δ6 subcloned into pPIC3.5K then transformed into *P. pastoris* could produce both GLA and STA. In addition, Wan et al. (2009) had expressed pHBM:Δ6 derived from *Cunninghamella echinulata* in *P. pastoris.* The result showed that the GLA was produced.

Normally, GLA (C18:3Δ6,9,12) is produced by addition of double bond at the 6^{th} carbon atom from the carboxyl end of the LA chain using Δ6 activity. However, the Δ6 not only introduce double bond at the 6^{th} position from the carboxyl end of LA chain (C18:3Δ9,12) but it is also act on the substrate C15, C16, C17, C18, C18:1, and tetracosapentaenoic acid (TPA, C24:5n-3) (Wan et al., 2009; Zhang et al., 2005). Their results suggested that Δ6 might have no specificity on fatty acid chain-length but have position specificity. Another yeast, *Saccharomyces cerevisiae* has also been used for expression of Δ6 (Tanomman et al., 2013; Kurdrid et al., 2005; Zhang et al., 2004; Laoeng et al., 2000). However, unlike *P. pastoris*, *S. cerevisiae* cannot produce endogenous substrate LA and ALA due to the lack of Δ12 desaturase (Δ12) and Δ15 desaturase (Δ15) activity (Zhang et al., 2005). Consequently, the expression by this model organism *S. cerevisiae* has to add the substrate LA and ALA into the culturing medium to determine the Δ6 activity. Moreover, Wan et al. (2011) expressed *R. stolonifer* Δ6 in *S. serevisiae* and showed that lower percentage of GLA was detected when compared with *P. pastoris*.

The recombinant *P. pastoris* containing pGAPZ:E5 and pGAPZ:E6 contained peaks similar to control group including LA and ALA (Figure 4.16-4.17) without any new peak detected in conventional Δ6 pathway (Figure 4.11). Since, the function of E5 is to convert ARA to docosatetraenoic aicd (DTA, C22:4n-6) (No. 7 and 9 in

figure 4.11) and from EPA to docosapentanoic acid (DPA, C22:5n03) (No. 8 and 10 in figure 4.11) in omega-6 and omega-3 PUFAs pathways, respectively. The function of E6 is to convert GLA to di-homo gamma-linolenic acid (DGLA, C20:3n-6) (No. 3 and 5 in figure 4.11) and stearodonic acid (STA, C18:4n-3) to eicosatetraenoic acid (ETA, C20:4n-3) (No. 4 and 6 in figure 4.11) in omega-6 and omega-3 PUFAs pathways, respectively. In this work, the recombinant *P. pastoris* containing pGAPZ:E5 and pGAPZ:E6 did not produced DPA, DTA, DGLA, and ETA due to the lack of the substrates ARA, EPA, GLA, and STA, respectively. Interestingly, the recombinant *P. pastoris* containing pGAPZ:E5 presented peak of eicosatrienoic acid (ERA, C20:3n-3) (No. 11 in figure 4.11), which is the product from E5 activity in the alternative Δ8 pathway (Figure 4.11). This result indicated that this E5 did not have only function for conversion from C20 to C22 (ARA to DTA or EPA to DPA) only but also showed activity on C18 PUFAs. Agaba et al. (2004) showed that zebrafish elongase (ZfELO: accession number AF532782) presented the multifunctional of elongation on C18, C20, and C22 PUFAs. Moreover, this ZfELO has preferred to convert C18 > C20 > C22 of omega-3 PUFAs more than omega-6 PUFAs. In addition, the result from BLAST and alignment showed high percent identity between ZfELO and E5 of this work. Therefore, this E5 might be contained the multifunctional similar to ZfELO.

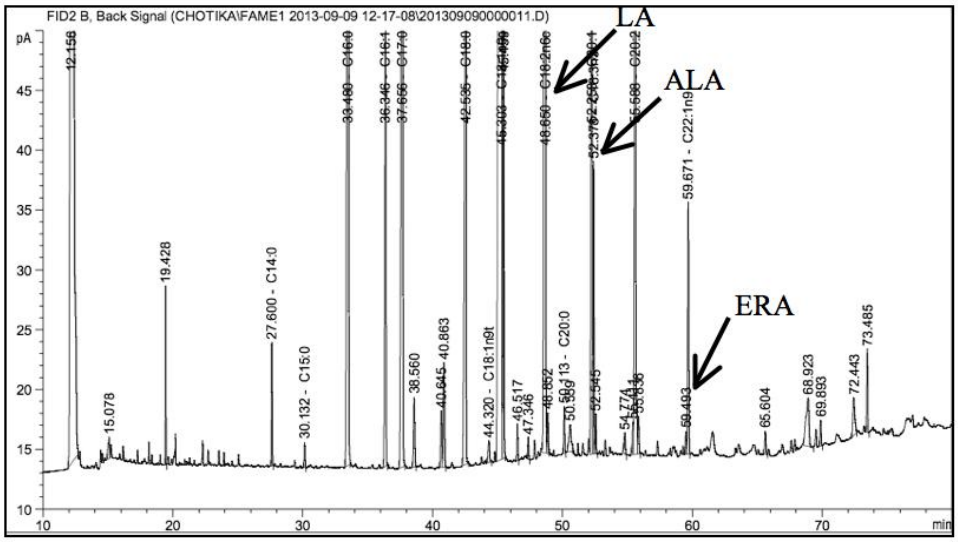

Figure 4.16 Identification of fatty acid profile of *P. pastoris* containing pGAPZ:E5. LA is Linoleic acid (LA, C18:2n-6) and ALA is alpha-linolenic acid (ALA, C18:3n-3).

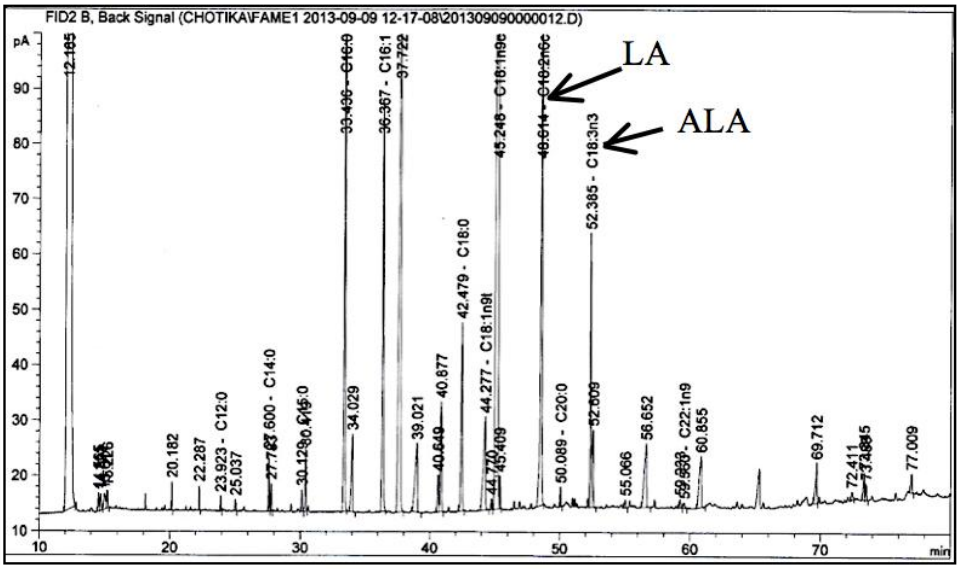

Figure 4.17 Identification of fatty acid profile of *P. pastoris* containing pGAPZ:E6. LA is Linoleic acid (LA, C18:2n-6) and ALA is alpha-linolenic acid (ALA, C18:3n-3).

In this work, we also aimed to construct recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E5:E6. Unfortunately, the E5 gene contained the *Bgl* II and *Bam* HI sites, therefore, the pGAPZ:E5 could not be used for the construction of expression cassette plasmid. Consequently, In this work, only recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6 was constructed then cultured and the fatty acid compositions was determined by GC. The result showed that this recombinant yeast also contained peaks of LA, ALA, and GLA (No. 1, 2, and 3 in figure 4.11) that are similar to the recombinant *P. pastoris* containing pGAPZ:Δ6 and pGAPZ:Δ6&Δ5. This yeast had 4.5% conversion from LA to GLA. Furthermore, the new peaks of arachidonic acid omega-6 PUFAs (ARA, C20:4n-6) and eicosapentanoic acid omega-3 PUFAs (EPA, C20:5n-3) (No. 7 and 8 in figure 4.11) were observed (Figure 4.18). Both of ARA and EPA are product of Δ5 activity. They were 0.4 and 0.5% of the total fatty acid, respectively. These results indicated that this recombinant *P. pastoris* contained the activity of the Δ6, Δ5, and E6. Therefore, we can stated that, in this Δ6&Δ5 and E6 recombinant *P. pastoris* the ARA and EPA biosynthesis was detected.

Several researches have studied the production of ARA and EPA in *P. pastoris*. Kajikawa et al. (2004) had constructed the *P. pastoris* containing co-expression of pPICZA:Δ6, pPIK3K:elongase-like, and pPIC6A:Δ5 that derived from *Marchantia polymorpha.* The result showed that recombinant *P. pastoris* containing all of three recombinant plasmids could reconstitute ARA 0.1% and EPA 0.03% of total fatty acid in cell. Furthermore, Li et al. (2009) had improved the production of ARA and EPA in *P. pastoris* by increase the copy number of co-expressed pAO:Δ6:E6:Δ5 derived from *Phaeodactylum tricornutum*. The result showed that recombinant *P. pastoris* containing one copy number of expression cassette recombinant plasmid could produce ARA 0.1% and EPA 0.05% of total

fatty acid in cell. When they increase into two copy numbers of expression cassette recombinant plasmid has improved the generation of ARA and EPA into 0.3 and 0.1%, respectively. Conclusion, this recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6 showed higher accumulation of ARA and EPA than two previous works. The reason might be come from several factors: (i) different promoter for driving the gene, both co-expressed pPICZA:Δ6, pPIK3K:elongase-like, and pPIC6A:Δ5 derived from *M. polymorpha* and pAO:Δ6:E6:Δ5 derived from *Phaeodactylum tricornutum* using alcoholdehydrogenase (AOX) promoter and our recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6 using GAP promoter. The constitutive GAP promoter always drive gene for encoding protein that converted the LA and ALA to ARA and EPA, respectively. In contrast, the inducible AOX promoter has to induce for protein expression. (ii) The period for culturing, the long period for culturing might be result in more accumulation of ARA and EPA. This work, recombinant *P. pastoris* was cultured for four days longer than the two previous works that cultured for three days. (iii) The multiple gene insertion, both of two previous works did not screen for multiple gene insertion. Therefore, the colony that used for fatty acid determination might be contained low copy number of recombinant plasmid. Consequently, that recombinant *P. pastoris* has contained low level of protein expression, therefore low level of ARA and EPA accumulation.

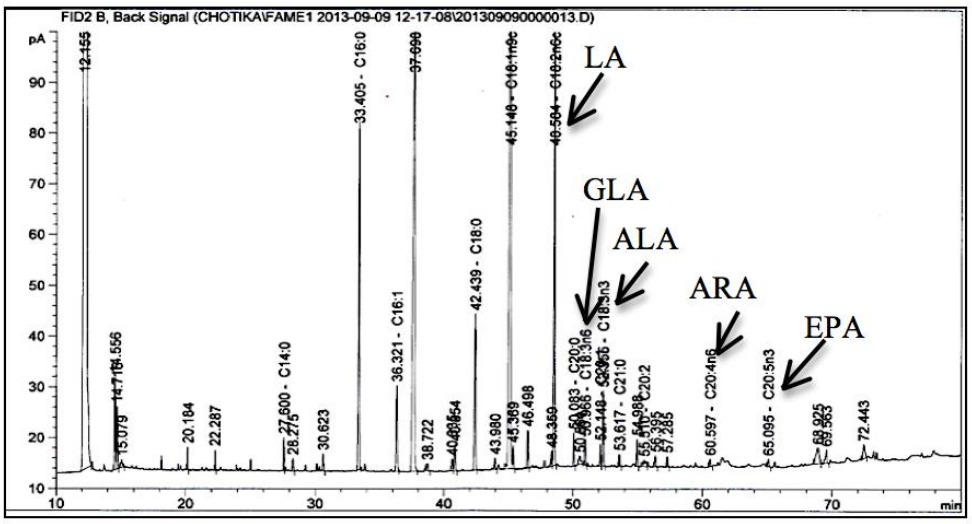

Figure 4.18 Identification of fatty acid profile in *P. pastoris* containing pGAPZ: Δ6&Δ5:E6. LA is Linoleic acid (LA, C18:2n-6), ALA is alpha-linolenic acid (ALA, C18:3n-3), GLA is gamma-linolenic acid (GLA, C18:3n-6), ARA is arachidonic acid (ARA, C20:4n-3), and EPA is eicosapentanoic acid (EPA, C20:5n-3).

CHAPTER V

CONCLUSION

Nile tilapia Δ6 desaturase (Δ6) and zebrafish bifunctional Δ6&Δ5 desaturase (Δ6&Δ5), Δ5 elongase (E5), and Δ6 elongase (E6) were cloned into pGAPZ plasmid then sequenced. The Δ6 contained 1,335 bp coded for 445 amino acids which showed 99% similarity with the Nile tilapia (AGV52807.1). The Δ6&Δ5 contained 1,332 bp coded for 444 amino acids that was 99% similar with zebrafish (NP_571720.2). The E5 was 873 bp in length and coded for 291 amino acids with 100% similarity with zebrafish (NP_956747.1). The E6 contained 798 bp coded for 266 amino acids that was 99% similar to zebrafish (NP_955826.1).

The amino acid alignments of both Δ6 and Δ6&Δ5 indicated several important regions, which are found in desaturase family. There were N-terminal cytochrome *b5* containing haem-binding motif (H-P-G-G), three histidine boxes which are active site of the enzyme, and two trans-membrane regions. The E5 and E6 contained important regions that are found in elongase family. These were multiple trans-membrane regions and a single HXXHH histidine box.

Recombinant *P. pastoris* were constructed by transformation of linearized recombinant plasmids. Each recombinant *P. pastoris* was confirmed for the present of the different recombinant plasmid by colony PCR technique. The pGAPZ forward and specific reveres primers were used for the confirmation of recombinant *P. pastoris* containing pGAPZ:Δ6. The specific forward and pGAPZ reverse primers

were used for checking recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5, pGAPZ:E5 and pGAPZ:E6. The results showed that each recombinant *P. pastoris* contained the band size similar to that of the reference sequence database from GenBank. The recombinant *P. pastoris* containing each of Δ6 and Δ6&Δ5 showed 1,500 bp bands, which includes the gene and the multiple cloning site of pGAPZ plasmid. The recombinant *P. pastoris* containing the E5 and E6 showed 1,000 bp bands, which includes the gene and the multiple cloning site of pGAPZ plasmid. Each recombinant *P. pastoris* was restreaked on YPD plate containing different concentration of Zeocin (100-500 μg/ml). Some of the recombinant *P. pastoris* clones could grow on YPD containing 500 μg/ml Zeocin. The ability to grow on high level of Zeocin indicates that these clones might have multiple copy of the inserted plasmid.

The *P. pastoris* (SMD1168H) and each recombinant *P. pastoris* containing pGAPZ (empty plasmid), pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E5, and pGAPZ:E6 were cultured and the fatty acid compositions were determined by GC. The results indicated that no new peaks were detected in the recombinant *P. pastoris* containing empty pGAPZ plasmid, pGAPZ:E5, and pGAPZ:E6 when compared to *P. pastoris* (SMD1168H). The GC analysis results indicated that the recombinant *P. pastoris* FAMEs containing pGAPZ:Δ6, pGAPZ:Δ6&Δ5, and pGAPZ:Δ6&Δ5:E6 contained new peaks that represented GLA omega-6 PUFAs. The recombinant *P. pastoris* containing pGAPZ:Δ6, pGAPZ:Δ6&Δ5, and pGAPZ:Δ6&Δ5:E6 have 10.6, 8.9, and 4.5% conversion from LA to GLA, respectively. The GC analysis of recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6 FAMEs contained new peaks of ARA omega-6 PUFAs, and EPA omega-3 PUFAs. Both ARA and EPA were about 0.4 and

0.5% of the total fatty acid, respectively. In the future, cultivation method should be developed to increase the accumulation of ARA and EPA in these recombinant *P. pastoris*. Moreover, these recombinant *P. pastoris* with high PUFAs can be used as supplement in economic animal feed, e.g. chicken and fish to increase their quality and price.

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BIOGRAPHY

Ms. Chotika Gosalawit was bone on June 13, 1986 in Sakaeo, Thailand. She graduated with a bachelor degree in Marine Science from Kasetsart University in 2008. First two semesters, she had supplied to study Master degree course in school of Biotechnology, Institute of Agricultural technology, Suranaree University of Technology with Dr. Parinya Noisa. Thereafter, Assoc. Prof. Dr. Mariena Ketudat-Cairns is her advisor. While studying, she received a Graduate scholarship from SUT to support her tuition and fee. Her research topic was production of *Pichia pastoris* with high polyunsaturated fatty acid. The results from some part of this study have been presented as poster and oral presentation at (1) The 24th Annual Meeting of Thai Society for Biotechnology, Ubon Ratchathani 29-30 Nov. 2012. (2) The 1st School of Biotechnology International Colloquium, Nakorn Ratchasima 18-19 Jul. 2012. (3) The 2nd School of Biotechnology International Colloquium, Nakorn Ratchasima 25-26 Sep. 2013. (4) The 25th Annual Meeting of Thai Society for Biotechnology, Bangkok 16-19 Oct. 2013.