MOLECULAR CLONING AND FUNCTIONAL CHARACTERIZATION OF A NOVEL ELOVL (ELONGASE) FAMILY FROM A FRESHWATER TELEOST (*Oreochromis niloticus*)

HAN WAN YIN

UNIVERSITI SAINS MALAYSIA

2017

MOLECULAR CLONING AND FUNCTIONAL CHARACTERIZATION OF A NOVEL ELOVL (ELONGASE) FAMILY FROM A FRESHWATER TELEOST (*Oreochromis niloticus*)

by

HAN WAN YIN

Thesis submitted in fulfilment of the requirements

for the degree of

Master of Science

June 2017

ACKNOWLEDGEMENT

It would not have been possible task to complete without these wonderful people around me. There aren't enough thank yous to express my gratitude to those who had given favor on every effort, advice and guidance to me throughout this project.

First of all, I would like to express the deepest appreciation to my supervisor, Professor Dr. Alexander Chong Shu-Chien, for his advices, guidance, and support through learning process of this project. Without him, I would not make it this far. A special gratitude goes to my two 'sifu', Ann and Meng Kiat, who have guided me, given me advices, encouragement and support throughout my ups and downs to complete this project. I appreciate their patience and valuable time for guiding me in this project.

Furthermore, I would also like to acknowledge with much appreciation to my lab mates: SH, YS, SS, BK, JW, JM, PT, MQ, SC, Kak Zatil, Kak Mela, Kak Ira, Us, Ikin, Kak Ame, and Anjali for their guides, helps, supports and encouragements throughout the project. I have lot of fun and laughter working together throughout the process. Also, I like to thank to lab assistants and school workers: Kak Nurul, Kak Shaba, and Puan Roziana for their helps throughout the research process. I am grateful to be given friendly helps by all of them.

In addition, I would like to acknowledge MyMaster and USM fellowship which have financially supported me throughout my study. Most importantly, an honourable mention goes to my family, boyfriend and friends for their understandings and supports in completing this project. I'm grateful to receive all the loves from them. Thank you very much.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ALA	α-linolenic acid
ANOVA	analysis of variance
ARA	arachidonic acid
ARP	B-actin acidic ribosomal phosphoprotein
BF_3	boron trifluoride
BLAST	basic local alignment search tool
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
DGLA	dihomo-γ-linolenic acid
dH_2O	autoclaved sterile water
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Ef1a	elongation factor 1-alpha
Elovl	elongation of very long chain fatty acids
EPA	eicosapentanoic acid
ER	endoplasmic reticulum
Fads	fatty acyl desaturase
FAME	fatty acid methyl ester
GC	gas chromatography

GLA	γ-linolenic acid
HUFA	highly unsaturated fatty acid
IPTG	isopropyl β-D-thiogalactopyranoside
JTT	Jones-Taylor-Thornton
KCl	potassium chloride
KCR	β-ketoacyl-CoA reductase
KCS	β-ketoacyl-CoA synthase
LA	linoleic acid
LB	Luria-Bertani
LCFA	long chain (saturated) fatty acid
LC-PUFA	long-chain polyunsaturated fatty acid
MCFA	medium chain (saturated) fatty acid
MEGA	Molecular Evolutionary Genetic Analysis
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MUFAs	monounsaturated fatty acids
n or ω	omega
NaCl	sodium chloride
NaOH	sodium hydroxide
OnElovl	*Oreochromis niloticus* (Nile tilapia) *Elovl*
ORF	open reading frame
PCR	polymerase chain reaction
pH	potential of hydrogen (scale of acidity)
PUFA	polyunsaturated fatty acid
qPCR	quantitative real-time PCR

RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S.O.C.	super optimal broth with Catabolite
SCD	stearoyl-CoA desaturase
SCFA	short chain fatty acid
SCMM-U	*S. cerevisiae* minimal medium without uracil
SFAs	saturated fatty acids
SPSS	Statistical Package for the Social Sciences
TBE	Tris-borate-EDTA
UV	ultraviolet
VLC-PUFA	very long-chain PUFA
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD	yeast extract peptone dextrose (medium for yeast growth)
Δ	position of the double bond from the carboxyl end of the molecule

PENGKLONAN MOLEKUL DAN PENCIRIAN FUNGSI SUATU FAMILI ELOVL NOVEL (ELONGASE) DARIPADA IKAN TELEOST AIR TAWAR (*Oreochromis niloticus*)

ABSTRAK

Kini, terdapat tujuh ahli famili ELOVL (ELOVL1-7) yang telah dikenalpastikan dalam mamalia. Dari segi fungsi, ahli famili *Elovl* boleh dibahagikan kepada dua kategori, iaitu famili *Elovl* yang boleh memanjangkan substrat asid lemak tepu dan asid lemak mono-tak tepu (MUFA), ataupun famili *Elovl* yang boleh memanjangkan substrat asid lemak poli-tak tepu (PUFA). Setakat ini, sekumpulan protein *Elovl* yang dinamakan sebagai *Elovl4-like* telah dikenalpastikan dalam ikan teleost. Namun demikian, kumpulan protein ini telah menunjukkan persamaan yang rendah dan pencirian fungsi yang berbeza berbanding protein *Elovl4* yang dilaporkan. Oleh itu, wujudnya keperluan untuk mengenalpastikan sama ada protein *Elovl* ini boleh dikategorikan sebagai satu ahli famili *Elovl* yang baharu ataupun subfamili daripada ahli famili *Elovl* yang telah dilaporkan dalam kajian lepas. Dengan ini, kajian ini telah dijalankan untuk mengklon dan menganalisis jujukan gen *Elovl* ini (XM_005479121.2) daripada ikan tilapia (*Oreochromis niloticus*), iaitu suatu ikan air tawar. Hasil analisis telah menunjukkan bahawa gen *Elovl* tilapia mempunyai persamaan yang amat rendah berbanding protein *Elovl* yang lain (*Elovl1-7*). Namun demikian, *Elovl* ini mempunyai persamaan yang tinggi dengan protein *Elovl* yang telah dinamakan *Elovl4-like* dalam GenBank. Hasil daripada analisis ini juga mencadangkan bahawa protein *Elovl* ini boleh diklasifikasi sebagai famili *Elovl* yang baharu. Oleh yang demikian, protein *Elovl* ini dinamakan *Elovl8* putatif dalam kajian ini. Suatu kajian *in-vitro* dengan menggunakan yis telah dijalankan untuk mencirikan fungsi *Elovl* baharu ini. Substrat asid lemak, iaitu C18:2n6, C18:3n3, C18:3n6,

C18:4n3, C20:4n6, C20:5n3, C22:4n6 dan C22:5n3 telah digunakan untuk menguji fungsi gen dalam kajian ini. Keputusan kajian telah menunjukkan bahawa gen ini mempunyai keupayaan untuk menukar MUFA dan C18 PUFA kepada produknya. Tambahan pula, pemilihan substrat oleh protein ini berbeza daripada ahli *Elovl* yang lain. Dengan penemuan ini, protein ini kemungkinan besar ialah protein ini dicadangkan untuk dinamakan sebagai *Elovl8*. Pengekspresan gen *Elovl8* didapati paling tinggi dalam tisu usus, diikuti dengan insang, mata, otak dan hati ikan. Sebagai kesimpulan, penemuan dalam kajian ini menekankan bahawa terdapat perbezaan yang ketara dalam *Elovl8* ikan tilapia daripada 7 ahli *Elovl* yang lain, dan dengan ini kumpulan protein *Elovl8* dicadangkan untuk diklasifikasikan sebagai ahli famili *Elovl* yang baharu dalam teleost.

MOLECULAR CLONING AND FUNCTIONAL CHARACTERIZATION OF A NOVEL ELOVL (ELONGASE) FAMILY FROM A FRESHWATER TELEOST (*Oreochromis niloticus*)

ABSTRACT

At present, seven members of ELOVL family (ELOVL1-7) have been identified in mammals. In terms of function, they are broadly divided into those that elongate the saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), and those that elongate the polyunsaturated fatty acids (PUFA). Recently, a group of *Elovl* proteins, termed as *Elovl4-like*, was discovered from teleost. However, these proteins have shown low identities and different in term of function with the typical identified *Elovl4* protein. An interest has arisen to investigate whether this group of *Elovl* proteins could be a new *Elovl* family member or as a subfamily of the existing *Elovl* member. This study was undertaken to clone and analyse this *Elovl* gene sequence (XM_005479121.2) from a freshwater fish, Nile tilapia (*Oreochromis niloticus*). Results showed that the sequence of Nile tilapia *Elovl* elongase revealed low identities with the other identified *Elovl* elongase (*Elovl1-7*) while high identities with a group of proteins termed *Elovl4-like*. The results had suggested that this group of *Elovl* proteins can be classified as a new member of *Elovl* family and it is designated as putative *Elovl8*. An *in-vitro* study was carried out to functionally characterize this new member of *Elovl* in yeast. Substrate C18:2n6, C18:3n3, C18:3n6, C18:4n3, C20:4n6, C20:5n3, C22:4n6 and C22:5n3 were tested in this study. Results showed that this gene has the ability to convert MUFA and C18 PUFA into their respective products which clearly revealed that its substrate selectivity is different from other *Elovl* members. Therefore, it strongly supports that the protein could be a new member of *Elovl* family and hereby named as *Elovl8*. At the tissue

level, the *Elovl8* gene expression was the highest in intestine, follow by gill, eye, brain and liver. Overall, results from present study strongly suggest that Nile tilapia *Elovl8* is distinct from the current seven identified *Elovl* family members, and therefore, this group of *Elovl8* proteins are proposed to be classified as a new *Elovl* members in teleost.

CHAPTER ONE: INTRODUCTION

1.1 Research background

Long chained polyunsaturated fatty acid (LC-PUFA) is a type of fatty acid with carbon chain length more or equal to 20 carbon atoms containing three or more double bonds (Lim *et al*., 2011; Tocher, 2003; Christie, 1982). LC-PUFA; an omega-6 polyunsaturated fatty acid (PUFA), arachidonic acid (ARA), two omega-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential compounds that play important roles in many metabolic and physiological processes ensuring normal cellular function (Monroig *et al*., 2009). LC-PUFAs are also components of cell membrane phospholipids, determining the membrane's fluidity, and activity of membrane proteins and enzymes that involved in transportation and signal transduction (Monroig *et al*., 2009; McMurchie, 1988).

LC-PUFA can be obtained through dietary intake or biosynthesis activities. Fish oil is a good source of omega-3 fatty acids, especially omega-3 LC-PUFAs (EPA and DHA) in human diet, which reduces the risk of coronary heart diseases (Swapna *et al.*, 2010; Gbogouri *et al.*, 2006). Thus, the demand of fish as food is increasing tremendously. This phenomenon has caused the global capture of fish is dropping due to overfishing (Bell, *et al.*, 2001). The increasing demand of fish has also raised interest in obtaining the enzymes involved in LC-PUFA biosynthesis pathway in many organisms.

LC-PUFA biosynthesis pathways involve reactions where shorter-length of fatty acids undergo processes of desaturation and elongation into intermediates prior to transformation into the respective LC-PUFAs (Tocher, 2003). Desaturase and elongase are two important enzymes that play the role in biosynthesis pathway of

LC-PUFAs. Desaturase is the enzyme introduces double bonds at specific positions of the fatty acyl chain while elongase is the enzyme catalyses the condensation step in the elongation pathway resulting in the addition of two-carbon unit to the pre-existing fatty acid (Guillou *et al.*, 2010). Their activities differ according to species and their dietary nutrition (Ghioni *et al*., 1999).

Not surprisingly, the enzymes (elongase and desaturase) have been extensively studied for several decades across many organisms especially in fish in order to understand the role of these enzymes in the biosynthesis pathway of LC-PUFA (Tanomman *et al.*, 2013; Mohd-Yusof *et al.*, 2010; Monroig *et al.*, 2009; Morais *et al.*, 2009; Agaba *et al.*, 2005). At present, seven fatty acyl elongase family members, designated as ELOVL1-7, have been identified from mammals that differ each other in their substrate specificity (Guillou *et al.*, 2010; Leonard *et al.*, 2004). Some studies have displayed tissue-expression distribution and fatty acid selectivity of these enzymes (Tamura *et al.*, 2009; Wang *et al.*, 2005).

Among these seven families, ELOVL elongases can be divided into those that elongate the saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), and those that elongate the polyunsaturated fatty acids (PUFAs) (Guillou *et al.*, 2010; Moon *et al.*, 2001). *Elovl2*, *Elovl4* and *Elovl5* elongases have been extensively studied so far in a range of fish, especially farmed species due to their importance roles in LC-PUFA biosynthesis pathway (Carmona-Antoñanzas *et al.*, 2011; Morais *et al.*, 2009; Agaba *et al.*, 2005). In additional, tissue-expression studies also demonstrated *Elovl* elongases can be expressed ubiquitously or specifically expressed in specific tissues (Guillou *et al.*, 2010).

1.2 Problem statement

Many enzymes which are related to the biosynthesis pathway of LC-PUFAs had been isolated and deposited into the GenBank. Recently, Kuah and his co-workers have discovered two *Fads* desaturases (GenBank: ACD70298.2 and AMY15661.1) and an *Elovl* elongase (GenBank: ACD02240.2) from a freshwater carnivorous fish species, striped snakehead (*Channa striata*) (Kuah *et al.,* 2016; Kuah *et al.*, 2015). Another *Elovl* elongase (GenBank: AGN95292.1) has been isolated and found that it is less than 50 % identities to any of the seven *Elovl* elongases family members. More importantly, it still incorporates the characteristic features of a fatty acyl elongase.

Interestingly, there are a group of elongase proteins deposited in the GenBank which termed as *Elovl4-like* that have shown high identities (>70 %) with the above mentioned striped snakehead *Elovl* elongase. However, none of the proteins have been studied and functionally characterized except for this striped snakehead *Elovl*. Kuah (2015) conducted a study to functionally characterize this gene from striped snakehead through *in-vitro* study and its tissue distribution in aldulthood of striped snakehead has also been identified. Therefore, an interest has arisen to further characterize this newly isolated *Elovl* elongase, to see if it can be categorised as a new *Elovl* family member or as a subfamily of the existed *Elovl* member.

To achive this, functional characterization of this group of proteins from more species is required. In this study, a freshwater omnivorous teleost, Nile tilapia (*Oreochromis niloticus*) which originated from Africa was chosen. This fish is now widely cultured in most of the tropical country as food fish, contributing highly for the development of freshwater aquaculture (Kocher *et al.*, 1998). Understanding this

Elovl4-like protein from Nile tilapia through molecular and functional characterization study will provide the insights if this is a new *Elovl* family member.

1.3 Objectives

The aims of the study are:

1. To isolate, clone and analyse homolog of the putative *elovl* gene sequence from Nile tilapia and construct a phylogenetic tree.
2. To study and functional characterize the e*lovl* gene from Nile tilapia through *in-vitro* assay in yeast.
3. To determine the expression distribution of the e*lovl* gene in brain, eye, gill, heart, intestine, kidney, liver, muscle, spleen and stomach tissues of Nile tilapia.

CHAPTER TWO: LITERATURE REVIEW

2.1 Lipid

Lipids are commonly known as fats and oils in general. They can be defined as organic compounds that cannot be dissolved in water; however, in contrary they are soluble in organic solvents, such as chloroform, ether, hexane, and benzene (Lim *et al*., 2011).

Along with proteins and carbohydrates, lipids are also one of the crucial energy sources for fish to ensure them to grow, develop, reproduce and move normally (Rainuzzo *et al*., 1997; Tocher, 2003). Besides that, lipids are also a major stuctural component of fish cell membrane. It helps to maintain, repair and synthesize cell membrane (Brasaemle, 2007).

Triacylglycerides, waxes, sterols, phosphoglycerides, and sphingolipids are five classes of lipids (Lim *et al*., 2011). For fish, lipids can be divided into two groups according to their chemical nature; polar lipids composed principally of phospholipids and neutral or nonpolar lipids composed principally of triacylgylcerols (triglycerides) (Tocher , 2003).

2.2 Fatty acids

Fatty acids are carboxylic acids that contain straight and unbranched hydrocarbon chain with a carboxyl group (COOH-) at one end while a methyl group (CH_3-) at the other end (Lim *et al*., 2011). Fatty acids can be represented by $CH_3(CH_2)_nCOOH$, where the n is usually an even number. Fatty acids usually can be occurred naturally.

The naming of fatty acids can be determined purely according to the number of carbon atoms and the number (if any) of the double bonds and their position relative to the carboxyl groups (Davidson & Cantrill, 1985). Fatty acids have both a systematic and a common name, for example octadecadienoic acid coressponds to linoleic acid (C18:2n-6) (Tvrzicka *et al.*, 2011).

Fatty acids also can be named follows an anbreviated system:

CN:pn-x

where CN (carbon number) represents the total number of carbon atoms, p represents the number of double bonds in the hydrocarbon chain, and x represents the carbon which is the first double bond appears numbering from the methyl end ($-CH_3$) (Tvrzicka *et al.*, 2011; Brett & MÜller, 1997; IUPAC-IUB, 1967). For example, the linoleic acid (LA), C18:2n-6 indicates that the fatty acids has 18 carbon atoms and has 2 double bonds in the chain, and the 6^{th} carbon counted from the methyl end is the first double appears in the hydrocarbon bond. C18:2n-6 could also be written as 18:2Δ9,12 whereby Δ signifies the position of the double bond from the carboxyl end of the molecule.

Fatty acids are either saturated or unsaturated carboxylic acids. Saturated fatty acids (SFAs) do not consist of any double bonds in the hydrocarbon chain such as C16:0 and C18:0 (Tvrzicka *et al.*, 2011). SFAs can be divided into three subgroups according to their chain length; which are short chain fatty acids (SCFA), medium chain fatty acids (MCFA) and long chain fatty acids (LCFA) (Tvrzicka *et al.*, 2011).

Unsaturated fatty acids can be divided into monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). MUFAs consist of single double

bond such as C18:1n-9 and C18:1n-7 (Tocher, 2003). MUFAs can take the form of the *cis*-configuration and *trans*-configuration. The *cis* term is used when the two hydrogens at the double bond are on the same side of the molecule as one other while the *trans* term is used when the hydrogens are on the opposite side to one another resulting in a non-curved structure (Figure 2.1) (Tvrzicka *et al.*, 2011).

In general, PUFAs refer to fatty acids that consist of two or more carbon-carbon double bonds in the carbon chain such as C18:3n-3. Carbon chain length which is 20 carbon atoms or more with three or more double bonds is often known as long-chain polyunsaturated fatty acid (LC-PUFA), or can also name as highly unsaturated fatty acid (HUFA) (Lim *et al.*, 2011; Tocher, 2003; Christie, 1982). Very long-chain PUFA (VLC-PUFA) refer to PUFA with two or more double bonds, with more than 24 carbon atoms. Figure 2.2 demonstrates structures and nomenclatures of SFA, MUFA and PUFAs.

The length of the carbon chain and the degree of unsaturation (number of double bonds) in fatty acids can directly determine its physical and chemical properties (Lim *et al.*, 2011). Very long SFAs have higher melting point and are in solid forms at room temperature whereas unsaturated fatty acids usually are in liquid state at room temperature (Tvrzicka *et al.*, 2011; Church & Pond, 1974).

2.2.1 Long-chain polyunsaturated fatty acids (LC-PUFAs)

Long-chain polyunsaturated fatty acids (LC-PUFAs) are essential for normal growth and development in all vertebrates (Hamosh & Salem, 1998). There are two families of LC-PUFA, the omega-3 (n-3 or ω-3) which is derived from ALA, and the omega-6 (n-6 or ω-6) which is derived from LA. The three most important LC-

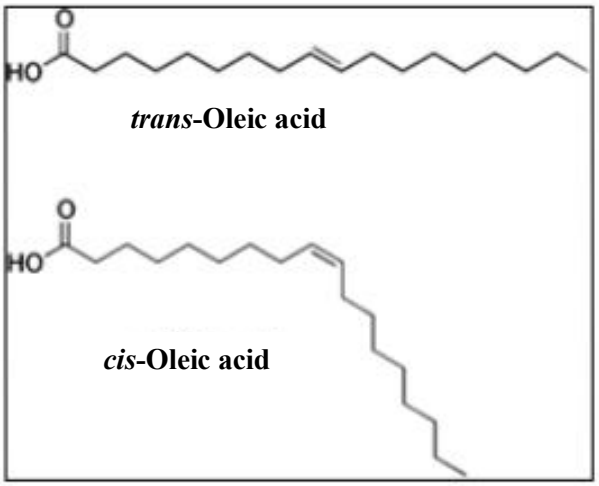
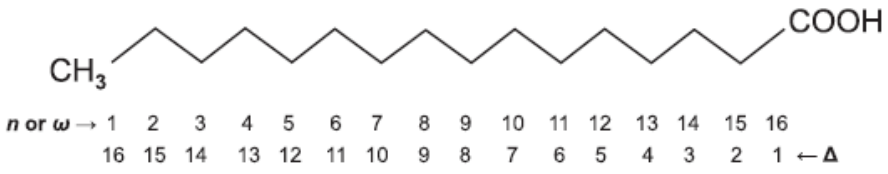
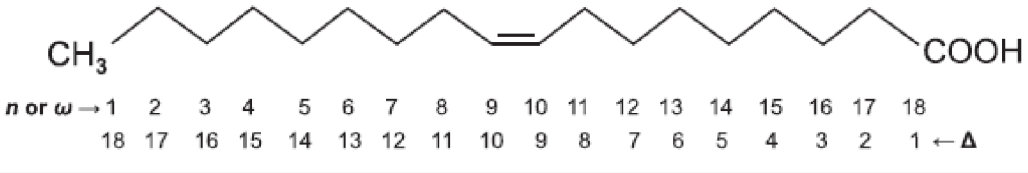


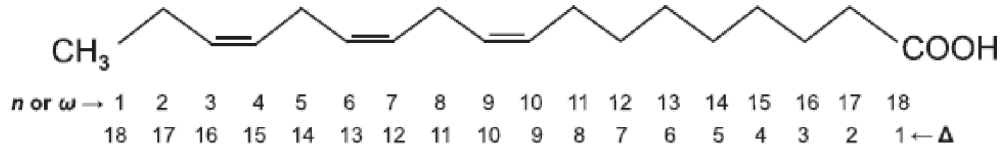
Figure 2.1: Sturctures of *cis* and *trans*-configuration of oleic acid.



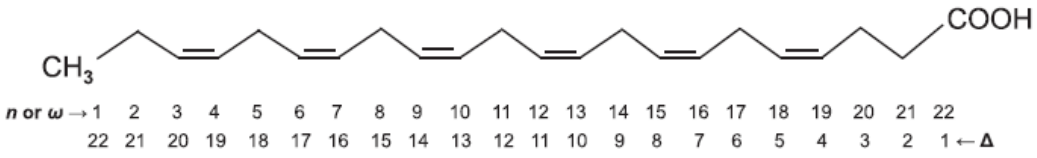
Hexadeconoic acid (palmitic acid; C16:0)



cis Δ9-octadecenoic acid (oleic acid; C18:1n-9; C18:1Δ9)



all *cis* Δ9, 12, 15-octadecatrienoic acid (α-linolenic acid; C18:3n-3; C18:3Δ9,12,15)



all cis Δ4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA; C22:6n-3; C22:6Δ4,7,10,13,16,19)

Figure 2.2: Stuctures and nomenclatures of the representative SFA, MUFA and PUFAs (adapted from Castro *et al.*, 2016).

PUFAs in vertebrates are an omega-6 PUFA, arachidonic acid (ARA, C20:4n-6), and two omega-3 PUFAs, eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) (Figure 2.3).

Studies have well reported that LC-PUFAs have beneficial effects in human health and medical conditions such as reducing cardiovascular diseases and inflammation, neural development and neurological conditions (Tocher, 2015; Swapna *et al.*, 2010; Calder, 2006; Schmidt *et al*., 2005a; Schmidt *et al.*, 2005b). Both ARA and EPA are also the main eicosanoid precursors that are involved in a great variety of physiological functions such as controlling the release of hormones and also regulate cerebral blood flow (Estévez *et al.*, 1999).

Fish especially its oil have been considered as the most important source of LC-PUFAs especially n-3 PUFAs such as EPA and DHA (Gbogouri *et al.*, 2006; Bell *et al.*, 2001). The benefits of LC-PUFA towards human consumers resulted in an increase of demand on fish consumption. Thus, it is not surprise that LC-PUFAs biosynthesis pathway has been an important study in many organisms especially in fish for several decades now.

2.3 Biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFAs)

All vertebrates including fish cannot synthesize ALA and LA *de novo* from SFA and MUFA due to the lack of the Δ12 and Δ15 desaturase enzymes that required to produce these PUFA from oleic acid (Figure 2.4), and thus ALA and LA are essential in their dietary intake (Tocher, 2003). On the other hand, LC-PUFAs can be synthesized by vertebrates, including fish, through a cascade reaction where shorter fatty acids are undergoing desaturation and elongation into intermediates

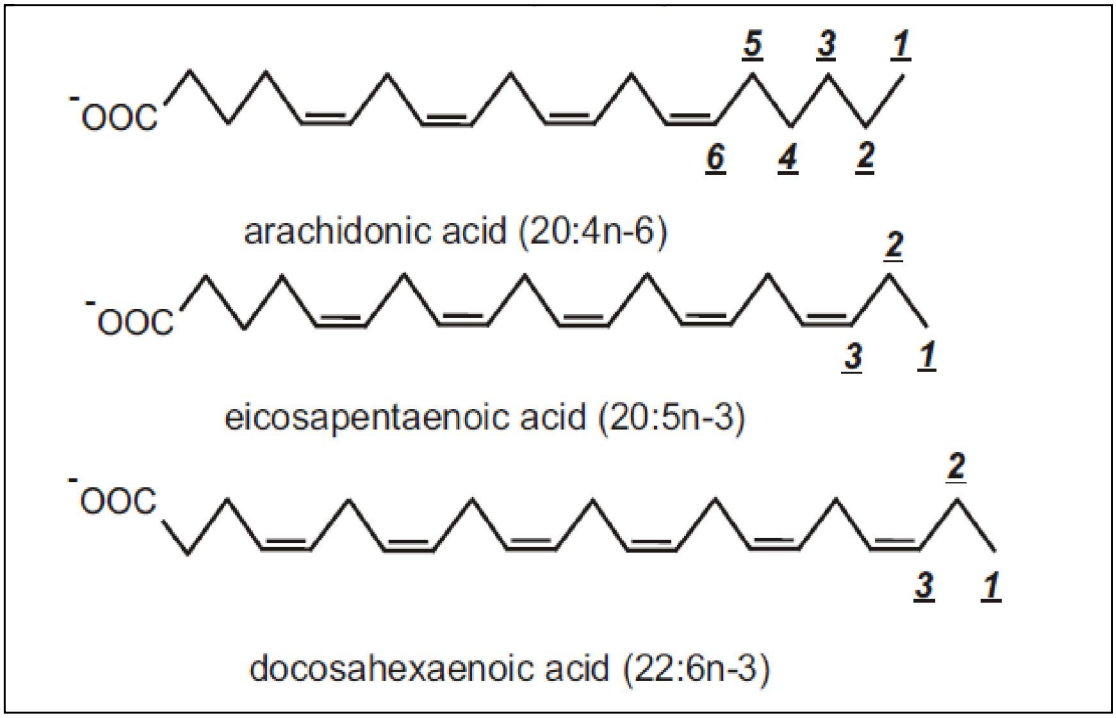


Figure 2.3: Structural formulas and types of shorthand notations of LC-PUFAs (adapted from Tvrzicka *et al.*, 2011).

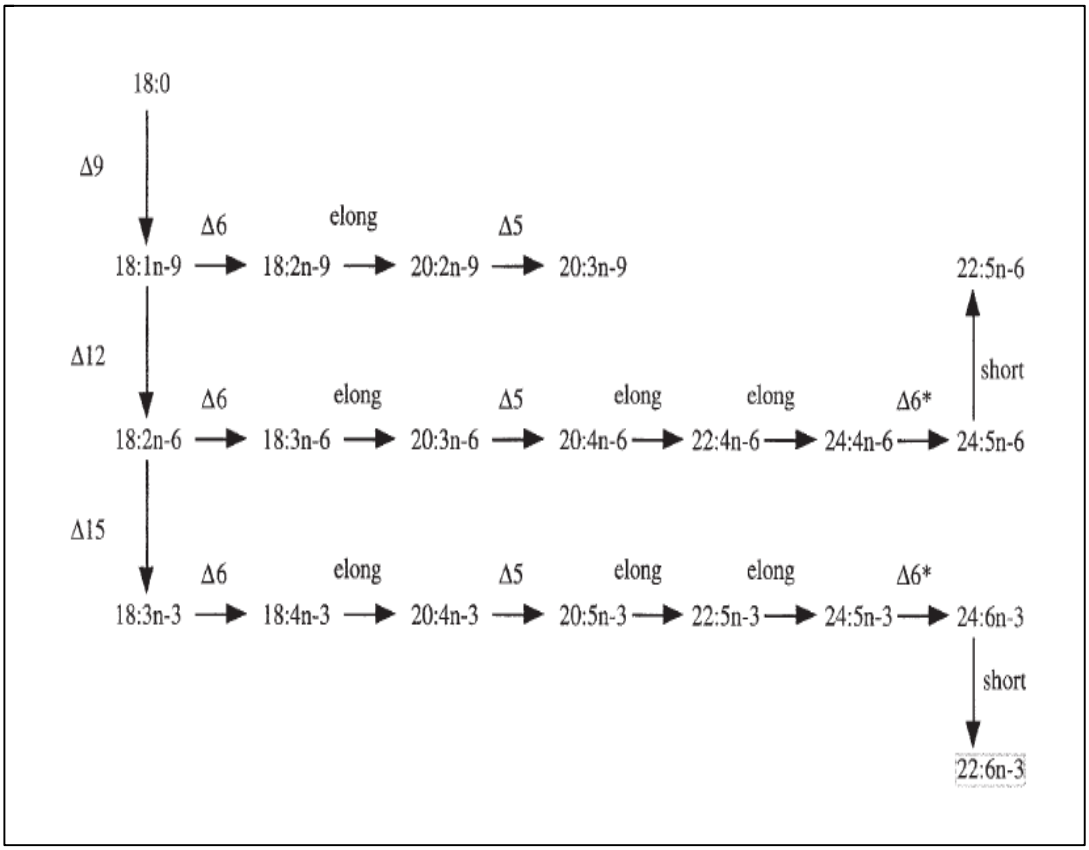


Figure 2.4: A general representation of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis pathways from n-3, n-6 and n-9 in vertebrates. Vertebrates are unable to synthesis LA (C18:2n-6) and ALA (C18:3n-3) as they do not possess the Δ12 and Δ15 fatty acyl desaturase. These two fatty acyl desaturases are only found in plants and some invertebrates. Δ9 represents stearoyl-CoA desaturase (SCD), Δ5 and Δ6 indicate fatty acyl desaturase activities. 'Elong' indicates the acitivity of PUFA elongation and 'short' indicates peroxisomal chain shortening (adapted from Tocher, 2003).

prior to transformation into respective LC-PUFAs (Figure 2.5). However, this capacity varies from species to species.

Most of the marine fish have limited abilities to synthesis essential LC-PUFAs from C18 PUFAs. This is because they are either lack of the Δ5 and Δ6 desaturase activity or limited C18 to C20 elongase activity (Ghioni *et al.*, 1999). With the high intake of LC-PUFAs in the diet, this had reduced the Δ6 desaturase activity (Buzzi *et al.*, 1996). However, unlike most of the marine counterparts, freshwater species have the ability to desaturate and chain elongate C18 PUFA to LC-PUFAs according to their needs. They can convert LA into ARA which is the n-6 pathway and ALA into EPA and DHA which is the n-3 pathway by an alternative series of chain desaturation and elongation reactions (Tocher, 2003).

Synthesis of ARA is achieved by Δ6 desaturation of LA (C18:2n-6) to produce γ-linolenic acid (GLA, C18:3n-6) and then it is elongated to dihomo-γ-linolenic acid (DGLA, C20:3n-6) followed by Δ5 desaturation. As for synthesis of EPA from ALA, it has almost a similar sequence of desaturation and elongation which happens in ARA production. However, DHA synthesis could occur via two separate pathways. The 'Sprecher pathway' where the DHA synthesis from EPA via C24 intermediates (Sprecher *et al.*, 1995). It involves two elongation steps from EPA to produce docosapentarnoic acid (DPA, C22:5n-3) and then tetracosapentaenoic acid (C24:5n-3), an additional Δ6 desaturation to form tetracosahexaenoic acid (C24:6n-3) and finally chain shortened via β-oxidation to produce DHA.

Another simpler and more direct pathway for biosynthesis of DHA from EPA would be via an elongation step and then followed by Δ4 desaturation activity. Previously, Δ4 desaturation was only found in lower eukaryoutes such as

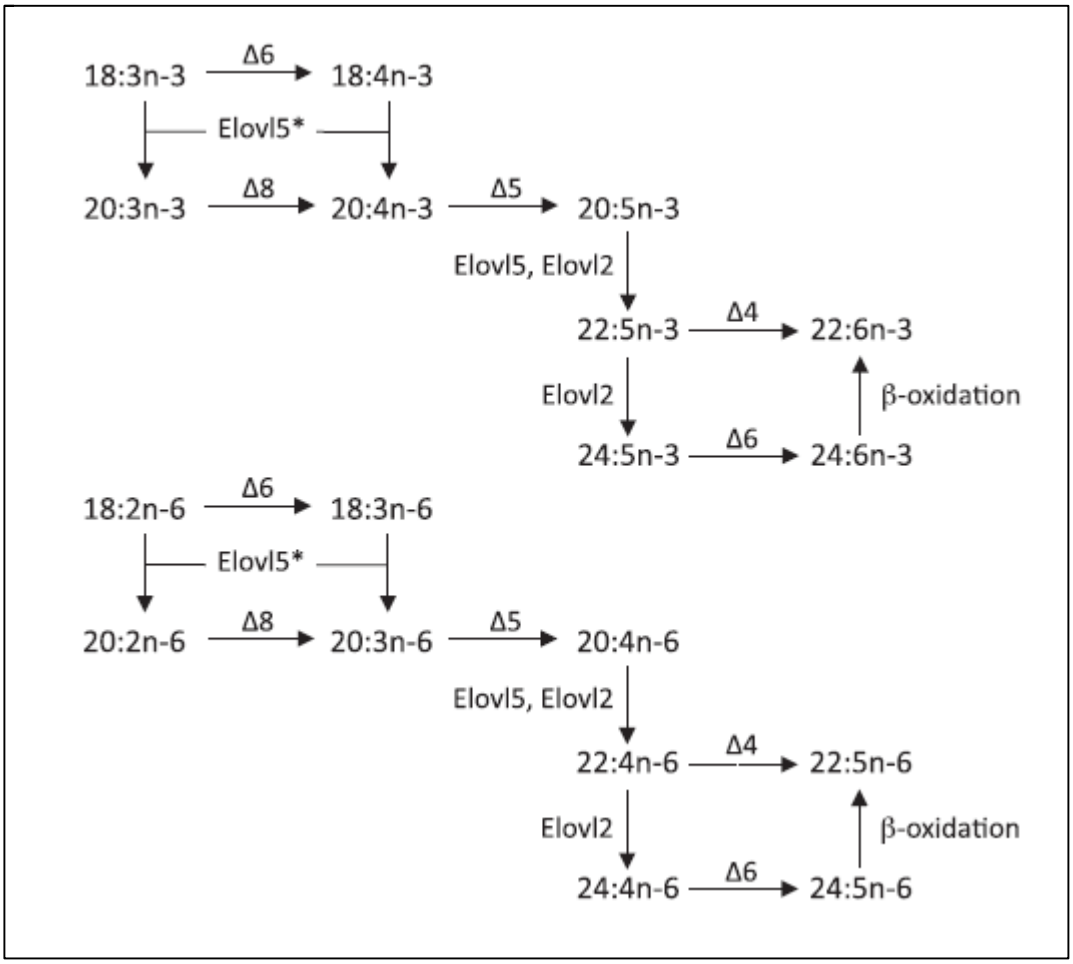


Figure 2.5: Long-chain polyunsaturated fatty acids (LC-PUFAs) biosynthesis pathway from C18 PUFA precursors in vertebrates. Pathway begins with ALA (C18:3n-3) for the n-3 pathway and LA (C18:2n-6) fot the n-6 pathway. Δ4, Δ5, Δ6 and Δ8 represent fatty acyl desaturases. *Elovl2* and *Elovl5* indicate elongation activity. B-oxidation is a chain shortening step (adapted from Monroig *et al.*, 2011a)

Thraustochytrium sp. and microalgae (Pereira *et al.*, 2004; Tonon *et al.*, 2003; Qui, 2001). In 2010, Li *et al.* has reported the presence of Δ4 desaturation in a marine herbivorous fish, rabbitfish (*Siganus canaliculatus*). Recently, it is also found that striped snakehead (*Channa striata*), a freshwater carnivorous fish, have also been observed to have Δ4 desaturation activity (Kuah *et al.*, 2015).

2.4 Enzymes involve in the LC-PUFA biosynthesis pathway

Elongase and desaturase are two important enzymes in the biosynthesis pathway of LC-PUFA. Desaturase is the enzyme that introduces double bonds at specific positions of the fatty acyl chain while elongase is the enzyme that catalysis the condensation process in the elongation pathway resulting in the addition of two-carbon unit onto the specific fatty acid precursor (Jacobi *et al.*, 2011; Guillou *et al.*, 2010).

Hashimoto *et al.* (2008) reported that desaturases are divided into four functional subfamilies and elongases are divided into two functional subfamilies. Desaturases are comprised of i) First Desaturase, introducing the first double bond into the saturated fatty acid chain; ii) Omega Desaturase, introducing a double bond between an existing double bond and the acyl end (contains Δ12 and Δ15 desaturases); iii) Front-End Desaturase, introducing a double bond between and existing double bond and the carboxyl end (includes Δ4, Δ5, Δ6 and Δ8 desaturases); and iv) Sphingolipid Desaturase whose sole function is the sphingolipid Δ4; while elongases are comprised of i) S/MUFA Elongase, elongating a saturated fatty acid or a MUFA; and ii) PUFA Elongase, elongating a polyunsaturated fatty acid (Hashimoto *et al.*, 2008).

Many studies and researches have been conducted to explain the functional characterization in substrate selectivity and tissue-expression distribution of both elongase and desaturase in many organisms due to the importance of LC-PUFAs on human health (Monroig *et al.*, 2011a; Iskandarov *et al.*, 2009; Agaba *et al.*, 2005). Study on elongase and desaturase slowly has shifted to invertebrates especially moluscs (Liu *et al.*, 2014a; Liu *et al.*, 2014b; Monroig *et al.*, 2013; Monroig *et al.*, 2012a) and recently there are studies conducted to understand the diversity and the functions of *Elovl* and *Fads* genes in chordates (Castro *et al.*, 2016; Monroig *et al.*, 2016).

2.4.1 Elongation of very long chain fatty acid (*Elovl*)

Elongase enzymes termed as elongation of very long chain fatty acids (*Elovl*) that are responsible for the addition of two carbon units to the carboxyl end of a fatty acid chain (Leonard *et al.*, 2004). The fatty acid elongation comprises with four separate enzymatic reactions: an initial condensation of the malonyl-CoA with the fatty acyl-CoA to yield β-ketoacyl-CoA by β-ketoacyl CoA synthase (KCS); reduction of β-ketoacyl-CoA to generate β-hydroxyacyl-CoA by β-ketoacyl CoA reductace (KCR); dehydration of β-hydroxyacyl-CoA to produce trans-2-enoyl-CoA by β-hydroxyacyl CoA dehydrase; and finally reduction of *trans*-2-enoyl-CoA to form the elongated acyl-CoA by *trans*-2-enoyl CoA reductase (Figure 2.6) (Guillou *et al.*, 2010; Jakobsson *et al.*, 2006; Leonard *et al.*, 2004).

To date, there have been seven fatty acyl elongase family members (ELOVL1 to ELOVL7) reported from mammals where ELOVL7 is the most recently identified elongase (Guillou *et al.*, 2010; Tamura *et al.*, 2009; Leonard *et al.*, 2004). All of the

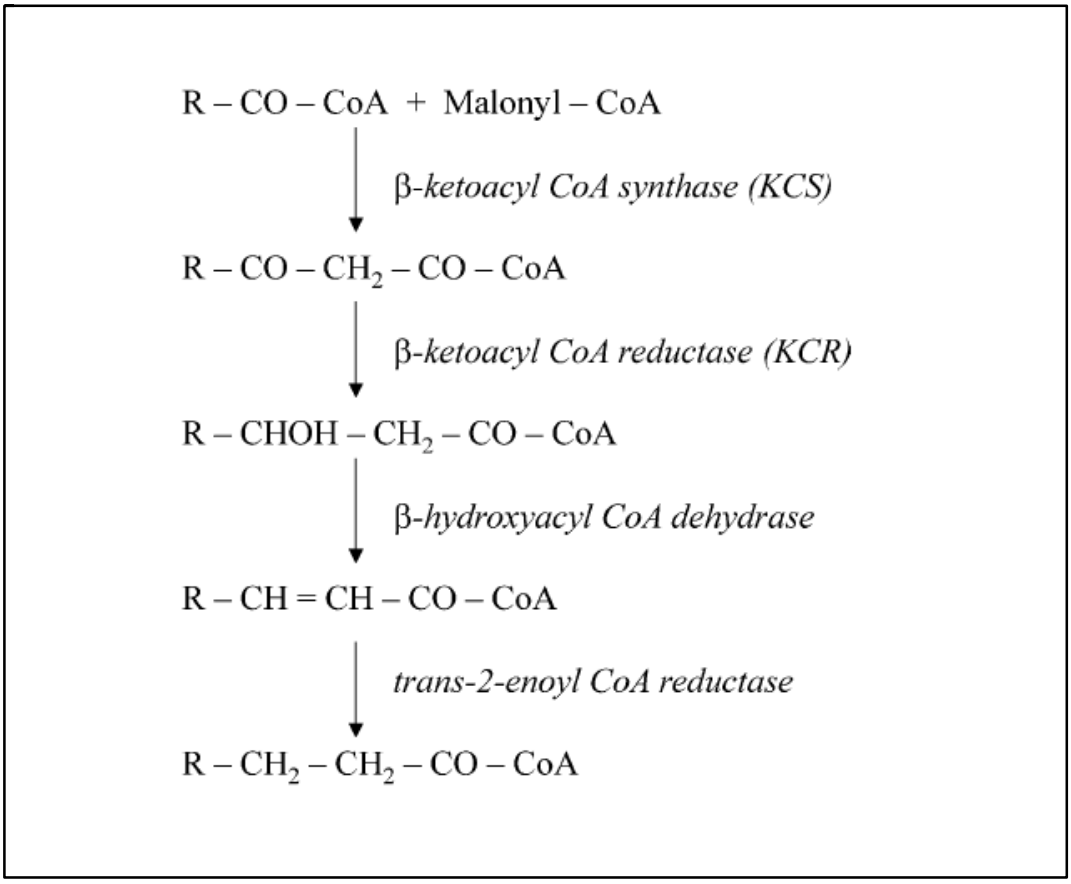


Figure 2.6: Brief demonstration on the four enzymatic reactions of fatty acid elongation (adapted from Leonard *et al.*, 2004).

fatty acyl elongase members are featured by a single histidine box redox centre motif (HxxHH), multiple transmembrane regions, four highly conserved motifs (KxxExxDT, QxxFLHxYHH, NxxxHxxNYxYY and TxxQxxQ) and an endoplasmix reticulum (ER) retention signal (carboxyl-terminal dilysine targeting signal) (Agaba *et al.*, 2005; Meyer *et al.*, 2004). They share common evolutionarily conserved motifs despite low overall sequence similarity.

Generally, ELOVL1, ELOVL3, ELOVL6 and ELOVL7 are preferable selecting SFAs and MUFAs while ELOVL2, ELOVL4 and ELOVL5 are preferable selecting PUFAs as substrate in elongation system (Guillou et al., 2010; Tamura *et al.*, 2009; Wang *et al.*, 2005; Moon *et al.*, 2001; Leonard *et al.*, 2000; Tvrdik *et al.*, 2000). A study has reported that ELOVL1 prefers to elongate C20-C22 SFA and MUFA substrates (Ohno *et al.*, 2010). ELOVL3 was observed to elongate SFA and MUFA containing up to 24 carbon (Westerberg *et al.*, 2006), while ELOVL6 is involved in the elongation of C12-C16 SFA substrates and is incapable to elongate substrates beyond C18 fatty acids (Moon *et al.*, 2001). There is a study suggested that ELOVL7 has the ability to elongate C18-22 SFA (Tamura *et al*., 2009).

In addition, study has reported that ELOVL2 has the ability to elongate C20-C22 PUFA substrates in mammals (Leonard *et al*., 2002). ELOVL4 is involved in the elongation of C22 PUFA substrates but it has also been reported to elongate SFA which is longer than C24 (Agbaga *et al.*, 2008). ELOVL5 is capable of elongating C18-C22 PUFA but not those with more than 22 carbons (Leonard *et al.*, 2000). Table 2.1 presented the substrates selectivity of each ELOVL elongase. In term of mRNA expression, Guillou *et al.* (2010) has reported that ELOVL1, ELOVL5 and ELOVL6 are expressed ubiquitously while ELOVL2, ELOVL3, ELOVL4 and ELOVL7 are specifically expressed in specific tissues.

Table 2.1: General fatty acid substrate selectivity of each identified ELOVL elongase.

ELOVL	FA substrate selectivity
1	C20-C22 SFA and MUFA
2	C20-C22 PUFA
3	≤ C24 SFA and MUFA
4	>C24 SFA and C22 PUFA
5	C18-C22 PUFA
6	C12-C16 SFA
7	C18-C22 SFA

2.4.2 Teleost *Elovl*

At present, *Elovl1*, *Elovl3*, *Elovl6* and *Elovl7* have not been characterized in fish so far while *Elovl2*, *Elovl4* and *Elovl5* have been extensively studied in a range of fish, especially farmed species (Carmona-Antoñanzas *et al.*, 2011; Morais *et al.*, 2009; Agaba *et al.*, 2005). In the previous study, the zebrafish elongase was reported that it had the ability to elongate SFA by the increased C18:0/C16:0 ratio in transformed yeast (Agaba *et al.*, 2004). There were no other studies have reported that the fish elongases were able to elongate SFA.

In 2004, Agaba *et al.* was first reported the presence of PUFA elongase in freshwater teleost which is zebrafish *Elovl5* (*Zf*ELO) and it is functionally characterized by heterologous expression in yeast (*Saccharomyces cerevisiae*) based on its sequence similarity with the *Mortierella alpina* elongase (GLELO). In the study, zebrafish *Elovl5* can efficiently elongate C18 (C18:4n-3 and C18:3n-6) and C20 (C20:5n-3 and C20:4n-6) PUFAs, while low conversion was showed towards C22 (C22:5n-3 and C22:4n-6) PUFAs substrates. Later, more other teleost species *Elovl5s* were cloned and characterized due to their important role in the LC-PUFA biosynthesis pathway of fish (Kuah *et al.*, 2015; Mohd-Yusof *et al.*, 2010; Morais *et al.*, 2009; Zheng *et al.*, 2009; Agaba *et al.*, 2005; Hastings *et al.*, 2004). Studies had confirmed that fish *Elovl5* has the ability to elongate C18 and C20 PUFA with n-3 generally being preferred as substrate over n-6 substrates.

Another *Elovl* family member *Elovl2* enzymes were cloned and functionally characterized in Atlantic salmon (Morais *et al.*, 2009), zebrafish (Monroig *et al.*, 2009) and rainbow trout (Gregory & James, 2014) to understand the fatty acid elongation pathways in fish. *Elov2* elongase has the ability to elongate C20 and C22

PUFAs substrates. Although C20 PUFAs can also be elongated by *Elovl5*, but *Elovl2* elongase has the ability to convert C20 substrates into DPA (C22:5n-3) and docosatetraenoic acid (DTA; C22:4n6) more efficiently as compared to *Elovl5* elongase. Its to be mentioned that the ability of *Elovl2* to elongate EPA to C24:5n-3 has been regarded as a key for the synthesis of DHA via the 'Sprecher pathway'.

Elovl4 is the latest *Elovl* family that has been investigated in fish. *Elovl4* elongases are involved in the elongation of SFAs and PUFAs with chain-lengths greater than C24 which has been succesfully demonstrated by Agbaga and co-workers (Agbaga *et al.*, 2008). Then, a few *Elovl4s* were isolated and funtionally characterized from different fish species such as Atlantic salmon (Carmona-Antoñanzas et al., 2011), zebrafish (Monroig *et al.*, 2010), cobia (Monroig *et al.*, 2011b) and rabbitfish (Monroig *et al.*, 2012b). As expected, the elongation function of *Elovl4* gene is conserved throughout the mammalian and the fish counterparts. In addition, Monroig *et al.* (2012b) had confirmed that the *Elovl4* elongase in fish can participate in the biosynthesis of DHA as they have the ability to elongate EPA to C24:5n-3.

2.5 Nile tilapia (*Oreochromis niloticus*)

Tilapia is the common name for around 70 species of perch-like fishes which is grouped under the Cichlidae family, native to the fresh waters of tropical Africa (Kocher *et al.*, 1998). These fishes have been introduced into nearly every tropical and subtropical country in the world for aquaculture. Nile tilapia (*Oreochromis niloticus*) (Figure 2.7) is one of the species of tilapias that is native in Africa. It was characterized by the female mouthbrooding behaviour (Baroiller *et al.*, 1995).

Figure 2.7: Nile tilapia (*Oreochromis niloticus*) (adapted from FishBase taken by Ueberschaer, B.).

Nile tiapia has considerable potential for aquaculture in many tropical and subtropical countries of the world (Fitzsimmons, 2000). It is rated as the most favored by farmers due to its suitability for farming in any condition of environments (Gupta & Acosta, 2004). The rapidly expanding of tilapia aquaculture, some studies have conducted to overcome some of the main problems associated with farming especially strains of Nile tilapia which is the major farmed species (Lara-Flores *et al.*, 2003; Likongwe *et al.*, 1996).

It is not surprised that Nile tilapia has become a model to study its biosynthesis pathway due to the global tilapia productions has increased year by year to provide a globally sustainable fish food. In 2005, Agaba and fellow cowokers have successfully isolated and functionally characterized *Elovl5* of Nile tilapia. *Elovl5* elongase of Nile tilapia has shown that it can efficiently elongate C18 and C20 substrates but not C22 substrates (Agaba *et al.*, 2005). Recently, Δ6 desaturase gene in Nile tilapia has been cloned and functionally characterized by heterologous expression in yeast (*Saccharomyces cerevisiae*) (Tanomman *et al.*, 2013). Result has reported that Δ6 desaturase gene of Nile tilapia (*Oni-FADS2*) displayed Δ6 enzymatic activity towards both LA and ALA and also significant Δ5 activity.

CHAPTER THREE: METHOD AND MATERIALS

3.1 Cloning and sequencing of ORF sequence of Nile tilapia elongase (*OnElovl*)

3.1.1 Primer design

A pair of specific primers was designed according to a nucleotide sequence of a Nile tilapia elongase cDNA (XM_005479121.2) deposited in the GenBank. The forward and reverse primers contained either a restriction site for *Hind* III or *Xho* I to amplify open reading frames (ORF) of elongase cDNA from Nile tilapia's liver tissue. The sequences of the primers were shown in Table 3.1.

3.1.2 Sample preparation

Wild Nile tilapias were caught from a river (Sungai Dua) in Universiti Sains Malaysia (USM). Livers of the Nile tilapia were obtained upon dissection. The tissue liver was chosen in the study due to higher expression of the gene. Then, the samples were soaked in RNAlater (20 mM EDTA, 25 mM sodium citrate, 5.3 M ammonium sulphate, pH 5.2) and kept in -80 ˚C freezer for further analysis.

3.1.3 Isolation and quantification of total RNA

Total RNA was extracted using TRI Reagent® (Molecular Research Center, Inc). Sample was homogenized in 0.5 ml of TRI Reagent®, and then another 0.5 ml of TRI Reagent® was added before centrifugation. Supernatant was transferred to a