

1. Introduction

Day by day awareness is increasing about the healthy food. People have started paying more attention towards their diet; most importantly fats and fatty acids. Few decades back emphasis on low fat, low energy diets has provided a major stimulus for development of products with low calories, low fat or fat substitutes. Though certain fats contribute to disorders like heart problems and obesity, not all fats are detrimental. Recent researches suggest that there is a need to consider the quantity as well as the quality of fat in our diet. Nutritional importance of omega-3 (n-3) polyunsaturated fatty acids (PUFAs) over n-6 PUFAs is now a day well accepted. Proper balanced intake of fats may provide number of health benefits (Simopoulos, 2008). Disruption in proper PUFA intake and metabolism (i.e. higher n-6: n-3 ratio in the diet) is associated with poor retinal/brain development, cognitive deficiency, coronary artery disease, hypertension, diabetes, inflammatory disorders, and cancer.

1.1 Fats and lipids

Lipids are derivatives of fatty acids and biosynthetically or functionally related compounds. Naturally all the important fats and oils of animal and plant origin consist of triacylglycerols (triglycerides) with structural formula as shown in figure 1.1, where R', R'', R''' represent same or different fatty acyl chains. Diacyl and monoacyl-glycerides are termed collectively "partial glycerides". They are rarely present at trace levels in animal and plant tissues (Gunstone, *et al.*, 2007).

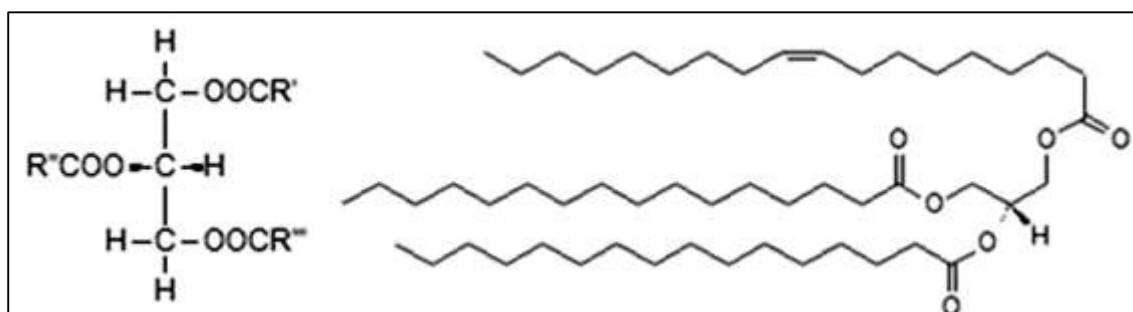


Figure 1.1 Structure of fats

1.2 Fatty acids and their classification

The fatty acids (FAs) are synthesized by chain elongation of an acetyl-CoA with malonyl-CoA (or methylmalonyl-CoA) groups that may contain a cyclic functionality and/or are substituted with heteroatoms (Fahy *et al.*, 2005). Free or unesterified fatty acids are ubiquitous in minor components of all living tissues (Gunstone *et al.*, 2007). They are classified into different groups by considering number of properties like degree of saturation, chain length, orientation of carbon chains on the sides of double bond, position of the first double bond within the acyl chain *etc.* Natural fatty acids usually contain 4 to 28 carbons (Most of the fatty acids in complex lipids contain 14 to 22 carbons). Depending upon chain length they are classified as short chain (2-6 carbons), medium chain (8-14 carbons) and long chain (16 carbons onwards) fatty acids.

1.2.1 Saturation Characteristics

Fatty acids are commonly classified as either saturated, monounsaturated, or polyunsaturated, based on the number of double bonds exist in their molecular structure. A 'saturated' fatty acid has the maximum possible number of hydrogen atoms attached to every carbon atom. The most abundant saturated fatty acid in nature is hexadecanoic or palmitic acid, (C16:0); purified from Palm oil and named after it. Palm oil contains mainly palmitate (43%–46%) and oleate (C18:0) (36%–41%) (Gunstone, 2002). A fatty acid with one double bond is said to be 'monounsaturated.' Common monounsaturated fatty acids (MUFAs) are palmitoleic acid (C16:1) and oleic acid (C18:1). Palmitoleic acid with 16 carbon atoms has the first double bond occurring at 7 carbon atoms away from the methyl group. Oleic acid has 18 carbon atoms with the first double bond occurring 9 carbon atoms away from the methyl group. Fatty acids having more than one double bond are called 'polyunsaturated fatty acids (PUFAs)'.

1.2.2 *cis* and *trans* orientations

Unsaturated fatty acids (MUFAs and PUFAs) are further classified as '*cis*' and '*trans*' fatty acids. In the '*cis*' configuration the hydrogen atoms at a double bond are positioned on the same side of the carbon chain and chains extend on the same side of the double bond (Figure 1.2). In fact, most of the naturally occurring fatty acids are of

this type. On the other hand *trans* fatty acids have the carbon chain extends from opposite sides of the double bond. Usually *trans* fatty acids are the product of food processing. They are found in partially hydrogenated fats, processed foods, meat, beef, dairy products *etc.* (Kris-Etherton *et al.*, 2012; Roe *et al.*, 2013). They are responsible for increase in Low Density Lipoprotein (LDL) cholesterol in blood and hence responsible for increase in risk of heart disorders and decrease in High Density Lipoprotein (HDL) cholesterol levels (Stender and Dyerberg, 2003). Vaccenic acid is a naturally occurring *trans* fatty acid found in the fat of ruminants and in dairy products such as milk, butter, and yogurt (Lawson *et al.*, 2001).

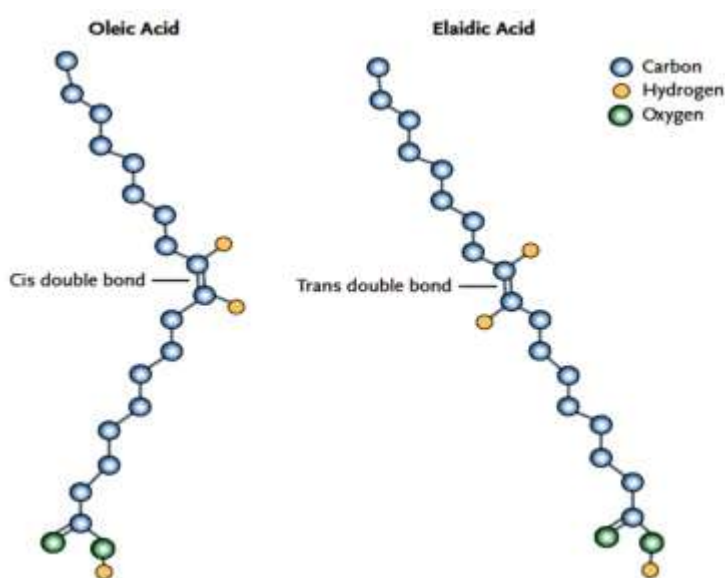


Figure 1.2 *cis* and *trans* conformation of oleic acid. The '*cis*' configuration the hydrogen atoms at a double bond are positioned on the same side of the carbon chain and chains extend on the same side of the double bond while *trans* fatty acids have the carbon chain extends from opposite sides of the double bond (Mozaffarian *et al.*, 2006)

1.2.3 Position of the double bonds

1.2.3.1 PUFAs with divinylmethane pattern

Most of the PUFAs show a divinylmethane pattern ($-C-C=C-C-C=C-$) in distribution of double bonds. These fatty acids have 2 or more *cis* double bonds that are separated from each other by a single methylene bridge ($-CH_2-$ unit). In PUFAs, the carbon on the methyl group of a fatty acid is called the omega (ω) carbon while that on the carboxyl end is called the delta (Δ) carbon. PUFAs are further classified as

omega-3 (n-3), omega-6 (n-6) and omega-9 (n-9) fatty acids named according to the position of the closest double-bond to the methyl group. In n-3 fatty acids first double bond towards methyl group is at C3 position, which is at C6 position in n-6 fatty acids and at C9 in n-9 fatty acids. Out of these n-3 and n-6 are the major groups of PUFAs while n-9 type of unsaturated fatty acids mainly consists of MUFAs like oleic acid, eicosanoic acid *etc.* Representative fatty acid structure of n-3 and n-6 PUFAs are as shown in figure 1.3.

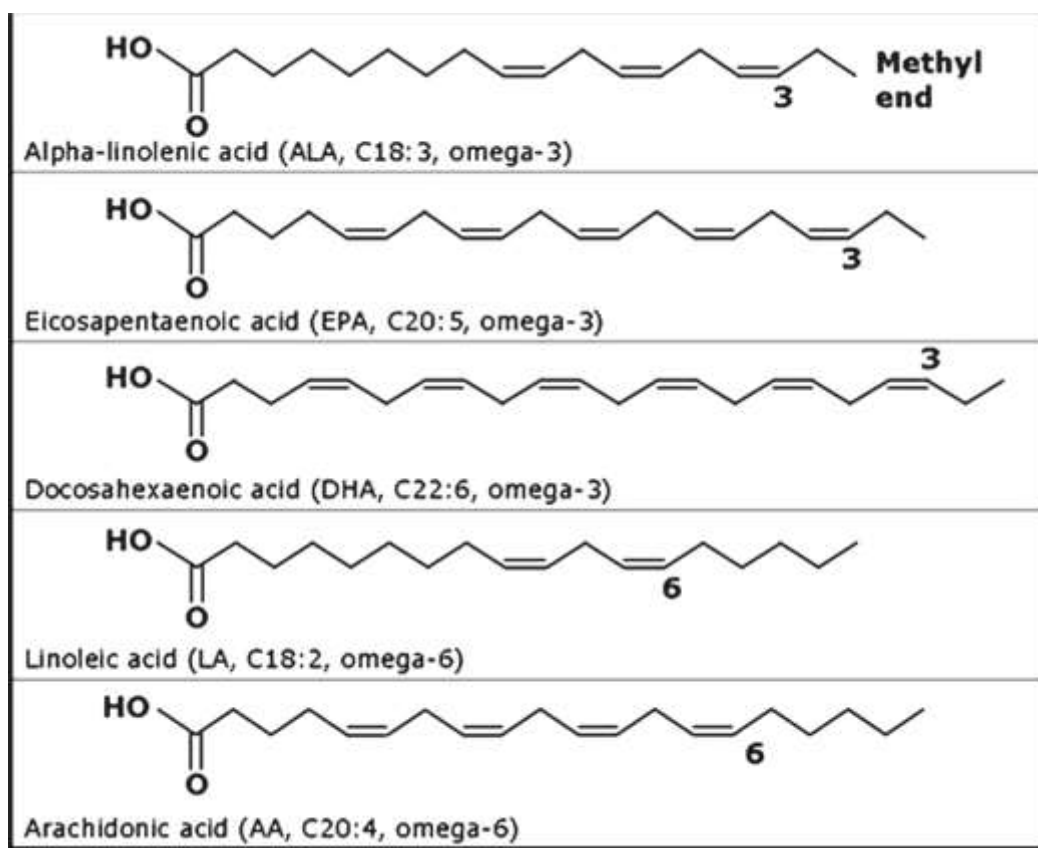


Figure 1.3 Structures of n-3 and n-6 PUFAs. n-3 fatty acids have an extra double at omega-3 position than that of n-6 fatty acids.

The PUFAs can be represented by a structural formula CX: y (n-Z); where, X stands for number of carbon atoms in the acyl chain; y is number of double bonds and n-Z indicates that the first double bond occurs at Zth carbon from omega end e.g. α -Linolenic Acid (ALA): C18:2 n-3; Linoleic Acid (LA): C18:2 n-6; and Oleic acid: C18:1 n-9. LA and ALA act as precursors for n-6 and n-3 PUFAs.

1.2.3.2 Conjugated fatty acids

There is a small group of unsaturated fatty acids with two or more conjugated double bonds ($-C=C-C=C-$). The conjugation of the double bonds is considered as an intermediate step in unsaturated fatty acid peroxidation. Thus, conjugated LA (CLA, a collective term used to designate a mixture of positional and geometric isomers of LA) is the first step in LA peroxidation (Gunstone *et al.*, 2007). The two double bonds in CLA are primarily at position 9 and 11, and 10 and 12 from the carboxyl end along the acyl chain. There can also be geometric changes (*cis* or *trans* configuration). Eight different CLA isomers of LA have been identified. Of these isomers, the *cis*-9, *trans*-11 form is believed to be the most common natural form with biological activity. The major CLA isomer found in natural products is "rumenic acid" (Kramer *et al.*, 1998). Food products from grass-fed ruminants (e.g. mutton and beef) are good sources of CLA, and contain much more of it than those from grain-fed animals (Aldai *et al.*, 2011).

1.2.4 Nutritional classification of fatty acids

Depending upon nutritional requirements and biosynthesis, fatty acids are classified as essential and non-essential fatty acids. Essential fatty acids are not synthesized by animals hence should be obtained from the diet (Simopoulos, 2008). Linoleic and α -Linolenic acid are the two essential fatty acid. Non-essential fatty acids constitute all the other fatty acids synthesized by animals.

1.2.4.1 Linoleic acid and α -linolenic acid as essential fatty acids

Human body can manufacture most of the fats it needs, but LA and ALA cannot be synthesized in our body from their precursor oleic acid (C18:1) due to lack of activities required for desaturation at n-3 and n-6 positions and thus must be obtained from dietary sources (Arterburn *et al.*, 2006; Simopoulos, 2008). These are termed as essential fatty acids. ALA and LA act as precursor for two distinct families of PUFAs i.e. omega-3 (n-3) family and omega-6 (n-6) family respectively. The physiologically active n-6 PUFAs are dihomo gamma linolenic acid (DGLA; C20:3 n-6) and arachidonic acid (AA; C20:4 n-6) while n-3 category belong to eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3).

A very small amount of ALA is converted into EPA (2%-8%) and DHA (0.2%-0.6%) in our body (reviewed in Barcelo-Coblijn and Murphy, 2009) hence these two nutritionally important fatty acid must be obtained from diet and are also categorized as essential n-3 PUFAs (Simopoulos, 2002). The principal n-3 fatty acids, ALA, EPA and DHA, are believed to each have their own importance in different physiological functions hence must be supplemented in our diet.

1.2.4.2 Linolenic acid (C18:2 n-6)

LA is the principal n-6 fatty acid which is not synthesized in animals but represents the most common PUFA, in plants and animal tissues. It has its closest double bond at 6th carbon atom from the methyl end; thus it is called an n-6 fatty acid. LA is the precursor of all the n-6 series formed by desaturation and elongation, firstly converted into γ -linolenic acid (GLA; C18:3 n-6) and then AA. Walnut, peanut, seeds of sunflower, grape, corn, sesame and soya contain large amounts of LA (Gunstone, 2002).

1.2.4.3 α -Linolenic Acid (C18:3 n-3)

In ALA, the double-bond closest to the methyl group is only 3 carbons away, so it is an n-3 fatty acid. ALA is the principal n-3 fatty acid; which a healthy human will convert into EPA (C20:5 n-3) (Schaeffer *et al.*, 2006), and later into DHA (C22:6 n-3). ALA is abundant in flax seed oil (35% - 40%) and is present in small quantities in walnut, soybean and canola oil (Gunstone, 2002). In fish oil, PUFAs of the n-3 series, especially EPA and/or DHA, are found in greater abundance while ALA is present in relatively less amount. Most of commonly found fatty acids are listed in table.1.1 along with their common name, structure and molecular weight (Gunstone *et al.*, 2007).

Table 1.1 Types of fatty acids

Systematic name	Trivial name	Formula	Mol.wt.
n-6 PUFAs			
9,12-octadecadienoic	Linoleic acid (LA)	18:2 (n-6)	280.4
6,9,12-octadecatrienoic	γ -linolenic acid (GLA)	18:3 (n-6)	278.4
8,11,14-eicosatrienoic	dihomo- γ -linolenic acid	20:3 (n-6)	306.5
5,8,11,14-eicosatetraenoic	Arachidonic acid (AA)	20:4 (n-6)	304.5
7,10,13,16-docosatetraenoic	Adrenic acid	22:4 (n-6)	332.6
4,7,10,13,16-docosapentaenoic	Osbond acid	22:5 (n-6)	330.6
n-3 PUFAs			
9,12,15-octadecatrienoic	α -Linolenic acid (ALA)	18:3 (n-3)	278.4
6,9,12,15-octadecatetraenoic	Stearidonic acid (SDA)	18:4 (n-3)	276.4
8,11,14,17-eicosatetraenoic	ETA	20:4 (n-3)	304.5
5,8,11,14,17-eicosapentaenoic	Timnodonic acid (EPA)	20:5 (n-3)	302.5
7,10,13,16,19-docosapentaenoic	Clupanodonic acid (DPA)	22:5 (n-3)	330.6
4,7,10,13,16,19-docosahexaenoic	Cervonic acid (DHA)	22:6 (n-3)	328.6
n-9 Unsaturated fatty acids			
9-octadecenoic acid	Oleic acid	18:1 (n-9)	282.46
11-eicosenoic acid	Eicosenoic acid	20:1 (n-9)	310.5
5,8,11-eicosatrienoic	Mead acid (ETE)	20:3 (n-9)	306.5
13-docosenoic acid	Erucic acid (EA)	22:1 (n-9)	338.6
Conjugated fatty acids			
9Z,11E-octadeca-9,11-dienoic acid	Remenic acid (RA) cis-9, trans-11 18:2 acid	18:2 (n-7)	280.44
8E,10E,12Z-octadecatrienoic acid	α -Calendic acid	18:3 (n-6)	278.43

1.3 Review of Literature

1.3.1 Biological importance of polyunsaturated fatty acids

In the body, fatty acids are used for generation of cellular energy and biosynthesis of membrane lipids and lipid mediators, modulate lipoprotein metabolism (Arterburn *et al.*, 2006). Poly-unsaturated fatty acids (PUFAs) are important components of cellular structures and functions. As integral components of cell membranes, PUFAs have strong effects on the fluidity and function of biological membranes mediated through lipid protein interactions. A Number of cellular processes including modulation of ion channels, endocytosis-exocytosis, activities of membrane-associated proteins *etc.* are controlled by presence of PUFAs (Spector and Yorek, 1985; Los *et al.*, 2013). They also serve as precursors to biologically active lipid mediators (C20 fatty acids and their metabolites) termed as eicosanoids that include prostaglandins, thromboxanes, leukotrienes and other oxygenated derivatives (Serhan *et al.*, 2008).

1.3.2 Importance of balanced intake of n-3 and n-6 PUFAs

Over the past few decades, many studies and clinical investigations have been carried out on the metabolism of PUFAs in general and on n-3 PUFAs in particular. Discovery of health benefits of n-3 PUFAs was started with an interesting fact that Greenland Eskimos consume large amount of fat from sea food, but displayed virtually no cardiovascular disease (Dyerberg *et al.*, 1975; Simopoulos, 2002). The high level of n-3 PUFAs and very less ratio of n-3 and n-6 PUFAs in diet consumed by the Eskimos results in reduced problems related with triglycerides, heart rate, blood pressure and atherosclerosis *etc.* (Dyerberg *et al.*, 1975; Bjerregaard *et al.*, 1997). The n-3 PUFAs have a number of beneficial roles in cell function and biology with different biological effects compared to other n-6 PUFAs as discussed below.

1.3.2.1 n-3 PUFAs and anti-inflammatory mediator production

In humans, PUFAs levels in various tissues mainly depend on dietary intake and have a major impact on health. The n-6 PUFAs act as precursors for certain prostanoids that are powerful promoters of inflammatory responses, platelet aggregation *etc.* while the n-3 PUFAs are found to be useful in managing chronic inflammatory conditions since these give rise to a family of anti-inflammatory mediators termed resolvins (Serhan *et al.*, 2008; Galli and Calder, 2009). Many anti-inflammatory pharmaceutical products of n-3

PUFAs inhibit the production of certain eicosanoids, cytokines, reactive oxygen species and the expression of adhesion molecules (Bloomer *et al.*, 2009).

Inclusion of flaxseed oil or fish oil in the diet resulted in decreased synthesis of the proinflammatory eicosanoids involved in inflammation related diseases like ulcerative colitis, Crohn's disease (Turner *et al.*, 2008; Turner *et al.*, 2009), autoimmune disorders, inflammatory bowels disease (Baumgart and Carding, 2007), osteoarthritis treatment (Zainal *et al.*, 2009), asthmatic bronchoconstriction. (Hageman *et al.*, 2012). Studies of healthy volunteers and rheumatoid arthritis patients have shown ~90% inhibition of cytokine production after dietary supplementation with fish oil (Calder, 2006). Use of flaxseed oil in domestic food preparation also reduced production of these cytokines. Thus, n-3 PUFAs are potentially anti-inflammatory agents and novel anti-inflammatory therapies can be developed that take advantage of positive interactions between the dietary fats and existing or newly developed pharmaceutical products.

1.3.2.2 n-3 PUFAs in fetal and infant early growth and development

The fetus depends completely on the maternal source of PUFAs (maternal tissues/stores and dietary intake) and infant obtains these PUFAs through the breast milk (Koletzko *et al.*, 2007). Both n-6 and n-3 PUFAs mainly AA and DHA are important in development of fetus and have different and specific roles in neural and behavioral functions. DHA is an essential component of cell membranes and is crucial for the function of rhodopsin for vision and postsynaptic receptors for neurotransmission (Crawford *et al.*, 2009). It is absolutely required for the development of the sensory, perceptual, cognitive and motor neural systems during the brain growth (McCann and Ames, 2005; Gould *et al.*, 2013). During the third trimester, large amounts of DHA accumulate in fetal tissue in the retina and brain, which may correlate with normal eyesight and brain function (Judge *et al.*, 2007). Pregnant women who consume n-3 fatty acids (DHA) have a low risk of low birth weight of infants, premature birth, infants with vision and nerve problems *etc.* (Helland *et al.*, 2008; Olsen *et al.*, 2007). Prophylactic diet therapy and n-3 PUFAs may prevent recurrent hypertriglyceridemia during pregnancy (Takaishi *et al.*, 2009). EPA and DHA supplementation to mothers during pregnancy and breastfeeding may protect their children against allergies (Palmer *et al.*, 2012).

1.3.2.3 n-3 PUFAs in preventing coronary heart disease

Metabolic and epidemiological studies as well as clinical trials have established a strong and consistent association between dietary fats and few diet related disorders like Coronary Heart Disease (CHD) (Peter *et al.*, 2013; Rizos and Elisaf, 2013), obesity, type 2 diabetes *etc.* (Hartweg *et al.*, 2009; Misra *et al.*, 2011). Appropriate intake of n-3 PUFAs (ALA, EPA and DHA) has been associated with a reduced risk of CHD and sudden cardiac death after an acute myocardial infarction mediated through low levels of high density lipoprotein (HDL) cholesterol and high levels of low density lipoprotein (LDL) cholesterol (Schiano *et al.*, 2008).

Circulating markers of inflammation, such as C-reactive protein (CRP), TNF- α , and some ILs (IL-6, IL-1), correlate with an increased probability of experiencing a cardiovascular event (Bloomer *et al.*, 2009). Intake of n-3 PUFAs resulted in a decreased expression of genes involved in inflammatory and atherogenesis related pathways, such as eicosanoid synthesis, scavenger receptor activity, adipogenesis, and hypoxia signaling. N-3 PUFAs supplementation also leads to decreased dyslipidemia, cholesterol delivery to the arterial wall, and arterial wall proinflammatory pathways and increased arterial wall anti-inflammatory markers (Bouwens *et al.*, 2009).

1.3.2.4 n-3 PUFAs and mental health

Adequate dietary intake of DHA and EPA is fundamental requirement for proper brain function, normal growth and development of brain (McCann and Ames, 2005; Uauy and Dangour, 2009). DHA/EPA are important throughout adulthood, as well as during the brain growth that characterize prenatal and postnatal development (Uauy and Dangour, 2009). Children whose mothers had taken DHA supplementation during pregnancy had significantly better problem-solving skills at 9 months old than those whose mothers had not taken DHA supplementation during pregnancy (Judge *et al.*, 2007). DHA deficiency has been associated with cognitive decline and the onset of Alzheimer's disease (AD) in adults (Tully *et al.*, 2003). In AD, the activation of multiple inflammatory cells in the brain leads to release of IL-1B, IL-6, and TNF- α from microglia cells may result into dysfunction of the neurons in the brain (Freund-Levi *et al.*, 2009). Dietary supplementation with DHA and EPA has proven beneficial for treatment of many of the known mental disorders like deficit/hyperactivity disorder like hypertension, major

depressive disorder (MDD), Alzheimer's disease, autism, dyspraxia, dyslexia, aggression, bipolar disorder, schizophrenia, personality disorder, accelerated cognitive decline-impairment, Parkinson's disease *etc.* (Bousquet *et al.*, 2011; Prior and Galduroz, 2012).

1.3.2.5 n-3 PUFAs and type 2 diabetes mellitus and obesity

Type 2 diabetes mellitus is the fourth leading cause of death in developed countries with a twofold excess mortality and a two to four fold increased risk of CHD and stroke. Compared to higher fat intakes, low fat-high carbohydrate diets result in a metabolic pattern that increases the risk of type 2 diabetes and CHD. Low consumption of n-3 PUFAs may results in dyslipidemia associated with metabolic syndrome (a risk factor for type 2 diabetes) elicits high serum levels of triglycerides, a reduction in serum HDL cholesterol and show higher responses in postprandial glucose and insulin concentrations (Misra *et al.*, 2011). Studies on experimental animals (Ghafoorunissa *et al.*, 2005; Hartweg *et al.*, 2009) and limited data in humans suggest that high intakes of either saturated or *trans* fatty acids may contribute to insulin resistance whereas n-3 PUFAs may prevent insulin resistance (Hartweg *et al.*, 2009). A few studies also showed that long-term dietary intake of n-3 PUFAs starting at 1 year of age lowers the risk of type 1 diabetes and islet autoimmunity (Norris *et al.*, 2007).

Overall systemic inflammation in severely obese nondiabetic patients and lipid metabolism was improved due to proper balanced intake of n-3 and n-6 PUFAs which may be beneficial in the long-term treatment of obesity which in turn nullifies risk for the development of type 2 diabetes and cardiovascular disease (Itariu *et al.*, 2012).

1.3.2.6 n-3 PUFAs and cancer

A strong inverse relationship is documented between n-3 PUFAs intake and some types of cancers. Supplementation of diet with n-3 PUFAs lowers the risk associated with different types of cancer (Wendel and Heller, 2009; Murphy *et al.*, 2012). N-3 PUFAs reduced prostate tumor growth and increased survival (Berquin *et al.*, 2007). Cancer cachexia is characterized by a progressive loss of muscle, resulting in functional impairment and shorter survival. EPA supplementation can be used as an anti-cachexia therapy (Murphy *et al.*, 2011). A clear role as an oncogene has been established for prostaglandin endoperoxide H synthase, (cyclooxygenase-2; COX-2), which play an

important role in conversion of AA to eicosanoids which are commonly linked to inflammation, tumorigenesis, angiogenesis, cell proliferation, and inhibition of apoptosis (Jones *et al.*, 2003). It is expressed at elevated levels during inflammation, in tumor cells and supplying blood vessels. The formation of eicosanoids derived from n-6 PUFAs (AA) is found to be decreased by supplementation with n-3 PUFAs and inhibit cell proliferation in cancerous cells and may thereby contribute to suppression of carcinogenesis (Howe, 2007; Kuriki *et al.*, 2007). Omega-3 PUFAs also enhance the sensitivity of tumor cells towards chemotherapeutic agents. (Tsuzuki *et al.*, 2007). Increased Protein Kinase B (Akt) activity in breast cancer cells can be down-regulated with DHA and EPA supplementation (DeGraffenried *et al.*, 2003). Similar results were obtained in case of prostate cancer cells also that the DHA and EPA were able to prevent progression of prostate cancer cells while AA promoted cell growth (Friedrichs *et al.*, 2011).

1.3.3 Sources of essential fatty acids in our diet

Fats and fatty acids are the major component of our diet. They are most concentrated source of energy and essential in the diet for absorption and further utilization of number of fat soluble vitamins, antioxidants *etc.* (Simopoulos, 2004; Gunstone *et al.*, 2007). Studies in Paleolithic nutrition and modern-day human populations reveal that human beings evolved consuming a diet with lower percentage of saturated fatty acids than today's diet. Furthermore, the diet also contained small and roughly equal amounts of n-3 and n-6 PUFAs (ratio of 1:1-2) and much lower amounts of *trans* fatty acids than does today's diet (Simopoulos, 2002). It is important to maintain an appropriate balance of n-3 and n-6 fatty acids (i.e. 1:5) in the diet as these two substances work together to promote proper health, growth and homoeostasis of inflammatory responses (Calder, 2006; Simopoulos, 2008). The human body has enzymes that can convert either LA or ALA into all the other different types of n-6 and n-3 fatty acids but cannot interconvert n-6 fatty acids to n-3 fatty acids. Thus, the ratio of these two in the diet essentially determines their ratio in the body. Hence a balance of n-6 and n-3 PUFAs in the diet is essential for proper health (Simopoulos, 2004; 2008). However, at present percentage of n-6 PUFAs with reference to n-3 PUFAs is higher in the human diet due to consumption of vegetable oils that are rich in LA, an altered ratio in favor of too much n-6 PUFAs can be

detrimental to one's health as described in section 2.2.1. According to the report by, Indian Council of Medical Research (ICMR), the current ratio of n-3 and n-6 PUFAs in Indian diets is ~1:25 while the ideal ratio is 1: 5.

1.3.3.1 Plant seed oils

The primary source of n-6 fatty acid in the diet is LA from the oils of seeds and grains. Sunflower (60%), safflower (70%) and corn oil (55%) are particularly rich sources of LA, which is at the root of the n-6 fatty-acid family but contains very less amount of ALA. Evening primrose oil and borage oil are rich in not only in LA, but also in n-6 derivative gamma-linolenic acid (GLA) (Gunstone, 2002).

Although ALA is present in dark green leafy vegetables (Spinach, Kale, Mustard greens, Collard *etc.*), often comprising over 50% of their fatty acids, these are not high in fat. The leaves and seeds of the perilla plant, avocado fruits, flaxseed oil (45% - 55%), soybean oil (5-7%), walnuts (7%) and canola oil (9%), purslane, hempseeds, sesame seeds, and other wild plants are the rich plant sources of ALA. Fatty acid composition of few routinely used plant seed oil in India, is as per given in table 1.2 (Kaur *et al.*, 2012).

1.3.3.2 Fishes and fish oil

Presently fishes are the main sources of long chain n-3 PUFAs for human consumption which include marine fishes, such as cod, eels, herring, mackerel, salmon, sardines, sharks, tuna, trout *etc.* (Gladyshev *et al.*, 2013; Tur *et al.*, 2012). They contain very little ALA, but are rich sources of n-3 derivatives EPA and DHA, derived from marine bacteria, microalgae, phytoplankton, zooplanktons and seaweeds *etc.* which are the primary producers of these fatty acids (Bell and Tocher, 2009; Gladyshev *et al.*, 2013). These organisms represent the starting point of the aquatic food chain by which PUFAs ultimately accumulate in fish. It is largely the cold water fish that serve as the major sources of EPA and DHA and even this show marked seasonal variability in their EPA and DHA content (Racine and Deckelbaum, 2007; Gladyshev *et al.*, 2013).

Table 1.2 Fatty acid composition of routinely used plant seed oils

Fats and Oils	Fatty acid amount in g/100 g				Predominant fatty acids
	Saturated	Mono unsaturated	LA	ALA	
Coconut	91	7	2	< 0.5	Saturated
Ghee	65	32	2	< 1.0	Saturated
Vanaspati	24	19	3	< 0.5	Saturated
Palm Oil	45	44	10	< 0.5	Saturated + MUFAs
Olive	13	76	10	< 0.5	MUFAs
Groundnut	24	50	25	< 0.5	MUFAs
Mustard	8	70	12	10	MUFAs
Sesame	15	42	42	1.0	MUFAs and PUFAs
Rice bran	22	41	35	1.5	MU and PUFAs
Cotton seed	22	25	52	1.0	PUFAs
Corn	12	32	55	1.0	PUFAs
Sunflower	13	27	60	<0.5	PUFAs
Safflower	13	17	70	<0.5	PUFAs
Soybean	15	27	53	5.0	PUFAs

Average content of LA (18:2 n-6) and ALA (18:3 n-3) in plant seed oils (g/100 g fat) (Kaur *et al.*, 2012).

1.3.3.3 Microorganisms as potential sources of n-3 PUFAs

Long chain n-3 PUFAs are synthesized mainly by marine phytoplankton, zooplankton and microalgae (Gladyshev *et al.*, 2013). High concentrations of EPA, observed in some planktonic fractions during cold periods, are typical of diatoms. They are eventually transferred through the food web and are incorporated into lipids of aquatic species such as fishes (Bell and Tocher, 2009). Alternatively lipids of liverworts, ferns, mosses, algae, yeasts and fungi can be utilized as oil source, which include ALA, EPA and DHA (Simopoulos, 2004). Microbial oil or single-cell oil (SCO) is a relatively new concept (Ratledge, 2004; Armenta and Valentine, 2013). Few microalgae and fungi that produce PUFAs very rich in DHA and EPA can be used to solve the problem of seasonal variation

and can provide a continuous supply of n-3 PUFAs. Till date commercial production of microbial oils is not achieved on large scale due to economic feasibility. There is no prospect that microbial oils similar to conventional oils could ever be produced economically. Therefore microbial single cell oil sources must be highly specific in their fatty acid composition and should contain currently expensive fatty acids which are not easily obtained from agricultural and animal sources (Ratledge, 2004, Xue *et al.*, 2013).

1.3.4 Dietary requirements and recommendations

In most countries daily consumption of PUFAs has been recommended with absolute values along with balanced ratio of n-3 and n-6 PUFAs. The World Health Organization (WHO) and the Food and Agricultural Organization (FAO) regulate the international recommendations on fats in human health. According to the report on dietary recommendations by Indian Council of Medical Research (Anonymous, 2009) adequate intake values of different n-3 PUFAs are as given in table 1.3.

Table 1.3 Recommended Dietary Allowances for PUFAs

Age Years	Adequate Intake (AI)		Total n-3 PUFAs (DHA+ DPA +EPA)
	Linoleic acid	α -linolenic acid	
Boys and girls			
1-3	5 g/day	0.5 g/day	40 mg/day
4-8	8 g/day	0.8 g/day	55 mg/day
Boys			
9-13	10 g/day	1.0 g/day	70 mg/day
14-18	12 g/day	1.2 g/day	125 mg/day
Girls			
9-13	8 g/day	0.8 g/day	70 mg/day
14-18	8 g/day	0.8 g/day	85 mg/day
Adults 18+ years			
Men	13 g/day	1.3 g/day	160 mg/day
Women	8 g/day	0.8 g/day	90 mg/day

1.3.5 Functional food and nutraceuticals

Functional foods and nutraceuticals which give specific medical or physiological benefit have gained much importance (Ozen *et al.*, 2012). These might inherently possess beneficial ingredients, or they may be fortified/modified and/or genetically altered so as to fulfill consumer's requirements e.g. Green tea can be considered as a natural functional food containing antioxidants which helps in the prevention of cancers, obesity *etc.* (Potenza *et al.*, 2007). A margarine spread, 'Benecol' containing stanol esters derived from wood pulp was first introduced in UK market in 1990s. The sitostanol ester in Benecol works by blocking the absorption of cholesterol from the gut thus decreases serum total LDL cholesterol concentrations by about 10 to 14 percent (Miettinen *et al.*, 1995; Raitakari *et al.*, 2008). Red wine also contains phenolic compounds and antioxidants (Micallef *et al.*, 2007). Fish oils are the routinely used n-3 PUFAs supplements and even tried for fortification of foods to fulfill n-3 PUFAs requirements (Kolanowski and WeiBbrodt, 2007). Most early developed and marketed functional foods include foods fortified with minerals such as vitamin C, vitamin E, calcium, folic acid, iron *etc.* (Menrad, 2003). The focus now shifted to foods fortified with various micronutrients such as phytosterol, specific fatty acids and soluble fiber, probiotics, prebiotics *etc.* (Ozen *et al.*, 2012). The highest growth in a nutraceutical market is likely to be seen in the use of probiotics which they ferment or biotransform and in n-3 PUFAs fortified foods and beverages (Betoret *et al.*, 2011; Makinen *et al.*, 2012).

Number of traditionally used fermented products, Dairy and non-dairy foods containing probiotic microorganisms are claimed to promote gut health. Tremendous researches have been done on utilizing *lactic acid bacteria* as probiotics, whereas rather limited emphasis was given towards yeast. Few types of yeast also have been explored to be used as probiotics when supplemented alone or in co-culture (Kumara *et al.*, 2004; Pennacchia *et al.*, 2008; Moslehi-Jenabian *et al.*, 2010). Among these, *S. cerevisiae var. boulardii* is the only yeast produced and used as a probiotics (Moslehi-Jenabian *et al.*, 2010). Some reports suggest a beneficial relationship between probiotics strains and yeast (Bladino *et al.*, 2003; Kedia *et al.*, 2007). By utilizing suitable yeast strain as a starter culture and using optimized food processing techniques, it is possible to produce functional foods that give nutritional enrichment as well as appropriate delivery of bio-active molecules (Moslehi-Jenabian *et al.*, 2010). Recently metabolic engineering of the oleaginous yeast

Yarrowia lipolytica resulted in a strain that produced EPA at 15% of dry cell weight which can be used as a sustainable source of n-3 PUFAS (EPA) (Xue *et al.*, 2013). Studies are being carried out to produce omega-3 enriched egg, meat, beef *etc.* by appropriate feeding (n-3 fatty acids in diets) (Antruejo *et al.*, 2011).

Novel yeast producing essential PUFAs can be tested for probiotic properties or recombinant yeasts producing essential PUFAs can be generated by expressing respective genes from those novel yeasts and can be explored for their utilization as functional food. These foods and beverages hold a very strong future as they can be personalized according to particular physiological condition or specific diseases and disorders arising out of the nutritional need of populations living in particular geographic areas.

1.3.6 Biosynthesis of PUFAs

Fatty acid synthesis occurs from acetyl-CoA and malonyl-CoA precursors through fatty acid synthases. Mammals can synthesize all fatty acids that it needs except ALA and LA. These essential fatty acids are metabolized in the animal body to produce long chain PUFAs; liver is the major site of fatty acid biosynthesis. Biosynthesis of PUFAs is carried out by a highly specific enzyme system involving number of desaturases and elongases. Both of this group of enzymes exhibits high substrate specificity and regioselectivity. The efficiency of the PUFAs biosynthetic pathway is always influenced by lipid pool present inside the cell and in the surroundings. The interplay between desaturases, elongases, and acyltransferases determines the overall fatty acid composition of the cell (Los and Murata, 1998).

1.3.6.1 Elongases

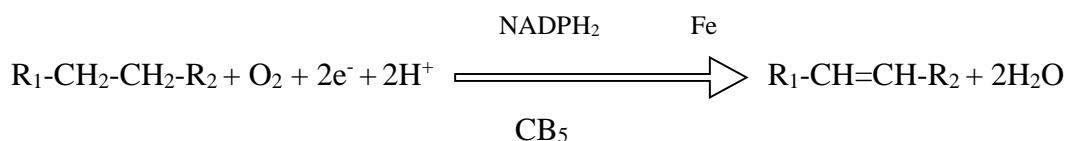
Elongation of lower fatty acids to long chain PUFAs is carried out by elongases. Many eukaryotic cells have capacity of 2-carbon chain elongation of both endogenously synthesized and dietary fatty acids, involving two primary systems *viz.* first one is in the endoplasmic reticulum (ER) and the other is in mitochondria (Jakobsson *et al.*, 2006). Peroxisomes also contain an acetyl-CoA dependent elongation system (Sprecher *et al.*, 1995). Large number of elongase genes were isolated and characterized from different organisms, e.g. rat, mouse, humans, yeast and other lower eukaryotes. Substrate

specificity of elongases varies with respect to fatty acids of different lengths and degrees of unsaturation (Leonard *et al.*, 2004; Jakobsson *et al.*, 2006).

1.3.6.2 Fatty Acid Desaturases

Fatty acid desaturases (FADs) are enzymes that introduce a double bond in a specific position of long-chain fatty acids (Los and Murata, 1998). FADs are non-heme, iron-containing, oxygen-dependent enzymes. FAD activity was first demonstrated in 1960 by Bloomfield (Bloomfield and Bloch, 1960), who described a “particulate enzyme” in yeast microsomes that required oxygen and NADPH to introduce a Δ -9 double bond into a saturated fatty acid substrate. They play an important role in the maintenance of the proper structure and functioning of biological membranes and are conserved across kingdoms. They are present in all groups of organisms including higher plants, green algae, diatoms, nematodes, fungi, and bacteria with the exception of archaea.

FADs are enzymes which catalyze the general reaction shown below:



1.3.7 Types of fatty acid desaturases

Fatty acid desaturases belong to two groups: soluble desaturases and membrane-bound desaturases (Nakamura and Nara, 2004). Acyl-Acyl Carrier Protein (Acyl-ACP) desaturase belongs to a group of soluble FADs that catalyze the insertion of a double bond into saturated fatty acids bound to acyl carrier protein e.g. stearyl-ACP desaturase EC 1.14.99.6 catalyzes the conversion of stearic acid (C18:0) to oleic acid (C18:1).

Membrane bound desaturases are further subdivided in two types; first is acyl-lipid desaturases which introduce double bonds to fatty acids that have been esterified to glycerolipids and second subgroup is acyl-CoA desaturases which are present in ER membrane and introduce double bonds into fatty acids that are bound to coenzyme A (fatty acyl-CoAs). Acyl-CoA desaturases are present in animals including insects and nematodes as well as in fungi. All mammalian desaturases that have been identified are acyl-CoA desaturases (Pereira *et al.*, 2003).

Desaturases are further classified according to their positional specificity or regioselectivity, i.e. which designates the preferred position for substrate modification. The specific site of desaturation in the fatty acid chain is defined by reference to the carboxyl terminus (Δ -position) or the methyl terminus (n-position) of the fatty acid.

Based on the position of the double bond insertion relative to a pre-existing double bond in a fatty acyl chain, desaturases can also be referred to as front-end desaturases or methyl-end desaturases.

1.3.7.1 Front end desaturases

These desaturases introduce a double bond between the pre-existing double bond and the carboxyl (front) end of the fatty acid, e.g. Δ -4, Δ -6; Δ -5; Δ -8 and Δ -9 FADs. The first gene encoding Δ -6 FAD (*desA*) was cloned in 1993 from cyanobacteria *Synechocystis spp.* strain PCC6803 (Wada *et al.*, 1993). Since then, front-end desaturases have been identified and characterized from a wide range of eukaryotic species including algae, protozoa, fungi, plants and animals including humans. They are remarkable for their structural similarity and functional diversity (Meesapyodsuk and Qiu, 2012).

1.3.7.2 Methyl-end desaturases

These introduce a double bond between the pre-existing double bond and the methyl-end, e.g. ' Δ -12' and 'n-3 desaturase'. Although both methyl-end desaturase and front-end desaturase are involved in the biosynthesis of long chain PUFAs, they are not uniformly present in all living organisms. The methyl-end desaturases are widely present in plants and microorganisms, while the latter mostly occur in animals and microorganisms (Pereira *et al.*, 2003).

1.3.8 Biochemistry of PUFAs biosynthesis

Long chain PUFAs are synthesized from monounsaturated fatty acids by the action desaturases, further elongation and subsequent desaturation reaction.

Fatty acid desaturases introduce double bonds at the Δ -5, Δ -6, Δ -9, Δ -12, n-3 positions of fatty acids. The order in which desaturases operate is very strictly determined. The first double bond is often inserted at approximately the middle position of saturated fatty acids with 18 carbons or 16 carbons in length by the Δ -9 desaturase (Nakamura and Nara, 2004). Fatty acids with different chain length and double bond position are generated

later by various fatty acid modifying enzymes. In C18 fatty acyl chain, the Δ -12 desaturases introduce a double bond into fatty acids that have a double bond at the Δ -9 position. The n-3 desaturase introduces a double bond into fatty acids that have a double bond at the Δ -12 position. Subsequent desaturation takes place after elongation of fatty acyl chain.

1.3.8.1 PUFAs biosynthesis in mammals

FADs are the enzymes which catalyze introduction of double bond at specific positions in an acyl chain. Higher animals including humans lack the Δ -12 and n-3 methyl-end desaturase activities (Pereira *et al.*, 2003). Consequently, they cannot synthesize LA (C18:2 n-6) and ALA (C18:3 n-3) from oleic acid (C18:1). Hence it becomes necessary to obtain these essential fatty acids from the diet. In humans, addition of a double-bond at the 9th carbon of LA results in gamma-linolenic acid (GLA: n-6). Like ALA, GLA has 3 double-bonds. But gamma-linolenic acid is an n-6, whereas ALA is an n-3 fatty acid. GLA further converted into AA (C20:4 n-6) which acts as precursor for biologically-important substances known as the eicosanoids including prostaglandins, thromboxanes, lipoxins and leukotrienes (Nakamura and Nara, 2004)

Important members of the n-3 family of PUFAs synthesized from ALA are; EPA (C20:5 n-3) and DHA (C20:6 n-3). EPA gives rise to its own class of n-3 family of eicosanoids, distinct from the n-6 eicosanoids derived from AA. In mammals, the conversion of EPA into DHA is complex, and occurs through the ‘Sprecher-the retro-conversion pathway’ (Sprecher *et al.*, 1995) which involves two rounds of chain elongation of EPA and another Δ -6 desaturation on the elongated product, followed by a single 2-carbon chain shortening (β -oxidation step) of the Δ -6 desaturated product in the peroxisome, giving DHA. The involvement of two different organelles in the compartmentalized biosynthesis of DHA implies that the intracellular movement of fatty acids occurs between the ER and the peroxisome (Sprecher *et al.*, 1995). In humans till date six members of the Elovl (elongation-of-very-long-chain-fatty acids) gene family encode for elongases have been identified, which are further grouped into two groups *viz.* first one responsible for the elongation of C18 and C20 PUFAs while second one with members exhibiting a substrate specificity for long-chain and involved in the Sprecher pathway of long-chain PUFA

biosynthesis (i.e., DHA) (Jakobsson *et al.*, 2006). The overall view of biosynthesis of PUFAs in humans is summarized in figure 1.4.

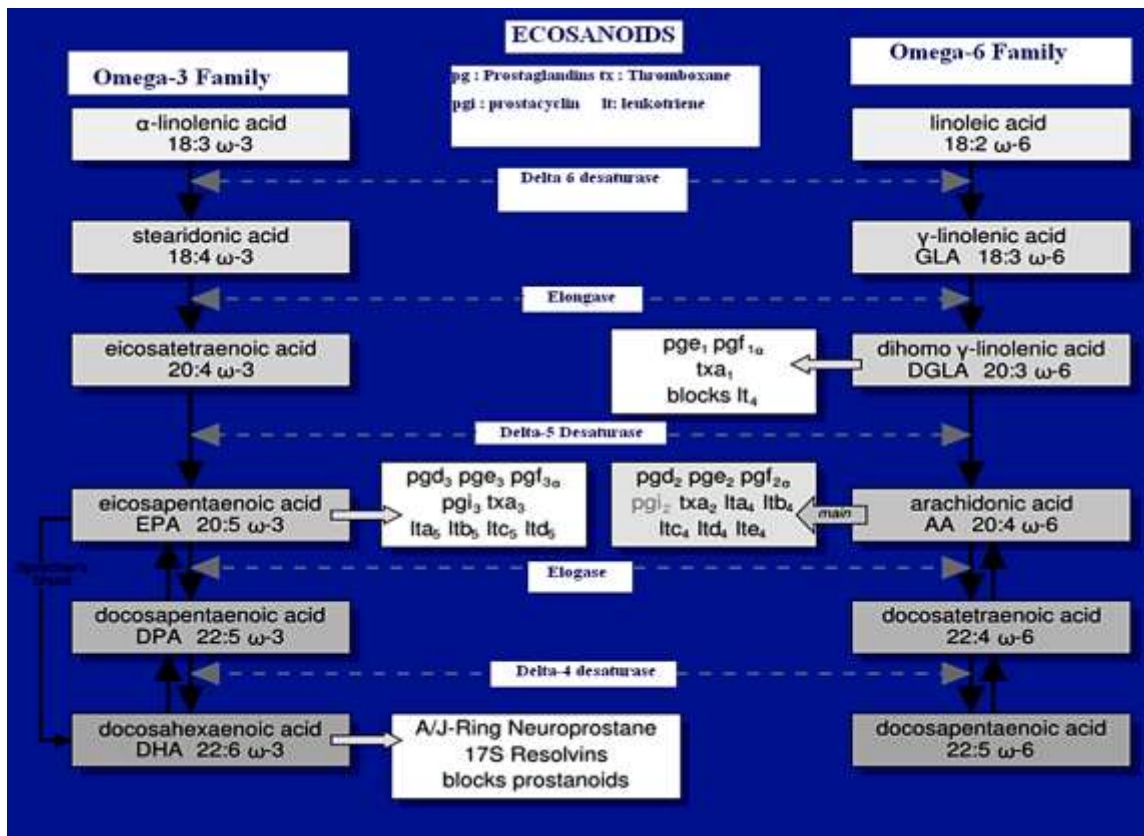


Figure 1.4 Biosynthesis of the n-6 and n-3 PUFA. The n-6 and n-3 families of LC-PUFAs are synthesized from their distinct precursors LA and ALA involves the same enzymes (desaturases and elongase). This creates a competition between both families to develop the metabolic derivatives eicosanoids including prostaglandins (pg), prostacyclins (pgi), thromboxanes (tx), leukotrienes (lt), lipoxins *etc.* (Adapted from, http://en.wikipedia.org/wiki/Essential_fatty_acid_interactions).

Higher animals including humans lack the Δ -12 and n-3 methyl-end desaturase activities (Pereira *et al.*, 2003). Human Δ -5- and Δ -6-desaturases, which catalyze the conversion of LA and ALA to AA and EPA respectively, are expressed highest in the liver (Cho *et al.*, 1999a, 1999b). These desaturases are encoded by the Fads1 and Fads2 genes respectively, which are localized in the fads cluster located on chromosome 11. Fads gene cluster also contain a third gene named Fads3 with an 1468 bp ORF that has been postulated as a front end desaturase (Figure 1.5).

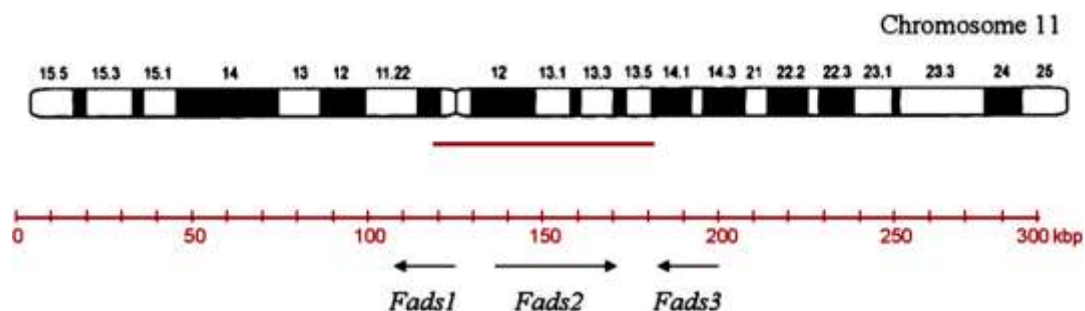


Figure 1.5 Chromosomal localization of the Fads cluster. In a 290-kbp region on the human chromosome 11, the Fads genes are localized within a 92-kbp zone and organized in a head-to-tail structure. The sizes are given in kilo base pairs (Blanchard *et al.*, 2011).

Splicing events during expression of Fads3 gene resulted in generation of 9 novel Fads3 alternative transcripts (Park *et al.*, 2009), showed the existence of different isomers of FADS protein in human cells but its biological function remains unknown (Pedrono *et al.*, 2010). Mammalian front-end desaturases operate on diet-derived PUFAs to synthesize long chain PUFAs. In a mammalian system, efficiencies of conversion of dietary ALA into LC-PUFAs are in strong associations with *snp* variants in the human Fads1 and Fads2 genes and with age, gender, and other factors (Schaeffer *et al.*, 2006; Malerba, 2008; Bokor *et al.*, 2010). It was also found that the demand for n-3 PUFAs is increased in old age, pregnancy and lactation. In certain disorders like schizophrenia, elevations in Δ -6 desaturase (FADS2) expression were seen that could have implications for central prostaglandin synthesis and proinflammatory signaling (Liu *et al.*, 2009). Thus in relation to genetic variants of desaturases, population subgroups may have different requirements of dietary PUFAs to achieve proper health and development.

1.3.8.2 Biosynthesis of PUFAs in plants

Plants synthesize a large variety of fatty acids but only a few of them are abundant and common. As discussed in earlier section 2.3.1, only a few plant species can produce long chain n-3 PUFAs (Gunstone *et al.*, 2007). In plants the de novo synthesis of fatty acids occurs primarily in the plastids of mesophyll cells in photosynthetic leaves, seeds and oil-accumulating fruits. The end products of fatty acid synthesis are usually the saturated fatty acids palmitate and stearate. These long chain acids are subjected to elongation, desaturation and further modifications.

The biosynthesis of PUFAs in plants is catalyzed by a series of enzymes with the first step carried out by an enzyme that converts oleic acid (18:1) to LA (18:2). An important enzyme for the production of PUFAs in plants is the oleate Δ -12 desaturase of the endoplasmic reticulum. The basic chemistry of fatty acid desaturation and elongation in plants is similar to that in animals. Most of the plant elongases are specific for saturated and monounsaturated fatty acids, but not PUFAs, hence plant oils lack long chain PUFAs (Leonard *et al.*, 2004).

1.3.8.3 PUFAs from yeast

Presence of n-3 PUFAs have been reported in variety of prokaryotic and eukaryotic microbes like bacteria, cyanobacteria, algae and fungi. Among these microorganisms yeast have better scope as they have relatively higher lipid content with similar fatty acids composition and energy value to plant oils thus can be utilized as an optimal and abundant source of PUFAs. EPA and DHA were found in a number of psychrophilic bacteria that inhabit in low-temperature deep-sea environments and in the intestines of sea fish and invertebrates (de Carvalho and Caramujo, 2012). As bacteria can produce some non-conventional fatty acids which might be toxic for consumption and total fatty acid content of bacteria ranging in between 2% - 5%; they are not considered as economical source of PUFAs (Russell and Nichols, 1999).

Yeast and fungi can form all required fatty acids *de novo* (Tehlivets *et al.*, 2007). Many of them also showed presence of PUFAs in their total fatty acid extracts. The molecular structures of the enzymes involved in fatty acid synthesis (acetyl-CoA carboxylase and fatty acid synthase), fatty acid elongation and desaturation (elongases and desaturases) are quite diverse between different pro- and eukaryotic species. In yeast, biosynthesis of saturated fatty acids from acetyl-CoA takes place in cytosol as well as in mitochondria while further elongation and desaturation of saturated fatty acid to produce long chain PUFAs takes place in endoplasmic reticulum at high energy load conditions at which cell possesses increased ATP/AMP ratio, elevated reduction equivalents (NADPH) and elevated acetyl-CoA pool (Tehlivets *et al.*, 2007). Thus, fatty acid synthesis may also be considered an efficient means to control cellular acetyl-CoA and NADPH levels.

The yeast *Saccharomyces* can produce only monounsaturated fatty acids and not the PUFAs as it has only one desaturase i.e. Δ -9 fatty acid desaturase enzyme encoded by

ole1 gene. The monounsaturated fatty acids in *Saccharomyces* and other fungi are formed from saturated fatty acyl CoA precursors by the OLE1 Δ -9-fatty acid desaturase (McDonough *et al.*, 1992; Wongwathanarat *et al.*, 1999). Few fungi also express membrane bound Δ -12 and Δ -15 desaturases *viz.* *Candida albicans* (Murayama *et al.*, 2006), *M. alpina* (Sakuradani *et al.*, 2005) *S. kluyveri* (Oura and Kajiwara, 2004). Some fungi will also elongate 18:2 and 18:3 and introduce additional double bonds through the activity of Δ -4,-5,-6- and Δ -7 desaturases to form very long chain (\geq C20) polyunsaturates such as arachidonic and EPA.

1.3.9 Structure of fatty acid desaturases

FADs are the members of a large superfamily of O₂-dependent, integral membrane proteins that catalyze a variety of oxidative modifications to lipids. Based on structural and catalytic similarities, membrane desaturases are included in a superfamily of oxidative enzymes along with alkane hydroxylase, xylene monooxygenase, carotene ketolase, and sterol methyloxidase (CrtR-like) and other related proteins.

The presence of a di-iron cluster in amino acid motifs of the membrane bound desaturases separates them from soluble desaturases. The castor Δ -9 desaturase is the first fatty acid desaturase enzyme for which an X-ray crystallographic structure has been determined (Lindqvist *et al.*, 1996) (Figure 1.6).

It has brought some insight into the structure/function relationship involved in the determination of substrate specificity and regioselectivity of soluble desaturase enzymes. Most of the front end desaturases studied at present (Δ -4, Δ -5, Δ -6, and Δ -8) has a cytochrome b5-like domain fused to the N-terminus of the main desaturation domain (Gostincar *et al.*, 2010). The majority of desaturase enzymes reside within membranes, due to that the membrane-bound desaturase activity and specificity of reaction are not well understood.

The primary sequences of a large number of membrane-bound desaturases have been discovered and organized into distinct clusters corresponding to the different regiochemistries of double bond introduction (Nakamura and Nara, 2004). However till date no three-dimensional structural information is available for these as membrane bound enzymes are difficult to purify.

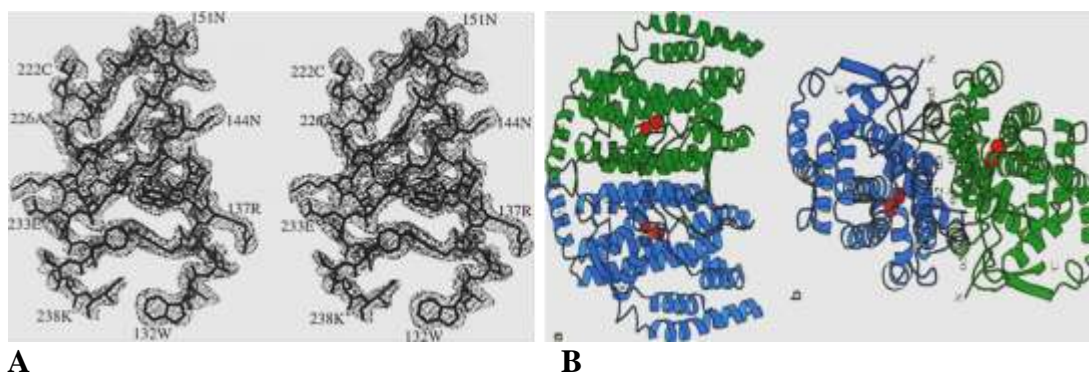


Figure 1.6 Crystal Structure of Δ -9 desaturase. A: Superposed refined model of Δ -9 desaturase, B: View of the dimer of Δ -9 desaturase. The two subunits are colored green and blue, respectively and the two iron centers are indicated by red spheres. (a) Viewed along the a-axis and (b) along a non-crystallographic 2-fold axis parallel to the b-axis (Lindqvist *et al.*, 1996).

2-D models based on hydropathy and topology analyses resulted in prediction of the four transmembrane domains and either one or two membrane-peripheral protein domains. Three highly conserved histidine-rich motifs are essential for catalysis. In all, primary structure similarity, especially the conservation of three histidine-rich motifs, and similarity of hydrophobicity patterns support their conservation. On this basis, a model for the membrane topology of this superfamily has been proposed as shown in figure 1.7 (Tocher *et al.*, 1998; Los and Murata, 1998).

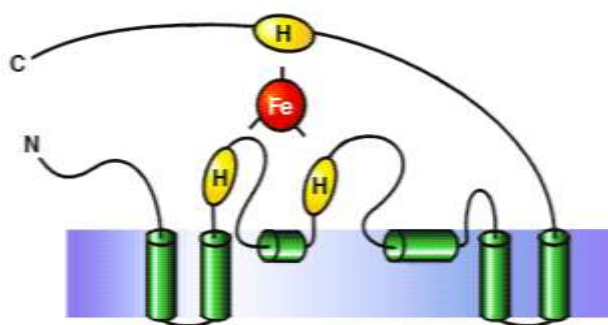


Figure 1.7 Current topological model for membrane-bound fatty acid desaturases. The predicted positioning of the membrane bound desaturase based on the localization of the conserved histidine clusters and iron atoms, which are assumed to constitute the catalytic center, at the cytoplasmic side. Fe represents a putative di-iron center and H represents histidine-rich structural motifs thought to be involved in coordinating the di-iron center (Los and Murata, 1998).

1.3.10 Structural determinants for activity and regioselectivity of desaturases

Membrane bound Desaturase enzymes are specific for their catalytic reaction with respect to the location, number, and stereochemistry of double bonds already present in fatty acids (Meesapyodsuk *et al.*, 2000). A Number of structural factors are responsible for the activity, substrate specificity as well as regioselectivity of the desaturases.

1.3.10.1 Types of regioselectivity

In case of membrane bound desaturases two major regioselective classes have been observed. First one is Δ -x, which introduces double bond in between x and x+1 carbon of the fatty acyl chain from carboxyl end. Second one is n-x, which desaturates fatty acyl chain at x and x+1 carbon from methyl end (Nakamura and Nara, 2004). Hence in order to get desaturation at particular position in a fatty acyl chain one has to rely on specific kind of desaturase only. The mechanism of fatty acid desaturation has been well-studied over the last decade. The current understanding of substrate specificity and regioselectivity of membrane-bound desaturases is based on mutant studies, biochemical topology analysis, and the comparison of related enzymes with divergent functionality (Meesapyodsuk and Qiu, 2012). This is because of the difficulties in expression and purification of the protein.

1.3.10.2 Cytochrome b5-like domain

Fatty acid desaturation requires cytochrome b5 and cytochrome b5 reductase for the electron transport in desaturation reaction (Tamura *et al.*, 1976). Most of the desaturases contain their own cytochrome b5 domain (Mitchell and Martin, 1995), while certain desaturases, including FAD-6 and FAD-3 do not have this domain and hence require an interacting cytochrome b5 complex (Petrini *et al.*, 2004). Cytochrome b5-like domains have been identified at N-terminal region in various desaturases from animals, fungi, plants and yeast. Due to presence of this domain NADH cytochrome b5 reductase transfers electrons to the catalytic site of these cytochrome b5 fusion desaturases directly and does not require an independent cytochrome b5. Co-expression of cytochrome b5 along with *fad-3* and *fad-6* genes, resulted in significant increase in PUFAs synthesis even at 30 °C, but did not affect the fatty acid synthesis so much at 20 °C (Yazawa *et al.*, 2010).

1.3.10.3 Histidine motifs and active center

Most membrane-bound FADs share a degree of sequence similarity and membrane topology; this includes three histidine rich sequence motifs which are thought to be important in forming a di-iron center at the active site. This enzyme domain family has extensive hydrophobic regions that would be capable of spanning the membrane bilayer at least twice. It has been seen that the soluble desaturases (Δ -9; stearoyl-ACP desaturase; EC 1.14.99.6) contain two conserved histidine motifs (HXXXH, HXXHH) (Shanklin and Cahoon, 1998) while membrane desaturases contained three conserved histidine-boxes and four trans-membrane domains (Los and Murata, 1998) that are likely to be involved in the catalysis of the desaturation reaction. Comparison of these sequences also reveals three regions of conserved histidine cluster motifs that contain eight histidine residues: HXXX(X)H, HXX(X)HH, and HXXHH which are reported to be catalytically essential

1.3.10.4 Substrate specificities and preferences

The genes encoding membrane-bound desaturases have relatively high degree of similarities, even though desaturases showed a broad range of catalytic diversity. Most of the characterized n-3-desaturases (Sk-FAD-3) are known to desaturate 18-carbon n-6 fatty acids (LA) into the n-3 PUFA (ALA); while they do not react with 20-carbon n-6 PUFAs (Oura and Kajiwara, 2004). In contrast, the fungal *Saprolegnia diclina* n-3-desaturase, SDD17, desaturates 20-carbon n-6 PUFA substrates, in preference to 18-carbon n-6 PUFAs, into the corresponding 20-carbon n-3 PUFAs (Pereira *et al.*, 2004). While, the nematode *Caenorhabditis elegans* n-3-desaturase, FAT-1, desaturates both 18-carbon and 20-carbon n-6 PUFAs; and it prefers 18-carbon n-6 PUFA substrates to 20-carbon ones (Meesapyodsuk *et al.*, 2000). A novel n-3 desaturase from the fungus *M. alpina* 1S-4, shows wide substrate specificity and can use both 18-carbon and 20-carbon n-6 PUFAs as substrates but shows a preference to 18 carbon substrates (Sakuradani *et al.*, 2005). N-3 desaturases from the filamentous fungi *Fusarium moniliforme*, *Fusarium graminearum*, and *Magnaporthe grisea* showed a bifunctional Δ -12 and n-3 desaturase activity with broad n-6 substrate specificity (Damude *et al.*, 2006).

1.3.11 Evolutionary relationship of FADs

Evolutionary analysis of available amino acid sequences of studied FADs elucidates their phylogenetic origin and catalytic diversity. Membrane desaturase genes are organized into three well resolved monophyletic groups (Lopez Alonso *et al.*, 2003). These groups are, the Δ -9 desaturase group, a second group comprising Δ -12 plus n-3 desaturases; and a functionally heterogeneous group including the ‘front-end’ desaturases; i.e. Δ -4, Δ -5, Δ -6, and Δ -8 desaturases. The evolution of front-end desaturases has undertaken a path different from that of the methyl-end desaturases.

Δ -9 desaturases are hypothesized to be the most ancestral group of desaturases as every other desaturase for its action requires existence of previous double bond at Δ -9 position. The Δ -9 desaturase is the only universally spread desaturase being present in all living beings groups; this observation also supports the hypothesis. The membrane Δ -9 desaturase cluster includes members of cyanobacteria, higher plants, fungi, invertebrates and vertebrates accordingly to its wide phylogenetic distribution. The remaining desaturases are missing in some of the evolutionary lineages (Lopez Alonso *et al.*, 2003). Monophyly of the Δ -12 and the n-3 desaturase group suggests a common origin of both kinds of desaturases groups i.e. Δ -9 desaturases (Lopez Alonso *et al.*, 2003). Phylogenetic analysis also revealed that n-3 desaturases arose by independent gene duplication events from a Δ -12 desaturase ancestor which also can be supported by functional activities of these enzymes as n-6 fatty acids are the substrate for n-3 desaturases. The divergence between Δ -12 and n-3 desaturases took place in the prokaryotic lineage. The Δ -12 Fatty Acid Desaturase (Δ -12-FADS)-like CD includes the integral-membrane enzymes; all of them show closest similarities between their respective histidine-boxes. These methyl end desaturases are absent in most of the animals, but present in lower eukaryotes, plants, cyanobacteria *etc.*

Functional Δ -5 and Δ -6 desaturases are found in a wide array of lineages including few yeast, fungi, microalgae, invertebrate protostomes and vertebrates, but they are mostly absent in plants. In humans, the Δ -5 activity is encoded by the *Fads1* gene, while *Fads2* codes for Δ -6 desaturase. Both the Δ -5 and Δ -6 desaturase enzymes have distinct, nonoverlapping substrate specificities (Cho *et al.*, 1999a, 1999b). In the nematode *C.*

elegans, Δ -5 and Δ -6 desaturation activities are also encoded by different Fads-like genes (Michaelson *et al.*, 1998b; Napier *et al.*, 1998). In contrast, teleosts (group of fishes e.g. rabbit-fish, zebra-fish *etc.*) showed distinct combinations of desaturase activities (e.g. bifunctional Δ -5/ Δ -6; Δ -4/ Δ -5 or separate Δ -5 and Δ -6 desaturases) (Hastings *et al.*, 2001; Li *et al.*, 2010). In case of humans, the Fads gene cluster has likely arisen during evolution through gene duplication as the exon organization is nearly identical in the three family members with each gene consisting of 12 exons and splice donor and acceptor sites interrupted at identical nucleotide positions within highly conserved codons (Marquardt *et al.*, 2000). On the basis of functional criteria, Δ -5 genes are thought to have evolved from a Δ -6 ancestor, since the action of the latter provides the substrate for Δ -5 desaturase in the LC-PUFA biosynthesis pathway (Lopez Alonso *et al.*, 2003). Thus it can be said that during the course of evolution a tandem gene duplication of Fads gene precursor (which may be encoding bifunctional desaturases), resulted in separate Δ -5 and Δ -6 genes and the process is still continued with further increase in genes in particular lineages e.g. Fads3 in humans (Castro *et al.*, 2012).

The presence of cytochrome b5 domain at the N-terminal to these enzymes reveals several distinct evolutionary events an example of co-evolution of chimeric proteins indicating some selective advantage. All of the enzymes that contain N-terminal Cytochrome b5 domain are involved in “front-end” modifications of lipid substrates. It is also clear from mutagenesis studies that the presence of N- and C terminal domains are essential for fatty acid desaturase activity (Libisch *et al.*, 2000), which can be inferred as the evolution of co-dependence of the two distinct domains of these enzymes.

1.3.12 Present status of the research on microbial *fad-3* genes

Major advances in the cloning and manipulation of fatty acid desaturase and elongase genes from various plants have been made over the last several years such as in the plants like *Arabidopsis thaliana*, *Brassica napus*, *Clorella vulgaris*, *Glycine max*, *Aleurites fordii* *etc.* (Alonso and Maroto, 2000; Warude *et al.*, 2006). Much of the *fad-3* genes characterized concentrate on desaturases that can convert C18 – C22 substrates to respective products in different major microorganisms including cyanobacteria, microalgae (Harwood and Guschina, 2009; Khozin-Goldberg *et al.*, 2011). A number of marine bacteria, belonging to genera *Shewanella*, *Colwellia* (Orikasa *et al.*, 2006; Lee *et*

al., 2009) and fungi like *Mortierella alpina* (Sakuradani *et al.*, 2005), *Saprolegnia diclina* (Tocher *et al.*, 1998; Pereira *et al.*, 2003; 2004; Warude *et al.*, 2006) have been studied for production of n-3 PUFAs.

There are fewer reports for yeasts, which include *S. kluyveri* (Oura and Kajiwara, 2004), *C. albicans* (Murayama *et al.*, 2006), *Kluyveromyces lactis* (Kainou *et al.*, 2006), *Pichia pastoris* (Zhang *et al.*, 2008) that are able to produce ALA to different extent. An 18-carbon PUFA-specific n-3 desaturase gene (*Sk-fad-3*) has been cloned from the yeast *S. Kluyveri* (Oura and Kajiwara, 2004). Kl-FAD-3 from yeast *K. lactis* has an overall identity of 79.3% to Sk-FAD-3 exhibited n-3 FAD activity. *Ca-fad-3* gene encodes n-3 desaturase in the dimorphic fungus *C. albicans*, which shares 63.2% similarity to Sk-FAD-3. Recently a complete CDS of the *Lk-fad-15* was obtained and characterized from oleaginous yeast *Lipomyces kononenkoae* (Yan *et al.*, 2013). This FAD is rather unique in having both n-3 and n-6 FAD activities.

A 20-carbon PUFA-specific n-3 desaturase gene (SDD-17) with a distinct preference for AA, whose product converts AA to EPA, has been cloned from the fungus *Saprolenia diclina* (Pereira *et al.*, 2004). A gene (*maw-3*) that encodes a novel n-3 desaturase has been isolated and cloned from the fungus *M. alpina* 1S-4. The enzyme has a wide substrate specificity and can use both 18-carbon and 20-carbon n-6 PUFAs as substrates but shows a preference to 18 carbon substrates (Sakuradani *et al.*, 2005). Damude *et al.* (2006), reported identification of n-3 desaturases from the filamentous fungi *Fusarium moniliforme*, *Fusarium graminearum*, and *Magnaporthe grisea* by their heterologous expression in *Y. lipolytica*. These enzymes are unique in having a bifunctional Δ -12 and n-3 desaturase activity with broad n-6 substrate specificity.

1.3.13 Regulation of fatty acid biosynthesis in yeast

The most intensively studied regulation of unsaturated fatty acid biosynthetic enzymes is the *Saccharomyces* Δ -9 desaturase. Fatty acid desaturase activities can be regulated by multiple factors, including the availability of nutrient carbon source, nitrogen source, oxygen levels, presence of unsaturated fatty acids in the surrounding growth medium, temperature *etc.* Temperature is one of the most important environmental factors.

1.3.13.1 Nutrient regulation of desaturase activity

Different yeast strains can grow in media having various carbon sources such as glucose, xylose, glycerol, starch, cellulose hydrolysates and variety of industrial and agricultural and domestic wastes. Lipids present in the culture media was found to be utilized as substrates for the production of cell mass and storage lipids (Botham and Ratledge, 1979; Ageitos *et al.*, 2011). When yeast cultures were grown in a media supplemented with either 16:1, 18:1, 18:2 and 18:3 fatty acids, it resulted in rapid insertion of those unsaturated fatty acids into the cell membranes and thus repression of desaturase enzymatic activity (Choi *et al.*, 1996; Martin *et al.*, 2007). When yeast cells were exposed to the monounsaturated fatty acids or 18:2, OLE1 mRNA levels rapidly dropped as much as 10-fold, followed by a loss of desaturase enzyme activity (McDonough *et al.*, 1992). However, in *S. kluyveri*, levels of Sk-OLE1 and Sk- FAD2 transcripts were unaffected by unsaturated fatty acids (Kajiwara, 2002; Watanabe *et al.*, 2004). A frequent response to nitrogen limitation is the accumulation of organic carbon compounds such as polysaccharides and PUFAs (Papanikolaou *et al.*, 2004).

1.3.13.2 Oxygen mediated regulation of desaturase activity

Fatty acid desaturase activity requires oxygen and NADPH to introduce a double bond into a saturated acyl CoA substrate (Bloomfield and Bloch, 1960). The transformation of saturated fatty acids into unsaturated fatty acids was found to occur when oil producing strains grow in oxygen abundant environments (Higashiyama *et al.*, 1999). In case of *S. cerevisiae* which is a facultative anaerobe can grow in the complete absence of oxygen if unsaturated acids and sterols are provided in the growth medium (Snoek and Steensma, 2006). In cells grown under low oxygen (hypoxic) conditions, OLE1 expression was found to be increased in order to compensate for its reduced enzymatic activity, thereby enabling the cells to synthesize appropriate levels of unsaturated fatty acids and thus enabling survival at low oxygen levels (Chellappa *et al.*, 2001).

1.3.13.3 Temperature mediated regulation of desaturase activity

The fatty acyl composition of phospholipid bilayers determines the fluid properties of cell membranes. Lower temperatures result in a decrease in the membrane fluidity (Los and Murata, 1998), which can be compensated by desaturation of membrane lipids by FADs.

The introduction of a *cis* double bond due to desaturation into a fatty acid moiety produces a rigid kink in the hydrocarbon chain that prevents the formation of van der Waals interactions between the methylene groups of neighboring fatty acids. Thus a phospholipid bilayer with highly unsaturated fatty acids will exhibit a higher level of lipid mobility, or fluidity (Los and Murata, 1998). On the other hand, higher temperatures results in fluidization of the membranes. Ultimately the expression of desaturases appears to be temperature dependent (Rodriguez-Vargas *et al.*, 2007). The expression levels of FAD3 and FAD2 in *S. kluyveri* increased at low temperatures (Watanabe *et al.*, 2004; Oura and Kajiwara, 2004). In *C. albicans* also, the proportion of PUFAs at 25 °C has been shown to exceed that at 37 °C (Brondz and Olsen, 1990), suggesting the Ca-FAD-6 and Ca-FAD-3 might be involved in membrane fluidity or some other functions.