A Review on Lipase Catalysed Synthesis of DHA Rich Glyceride from Fish Oils

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Abstract: **- Docosahexaenoic Acid (DHA) is one of the most useful polyunsaturated fatty acid (PUFA) with pharmaceutical and neutraceutical potential and important for the prevention & control of various human diseases and disorders such as cardiovascular disease, inflammation, allergy, cancer, immune response, diabetes, hypertension and renal disorders. DHA is also known as "brain food" as it is highly concentrated in the membranes of brain cells and retinal cells of eye. The most widely available source of DHA is cold water fishes such as tuna, sardine, salmon, cod and herring etc. However, the concentration of DHA in fish oil is available in a small quantity. DHA can be isolated from fish oil and concentrated in glyceride form by using different methodologies such as selective hydrolysis, selective esterification and transesterification by using different lipases. Although this paper focusses on synthesis of DHA rich glycerides from fish oil but an additional section has been incorporated including new developments in the field of PUFA synthesis, using different micro-organisms (fungi, algae and bacteria). In the present paper, the published research work on the above aspects has been reviewed & summarized.**

Key words: **Docosahexaenoic acid, fish oil, lipase, hydrolysis, esterification, microorganism.**

1. INTRODUCTION

Human health and nutrition has always been a major challenge for researchers. Therefore, the need is to focus on the synthesis of nutritionally valuable components which not only prevent the onset of disease but also provide the means of treatment. Such nutritionally important food ingredients include various long chain polyunsaturated fatty acids (PUFAs) in tri-glycerides form which are easy to consume in diet. One of the essential ω-3 PUFAs include DHA. Fish oils such as tuna, salmon, cod liver, herring etc., are rich source of these ω -3 fatty acids. The application of lipase for the synthesis of DHA rich glycerides is reported as a promising method as few of them are highly selective for enrichment of PUFA.

DHA is an even numbered, straight-chain, ω-3 PUFA which is an essential fatty acid because it cannot be synthesized in the human body [1]. DHA ($C_{22}H_{32}O_2$) is designated as C22:6 ω3 means DHA contain 22 carbon atoms, 6 double bonds and the term "ω" refers to the position of the double bond of the fatty acid closest to the methyl end of the molecule. All double bonds are in cis-configuration. The chemical structure of DHA is shown in Fig 1.

Fig 1.Chemical Structure of DHA

The natural source of DHA is fish oil which contains very little alpha-linolenic acid (ALA), but is rich in the EPA and DHA. Fish oils with the highest levels of EPA and DHA include tuna, sardine, cod and salmon etc. However, the concentration of DHA in various fish oils which has been reported to be approximately in the range of 10-30%. Several marine organisms like algae (*Thraustochytrium aureum, Thraustochytrium roseum, Schyzochytrium,* microalgae, *Crypthecodinium cohnii, Gyrodinium nelsoni, Amphidinium carteri, Gonyaulax),* bacteria (*Vibrio spp., Rhodopseudomonas spp*.) produce DHA, especially a triglyceride DHA [2]. The synthesis of DHA and EPA from fish oils has been reported with both chemical and enzymatic methods [3-5]. Enzymatic methods are largely preferred over conventional chemical method because of reduced cost of solvents [6-9], mild reaction conditions of pH, temperature, high enzyme specificity, ease of reaction [10-11]. This makes the enzymatic process much cleaner and energy efficient than the conventional thermal fat splitting alternative, which requires operation at elevated temperature and pressure.

Lipases (EC 3.1.1.3 triacylglycerol acylhydrolyase) can catalyze both hydrolysis and esterification reaction [12-13] of oils with a reasonable rate, depending upon their type such as *Candida rugosa*, *Candida antarctica*, *Pseudomonas*, *Mucor miehei*, *Rhiopus oryzae etc* [14-19]. For the synthesis of DHA rich glycerides, lipase catalyzed hydrolysis of oil is reported as the first step to release free fatty acids from the breakdown of ester bonds of triglycerides in fish oil. *Candida antarctica* lipase-B has been categorized as a selective lipase for long

chain fatty acid because it has a very narrow and deep substrate binding site in the range of 10 x 4 Å wide and 12 Å deep in comparison to other lipases. This small space inside the binding site causes to allow only very specific substrate to bind with it and hence result into high degree of selectivity [20-22]. According to Uppenberg and coworkers, hydrolysis with lipases can be carried out both selectively and non-selectively depending upon the nature and the overall shape of the acyl-chain binding active site in lipases [22]. Nowadays, practice is to use immobilized lipases than free lipases because of the advantages offered by immobilized lipases. According to the available literature, the immobilized lipases are more stabilized over a broader pH range & at elevated temperature, highly specific in nature and are able to retain their activity even after multiple cycles of reuse [23-24]. In addition to this, immobilized lipases are easy to recover and hence reduce the cost of the enzymes [8, 25].

The concentration of DHA can be enhanced into glycerides by selective splitting of fatty acids other than DHA. The concentration of DHA can also be enhanced by non-selective hydrolysis of TG oil and by isolation of DHA followed by transesterification. These reactions are catalyzed by enzymes commonly known as lipase [26].

II. BIOLOGICAL SIGNIFICANCE OF DHA

Fish has been considered to be an essential part of a healthy diet ever since it was suggested by the ancient Greek philosopher Plato in the 5^{th} to 4^{th} century BC [27]. The use of marine lipids for medical purposes in the form of cod liver oil as a treatment for arthritis has been documented in the scientific literature in the 1780s [28]. In the early 1970s, Dyerberg *et al.* [29-31], analysed the fatty acid composition of the plasma lipids in Greenland Eskimos and found a strong correlation between their diet rich in fish and the low incidence of heart diseases. Since the 1970s numerous studies have shown that the PUFA found in fish oil exhibit many health benefits [32-33], especially suggesting that EPA and DHA have beneficial effects. Fats are a principal and essential constituent of the human diet along with carbohydrates and proteins. Some fatty foods are sources of fat-soluble vitamins and the ingestion of fat improves the absorption of these vitamins regardless of their source. Fats are vital to a palatable and well-rounded diet and provide the essential fatty acids such as linoleic acid (LA) and ALA [34]. PUFA obtained from marine sources are mainly consumed in the form of either TG or fatty acid ethyl ester (FAEE). Studies conducted in the 1980s have shown that absorption of EPA and DHA was lower when ingested as FAEE compared to TG [35]. However, in later studies the absorption of PUFA in TG and FAEE forms by the human body was found to be similar. In the case of TG ingestion, EPA and DHA are more readily absorbed when located in the middle (*sn*-2) position of the triacylglycerol [36-39].

ω-3 and ω-6 fatty acids are important in the normal functioning of all tissues of the body. Deficiencies are responsible for symptoms and disorders including abnormalities in the liver, kidney, changes in the blood components, reduced growth rates, decreased immune function, depression and skin changes, including dryness and scaliness [40]. Prevention of atherosclerosis, reduced incidence of heart disease, stroke and relief from the symptoms associated with ulcerative colitis and joint pain have also been reported [41-42].

DHA is the most abundant and essential PUFA in the brain and retina. It has been called "brain food", as it is highly concentrated in membranes of brain synapses and in the retina of the eye. It comprises 40% of the PUFAs in the brain and 60% of the PUFAs in the retina. 50% of the weight of neuron's plasma membrane is composed of DHA. It declines in brain cell (neuron) membranes with aging may result in declining mental function [43-44]. DHA has the largest effect on brain PUFA composition of all the fatty acids [45]. DHA is found in three phospholipids: phosphatidyl-ethanolamine, ethanolamine plasmalogens, and phosphatidylserine. It modulates the carrier-mediated transport of choline, glycine, and taurine. The function of delayed rectifier potassium channels and the response of rhodopsin contained in the synaptic vesicles are included among many other important functions of DHA inside body [46]. DHA deficiency is associated with cognitive decline [47] and it gets depleted in the cerebral cortex of severely depressed patients [48].

It has been found to be very useful in arresting cardiovascular diseases and also in treating the children affected by Dyslexia and Dyspraxia. Both DHA and EPA lower triglycerides by reducing the rate of fatty acid synthesis in the liver [49]. DHA increases red blood cell membrane fluidity, thereby increasing the deformability of the blood cells so that they can move through capillaries more easily and thereby lower blood viscosity [50]. DHA may also reduce blood pressure by lowering cortisol [51]. Epidemiological studies have shown that consumption of DHA is associated with reduced risk of Alzheimer disease. It has been used for its anti-inflammatory effects in rheumatoid arthritis [52]. DHA plays an important role in the regeneration of the visual pigment rhodopsin, which has a critical role in the visual transduction system that converts light hitting the retina to visual images in the brain [53]. ω-3 fatty acids may regulate gene expression by interacting with specific transcription factors, including peroxisome proliferator-activated receptors and liver X receptors. DHA has also been shown to induce a 10-fold increase in transcription of the amyloid-ß-scavenger transthyretin [54]. Over the years, ω-3 PUFA in fish oils are considered to have very important bioactivities [55-59]. The dietary supplementation of PUFA has several benefits on cardiovascular disorders, autoimmune and inflammatory diseases, and cancer [56, 60]. In recent times, PUFA have gained the breakthrough attention amongst health care professionals because of the beneficial effects of ω-3 fatty acids derived from fish oils. In the early stages of life only, DHA is required in high levels in the brain and retina as a physiologically vital nutrient for optimal neuronal functioning

such as learning ability, mental growth and visual acuity. In general, DHA has been reported useful in preventing a significant number of health disorders [61-66]. Clinical studies have indicated that DHA available in fish oil is beneficial to human health, particularly in reducing the risks of cardiovascular mortality [67-68] and prostate cancer [69].

According to the various worldwide organizations, some guidelines have been formed for the consumption of DHA and EPA directly or in diet for different age group persons. These guidelines were formed to monitor the effect of intake of DHA and its association with the onset of various diseases. For example, the Institute of Medicine of the National Academies in 2002 established the first recommended daily intake (RDI) values for LA as 17 grams for adult men and 12 grams for adult women. The RDI for ALA was set at 1.6 grams for adult men and 1.1 grams for adult women. RDI's were also established for children, pregnant and lactating women [70]. Various international level proficient groups have published recommendations for the optimum use of DHA and arachidonic acid (AA) supplementation in term infant formula 0.8-10 g per day. The general recommendations of 0.2% to 0.4% fatty acids for DHA and 0.35% to 0.7% fatty acids for AA have been suggested according to the median worldwide range of DHA and AA concentration in breast milk [71]. The American Heart Association recommends a minimum daily consumption of 220 mg of DHA/EPA for those at risk of cardiovascular diseases [72]. Presently, the market for DHA is growing at a fast rate, the consumer market for DHA-fortified infant formula will reach $14.2 billion in 2016 [73] and even expanding day by day everywhere in world, as the awareness about DHA in diet has increased due to the health benefits provided by it. DHA is found to be compatible with various food products and beverages. Therefore available with bread, cereals, yogurt, cheeses, milk and juices also. DHA supplemented tablets, capsules and liquid gels of various brands are also available in the market, according to demand of the consumers. Some well-known brands for DHA enriched food are Trabi DHA, Horlicks, and juices such as Minute Maid, Pomegranate Blueberry flavoured fruit juice blend and baby food such as Gerber Baby Food with DHA. Market Life Sciences (NYSE:MATAK) had taken the initiative for producing the DHA from an non-GMO (Genetically Modified Organism) biomass (algal) source (Life's DHA) and supplemented a number of dairy products with this formula such as Horizon Organic's Milk+DHA. A Swiss based company (Water4Life) has also offered a product named V-Pure, a vegan supplement containing algae derived DHA and EPA [72].

III. METABOLIC PATHWAY FOR DHA AND EPA SYNTHESIS

ALA serves as the precursor of a family of ω-3 fatty acids that is formed by desaturation and chain elongation [74]. Once ingested by mammals, ALA is elongated and desaturated into long-chain PUFAs i.e. EPA and DHA. However, the conversion from parent fatty acids into very long-chain PUFAs occurs slowly in humans. Cold-water fish has traditionally been the primary food source of EPA and DHA [43, 75]. Mammals lack desaturases enzyme and therefore require exogenous supply of ALA, a precursor of DHA biosynthesis via the δ-4desaturase-independent pathway. The details of metabolic steps (desaturation plus elongation reactions) by which ALA is converted to the long chain products including EPA and DHA, are explained by Akoh [74] and Burdge [76].

IV. ENZYMATIC METHODS FOR THE SYNTHESIS OF DHA RICH GLYCERIDES

4.1 Selective Hydrolysis of fish oil/TGs

The advantage of the specificity of lipases can be taken in splitting the acyl chain of fatty acids other than DHA and EPA in glycerides and finally resulting in glycerides rich with DHA and EPA. Therefore, TG would decrease and DHA rich diglycerides (DG) and monoglycerides (MG) would increase in reaction mixtures with increase in conversion. But it is reported that TG levels remained high. The reason for this is that the saturated and monoenoic acids in TG that did not contain DHA were more easily hydrolyzed with lipase than those in TG that contain DHA as reported by Tanaka *et al.* [77]. The proposed reaction mechanism is that the PUFA-partial glycerides generated by the hydrolysis were converted to PUFA-TG by condensation & transacylation reactions. As PUFA-TG formed, were the poor substrates of lipase they were accumulated in the reaction mixture [78]. The lipases that are reported to be useful for selective hydrolysis are *Candida cylindracea* [14; 77 & 79] and *Aspergillus niger* [77] which selectively hydrolyse fatty acids other than DHA and EPA from the ester bond of oil while *Mucor meihei* lipase [80] is reported to be useful for transesterifying EPA with ethanol from glyceride mixture as shown in Fig. 2 [79].

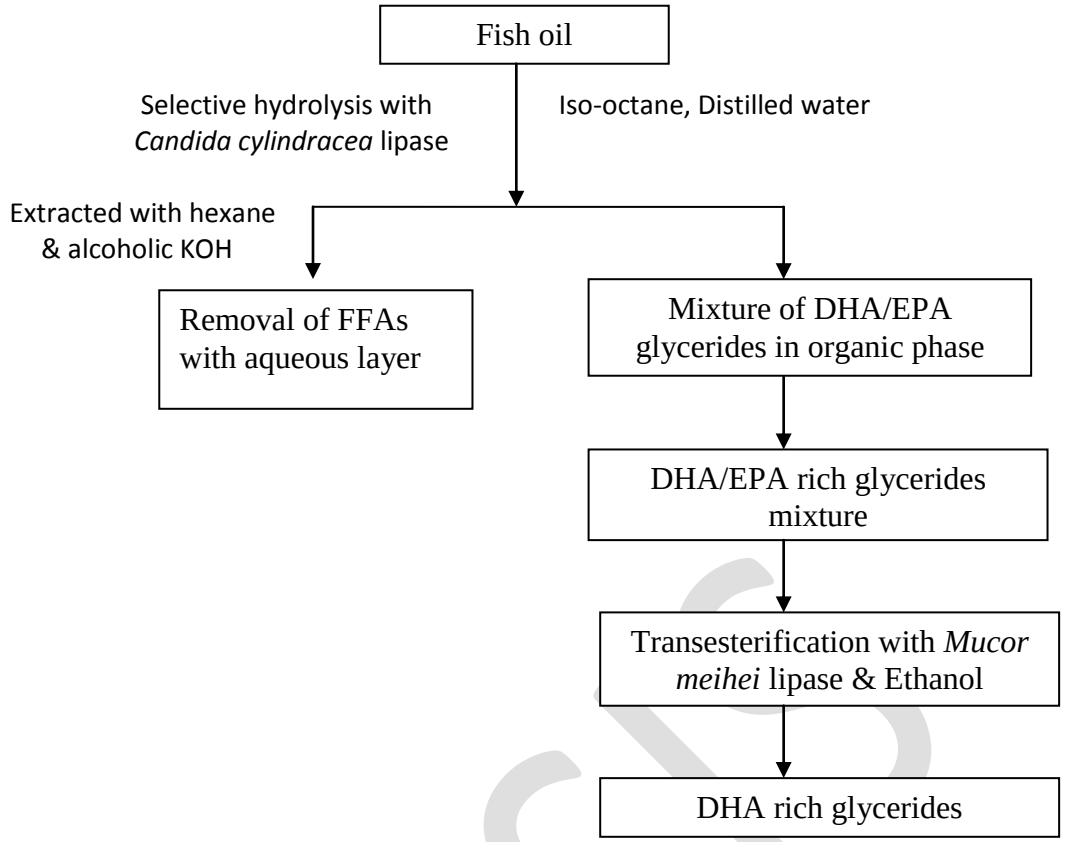

Fig. 2 Synthesis of DHA rich glycerides by selective hydrolysis of oil & esterification of PUFA [79]

Hydrolysis of (cod liver oil and refined sardine oil) was examined by Hoshino *et al.* [15] with six lipases (*Candida cylindracea*, *Aspergillus oryzae*, *Aspergillus niger*, *Rhizopus delemar*, *Geotrichum candidum and* Porcine pancreas) to concentrate the ω-3 PUFA. *Candida cylindracea* and *Aspergillus niger* lipases gave glycerides with a more than two-fold increase in ω-3 PUFA content over the original fish oils. *Candida cylindracea* lipase seems to be most promising with respect to recovery of TGs while *Aspergillus niger* lipase not only increases the DHA content but also EPA and docosapentaenoic acid (DPA). When hydrolysis of tuna fish oil was examined by Tanaka *et al.* [81] with six lipases (*Candida cylindracea*, *Aspergillus niger*, *Rhizopus delemar*, *Rhizopus javanicus*, *Pseudomonas sp. and Chromobacterium viscosum*), the DHA content in the glyceride mixture was found three times more than that in the original fish oil after 70% hydrolysis with *Candida cylindracea* lipase. Moore and McNeill [82], reported that by controlling the degree of hydrolysis of Chilean fish oil, two products were obtained, one enriched in total ω-3 (50%), the other enriched in DHA & depleted in EPA (DHA 40%, EPA 7%). Wanasundara and Shahidi [83], reported that the highest concentration of ω-3 PUFA was obtained by *Candida cylindracea* lipase; 43.5% in Seal Blubber Oil including (9.75% EPA, 8.61% DPA and 24% DHA) and 44.1% in Menhaden Oil (18.5% EPA, 3.62% DPA and 17.3% DHA) after 40 h hydrolysis. Carvahlo *et al.* [84] reported that when Brazilian sardine oil was hydrolyzed with *Candida cylindracea* lipase for 16 h, 60.0% hydrolysis was achieved, resulted in an increase in the DHA content from 10.2% in the original oil to 22.5% (2.20-fold enrichment) in the acylglycerol.

Enrichment of DHA was observed in triglyceride by Koike *et al.* [85] with the aid of lipase used for hydrolysis reaction in water in oil (W/O) micro-emulsion formed by soybean lecithin. The molar fraction of DHA in triglyceride was enriched from 70% to 97%. Lipase reactivity in water in oil (W/O) micro-emulsion systems has been investigated as highly promising and an attractive way to the application of enzymes that catalyze the water-insoluble substrates and it also provide enormous interfacial area to associate enzyme and substrate for the hydrolysis of fats and oils [86-87]. Mbatia *et al.* [88] reported the enrichment of DHA in the glyceride fraction during hydrolysis of Nile pearch viscera oil with lipases from *Candida rugosa*, *Thermomyces lanuginosus*, *and Pseudomonas cepacia*. Hydrolysis with *Candida rugosa* lipase enriched EPA from 3 to 6 mol% and DHA from 9 to 23 mol%. Yan *et al.* [89], observed the increased degree of hydrolysis from 12% with the free lipase to 40% with immobilization of lipase in two phase medium composed of buffer and octane, coupled to fish oil treatment. The contents of EPA and DHA were increased from 6.94% and 0.97% in original fish oil to 12.65% and 3.85% in hydrolyzed glycerides. Kahveci *et al.* [90]

reported that the *Candida rugosa* lipase had shown (91.89%) the highest degree of hydrolysis after 24 h. The final value of ω-3 PUFA was increased from 13.77% to 27.81% (wt %). Around 2.15-fold increase of the original ω-3 PUFA content by hydrolysis of salmon oil was found in the presence of *Candida rugosa* lipase. DHA content increased from 7% to 19.39% on glyceride fraction.

For enrichment of PUFA on glyceride backbone, by selectively hydrolyzing FA's other than PUFA, *Candida cylindracea* lipase has been reported to be the most suitable. More than 3 fold increases in DHA content on glyceride backbone has been reported by Tanaka *et al.* [81].

4.2 Hydrolysis and Selective Esterification

PUFA containing oil can be hydrolyzed by heating with ethanol under alkaline conditions which requires a large-scale reactor and carries the risk of isomerization of PUFA. It further needs to bring down the pH of the reaction mixture to acidic conditions for recovery of PUFA. These problems can be easily overcome in enzymatic processes. Therefore, enzymatic methods have drawn considerable attention where enrichment of PUFAs can be done by hydrolysis of fish oil and selective esterification of PUFAs. During non-selective hydrolysis as shown in Fig.3 [79], *Candida rugosa* lipase is used to hydrolyze all the fatty acids from oil in the biphasic solvent system. These fatty acids can further be enriched in DHA content by selective esterification with *Rhizopus oryzae* lipase in presence of alcohol and causes removal of undesired fatty acid [91].

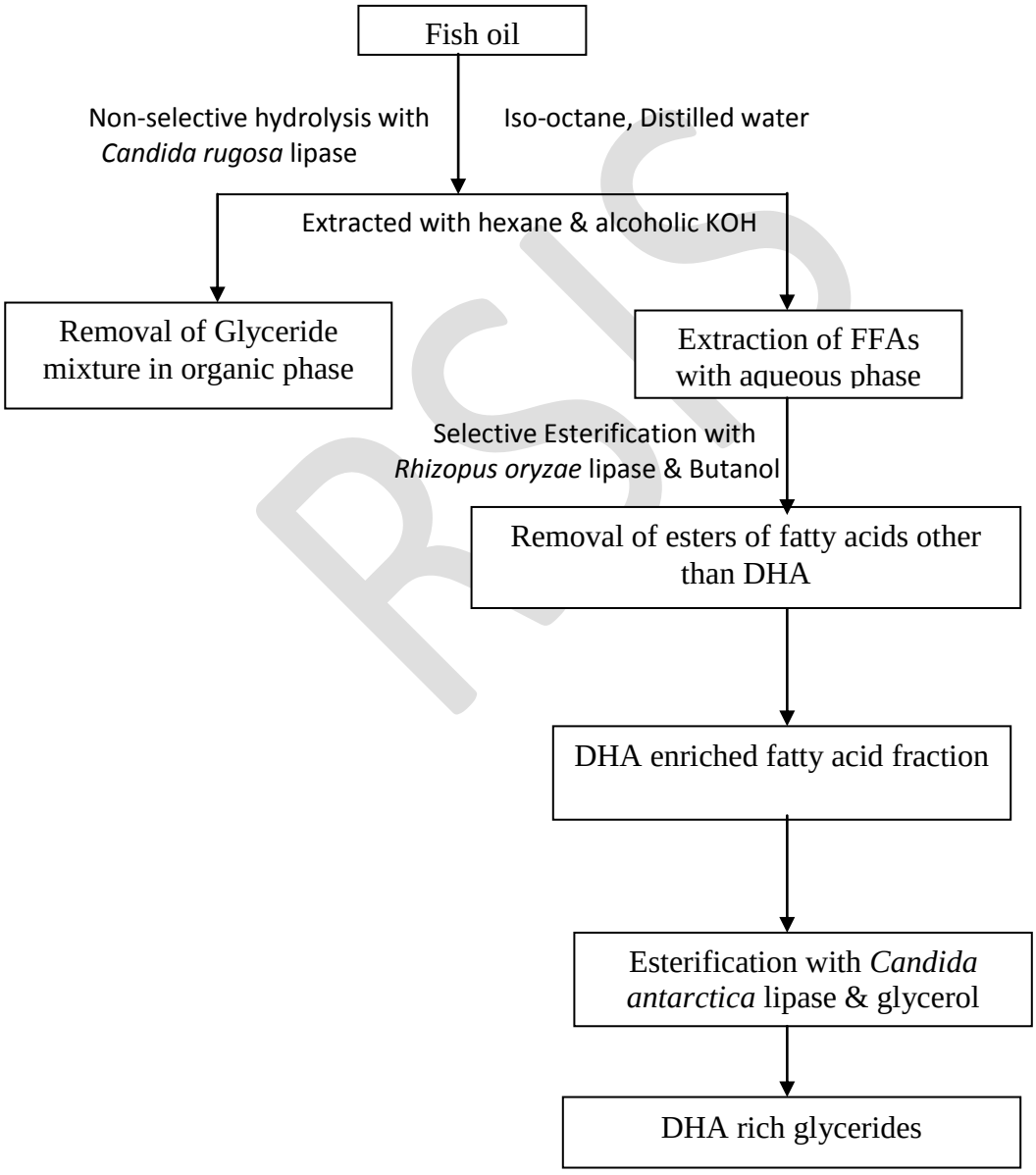

Fig. 3 Synthesis of DHA rich glycerides by Non-selective hydrolysis of oil, Selective esterification of PUFAs and further esterification with glycerol [79]

When hydrolysis of tuna oil using different lipases (*Pseudomonas aeruginosa*, *Candida rugosa* and *Rhizopus delemar*) was employed by Shimada *et al.* [92], it was reported that *Pseudomonas* lipase hydrolyzed the DHA ester more strongly than the EPA ester, and DHA was recovered in the FFA fraction with highest yield. Hydrolysis of tuna oil by lipase Enzylon PF obtained from *Pseudomonas fluorescens* also resulted in hydrolysis of 80% DHA and 75% EPA as FFA with the concentration of 24.5 and 6.7% after 48h of hydrolysis [93]. Bhandari *et al.* [94] reported 85.9% hydrolysis of tuna oil using *Candida rugosa* lipase.

Shimada *et al.* [95] reported that when FFAs originating from PUFA-containing oil were selectively esterified with lauryl alcohol using lipases such as *Pseudomonas aeruginosa*, *Candida rugosa* and *Rhizopus delemar*, PUFA was efficiently enriched in the FFA fraction and *Rhizopus delemar* lipase was selected as the most effective enzyme which brings about 68% esterification of tuna-FFA and the purity of DHA was raised to 90% in two step of selective esterification. Haraldsson and Kristinsson [96] reported that during esterification of tuna-FFA with ethanol using *Rhizomucor miehei* lipase, DHA content was increased from 23% to 74% in the form of FFAs in 24 h while during esterification of sardine oil fatty acids with butanol, DHA content was raised from 12% to 70% as FFAs in 24 h. Halldorsson *et al.* [97] obtained an increase in DHA content from 24.5% to 71% in FFA after 24 h during esterification of tuna-FFA with glycerol using *Rhizomucor miehei* lipase. Bhandari *et al.* [91] reported that when FFAs obtained from tuna oil was esterified with butanol using *Rhizopus oryzae* lipase, 76.2% esterification was achieved in 24 h and DHA was purified from 26% to 86.8% using single step of esterification.

For enrichment of DHA in FFA, by selectively esterifying FA's other than DHA, *Rhizopus oryzae* lipase has been reported to be the most suitable. Using single step of esterification, maximum DHA concentration (i.e. 86.8%) in FA mixture has been reported in biphasic solvent system by Bhandari *et al.* [91].

4.3 Esterification of DHA rich fatty acids/PUFA with glycerol

FFAs rich in DHA when reacted with glycerol in presence of *Candida antarctica* lipase [98, 99] and *Rhizomucor miehei* lipase [82] mixture of glycerides are obtained. Li and Ward [100] reported enzymatic synthesis of glycerides from glycerol and ω-3 PUFA concentrate from cod liver oil. During 24 h of reaction time, the concentration of MG, DG and TG obtained was 23.8, 40.6 and 18.1%. Haraldsson *et al.* [101] reported the synthesis of homogenous TGs of DHA using *Candida antarctica* lipase during esterification of glycerol with stoichiometric amount of EPA or DHA as ethyl esters and free fatty acids under vacuum at 65°C in absence of solvent. Cerdan *et al.* [99] reported that when PUFA concentrate obtained from cod liver oil was esterified with glycerol using *Candida antarctica* lipase, TG yield of 84.7% containing, 27.4% EPA and 45.1% DHA was obtained in 24 h. Medina *et al.* [102] synthesized TGs using enzymatic esterification of glycerol with PUFA concentrates obtained from cod liver oil and found TG yield of 96.5% after 72 h of reaction time. Halldorsson *et al.* [97] reported that when tuna oil FFA was esterified with glycerol, 90% conversion into glycerides was obtained after 48 h, with 79% DHA and 91% EPA recovery in glycerides. When Sardine oil FFA was esterified, 80% conversion into glyceride was obtained after 28 h. Liu *et al.* [103] reported the synthesis of glycerides from glycerol and ω-3 PUFA concentrates obtained from tuna oil using Lipozyme. After 24 h, degree of esterification obtained was above 90% with 12.1% MG, 56.1% DG and 31.3% TG. Byun *et al.* [104] synthesized MGs (68% w/w) in 72 h of reaction time during enzymatic esterification of glycerol with ω-3 PUFA obtained from sardine oil. Nagao *et al.* [105] reported the synthesis of TGs during esterification of FFAs with glycerol using immobilized *Rhizomucor miehei* lipase. Regiospecific analysis of the resulting TGs showed that the content of DHA at the sn-1(3) position (51.7 mol %) was higher than the content of DHA at the sn-2 position (17.3 mol %). Wang *et al.* [106] synthesized EPA and DHA rich glycerides using Novozym 435 and reported that 5.5% EPA and 74.6% DHA got incorporated into glycerides, which was 1.21 and 2.71 times more than that found in the original fish oil. During esterification of DHA rich fatty acids with glycerol using immobilized Candida antarctica lipase, 75.4% esterification was achieved by Bhandari et al. [98] with 30% TG, 16.1% DG, and 29.3% MG in the glyceride mixture. The concentration of DHA in the glyceride mixture obtained was 87%. Immobilized *Mucor miehei* lipase was used by Millar et al. [107]; Li and Ward [100] and Mukesh et al. [108] during esterification of glycerol with ω-3 PUFA. Li and Ward [100] studied the enzymatic synthesis of glycerides using glycerol and ω-3 PUFA obtained from cod liver oil in presence of organic solvent. The concentration of MG, DG and TG obtained was 34.5, 40.5 and 12.6 % respectively during 24 h of reaction time. He and Shahidi [109] have reported that 13.8, 43.1 and 37.4 % MG, DG and TG were synthesized in 48 h of reaction time using lipase *Chromobacterium viscosum*.

Haraldsson et al. [101] synthesized homogenous TG of DHA while using 99% pure DHA ethyl ester while other authors; Byun et al. [104], Liu et al. [103], Medina et al. [102], Cerdan et al. [99] and Li & Ward [100] reported synthesis of glycerides by enzymatic esterification of glycerol with PUFA obtained from hydrolysis of oil. Under these conditions glycerides that were synthesized contained both EPA & DHA. DHA rich fatty acids isolated from tuna-FFA was used by Bhandari *et al*. [98] for the synthesis of DHA rich glycerides and 87% DHA got incorporated into glycerides.

4.4 Enzymatic Transesterification

It is a reaction that exchanges carbonyl groups of fatty acids within and between TG molecules and it is used to modify the structure and composition of oils to improve the physical and nutritional properties of TG. The TG form of ω-3 PUFAs is considered to be nutritionally more favorable than methyl or ethyl esters of fatty acids due to impaired intestinal absorption of methyl or ethyl esters of ω-3 PUFAs in animals as reported by Wanasundara and Shahidi [83]. During (ethanolysis) of tuna fish oil with *Pseudomonas* lipase, an enrichment of 74% of DHA and EPA in glyceride mixture in 24 h of reaction time was achieved by Rakshit *et al.* [93].

Haraldsson and Kristinsson [96] reported that when transesterification of tuna fish oil was carried out with ethanol using immobilized *Rhizomucor miehei* lipase under anhydrous solvent-free conditions, it resulted in a good separation of EPA and DHA. After 24 h of reaction time, 65% conversion was obtained and the residual glyceride mixture contained 49% DHA. Breivik *et al.* [80] prepared concentrates of EPA and DHA via 2 step process. In the first step, transesterification of fish oil with ethanol was carried out in presence of *Pseudomonas* lipase and at 52% conversion, a concentrate of 46% of EPA and DHA was obtained as a mixture of MG, DG and TG which latter on converted into ethyl esters using immobilized *Candida antarctica* lipase. Senanayake and Shahidi [110] incorporated DHA into borage oil via lipase catalyzed acidolysis of borage oil with DHA. Incorporation of total ω-6 and ω-3 PUFA in glycerides after 24 h of reaction time was 44 & 27.6%. Irimescu *et al.* [111] synthesized symmetrically structured TG rich in DHA and caprylic acid via 2-MG in two step. The first step involves ethanolysis of bonito oil TG to 2-MG yielded 92.5% 2-MG with 43.5% DHA content in 2 h. Second step involves esterification of 2-MG with ethyl caprylate. The final structured lipids comprised 85.3% TG with two CA residues & one original fatty acid residue, 13% TG with one CA residues & two original fatty acid residues and 1.7% tricapryloylglycerol. To enrich the ω-3 PUFA content of TG in Menhaden oil, Lin *et al.* [112] employed the immobilized *Mucor miehei* lipase to catalyze the transesterification reaction under supercritical carbon dioxide and found 40wt% higher conversion as compared to n-hexane. Wongsakul et al. [113] synthesized 2 MG by alcoholysis of tuna oil with ethanol using Pseudomonas lipase and obtained a yield of 81% 2-MG containing 80% PUFA. Esteban *et al.* [114] reported the synthesis of MGs by enzymatic alcoholysis of cod liver and tuna oil and found incorporation of 40% and 45% combined EPA and DHA content in cod liver and tuna oil respectively. Eom *et al.* [115] studied the synthesis of DG by glycerolysis of glycerol with tuna oil using *Rhizomucor miehei* and *Candida antarctica* lipase and investigated the effect of DG on body weight and biochemical markers of obesity in mice. Valverde *et al.* [116] synthesized DHA rich glycerides from tuna fish oil by selective ethanolysis using lipases and obtained 45% DHA concentration with immobilized *Thermomyces lanuginosus*.

Most of the work as mentioned above synthesized PUFA rich glycerides (TG, DG and MG) using transesterification studies while Haraldsson and Kristinsson [96] and Valverde *et al.* [116] synthesized DHA rich glycerides and maximum DHA concentration incorporated into glyceride obtained was 49% as reported by Haraldsson and Kristinsson [96] using *Rhizomucor miehei* lipase which resulted in a good separation of EPA and DHA.

New developments in the area of production of DHA/PUFA rich glycerides

Production of PUFA using microorganisms has been reported as an alternative to chemical and enzymatic processes [117-120]. Although the contribution of microbial lipids to the oil industry is nearly negligible, but there are several reasons for using them:

1) Active lipids synthesizing apparatus makes oleaginous microorganisms perspective oil sources.
2) Their extremely high growth rates on wide varieties of substrates allow utilizing cheap or zero- cost materials.
3) There is no seasonal or climatic dependence.
4) Microbial competence to carry out numerous transformation reactions (e.g. oxidation, desaturation and hydrogenation) enables the upgrading of PUFA structures and allows simultaneous formation of lipid and other products.

Certain fungi, marine bacteria, heterotrophic and phototrophic microalgae and mosses contain various PUFAs, and may thus represent suitable sources of them [117-119]. Unagul *et al.* [120] studied the effect of coconut water (CW) on biomass and DHA formation by *Schizochytrium mangrovei* Sk-02 in a yeast extract-diluted sea water medium. The inclusion of coconut water increased biomass from two to three fold with concomitant increase in the DHA level. Chi *et al.* [121] investigated the potential of using the crude glycerol to produce DHA through fermentation of the microalgae *Schizochytrium limacinum.*

Metabolic engineering of the yeast *Yarrowia lipolytica* was done by Xue et al. [122] and the resulted engineered yeast lipid comprises EPA at 56.6% by weight. High EPA yield was obtained due to inactivation of the peroxisome biogenesis gene *PEX10* which also increases the yield of other commercially desirable lipid-related products. Blazeck *et al.* [123] reported genotypic and phenotypic optimization of *Yarrowia lipolytica* with significant lipogenesis capability via rewiring its metabolism for superior de novo lipogenesis by coupling combinatorial multiplexing of lipogenesis targets with phenotypic induction. Metabolic control resulted in saturated cells containing 90% lipid content and titres exceeding 25 g/ l lipids, which represents a 60-fold improvement over parental strain. Gemperlein *et al.* [124] reported for the first time the characterization of PUFA synthase from terrestrial origin. Two distinct types of PUFA biosynthetic gene clusters were

discovered, originating from linoleic acid producing myxobacteria of the genus *Sorangium* as well as from species of the recently discovered myxobacterial genus *Aetherobacter*, that turned out to be prolific producers of EPA and DHA. Using biotechnology approaches, they established heterologous production platform in the myxobacterial model strain *Myxococcus xanthus*.

V. CONCLUSIONS

The demand of PUFA in human nutrition and healthcare applications is rapidly growing. Lipase catalyzed hydrolysis of fish oil and esterification of PUFAs, holds a promising potential for production of DHA rich glycerides. . Microbial fermentation method is an alternative source for DHA production by fermentation. People suffering from depression, inflammatory diseases, vision problems, and heart ailments may benefit greatly by increasing their DHA intake. So, DHA supplementation may play an important role in both maintaining and improving the human health.

Abbreviations

DHA Docosahexaenoic acid
DPA Docosapentaenoic acid
EPA Eicosapentaenoic acid
FAEE Fatty acid ethyl ester(s)
LA linoleic acid
GLA Gamma-linolenic acid
ALA Alfa-linolenic acid
AA Arachidonic acid
CA Caprylic acid
PUFA Poly unsaturated fatty acid(s)
TG Triglyceride
DG Diglyceride
MG Monoglyceride

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