ENRICHMENT OF POLY UNSATURATED FATTY ACIDS (PUFA) IN FISH OIL AND MARINE ALGAE USING MICROBIAL LIPASE AND THEIR OXIDATIVE STABILITY STUDIES USING MEDICINAL PLANT EXTRACTS

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Under the Faculty of Marine Sciences

by

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Certificate

This is to certify that the Doctoral Thesis entitled **"Enrichment of poly unsaturated fatty acids (PUFA) in fish oil and marine algae using microbial lipase and their oxidative stability studies using medicinal plant extracts"** is an authentic record of the research work carried out by **Mr. Jithu Paul Jacob**, under my supervision and guidance at the School of Industrial Fisheries, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy and no part thereof has been submitted for any other degree at any other institution. I further certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate have been incorporated in the thesis. Plagiarism has been checked and found to be 6% and there for well within limits.

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Prof. (Dr.) Saleena Mathew
Supervising Guide

Declaration

I Mr. Jithu Paul Jacob do hereby declare that the Doctoral Thesis entitled "**Enrichment of poly unsaturated fatty acids (PUFA) in fish oil and marine algae using microbial lipase and their oxidative stability studies using medicinal plant extracts**" is an authentic record of the original research work carried out by me under the guidance and supervision of **Prof. (Dr.) Saleena Mathew,** School of Industrial Fisheries, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Cochin university of Science and Technology and that no part has been submitted earlier for award of any other degree, diploma or other similar title of this in any University or Institution.

Kochi-18 **Jithu Paul Jacob**
March 2018

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Abbreviations

4-HN	-	4-hydroxyl-2-nonenal
AAS	-	Atomic Absorption spectroscopy
ABS	-	Absorbance
ALA	-	Alpha linoleic acid
ANOVA	-	Analysis of Variance
AOAC	-	Association of the Official Analytical Chemists
API	-	Ayurvedic pharmacopoeia of India
As	-	Arsenic
BHA	-	Butylated hydroy anisole
BHT	-	Butylated hydroxyl toluene
C:M:W	-	Chloroform:Methanol:Water
CC	-	*Candida cylindracea*
Cd	-	Cadmium
Cu	-	Copper
DHA	-	Docosahexaenoic acid
DPPH	-	2,2'-diphenyl-l-picryl hydrazl
DSAC	-	degree of acetylation
Dv	-	*Diacronema vlkianum*
EDTA	-	Ethylene diamine tetra acetic acid
EEG	-	Electroencephalographic
EPA	-	Eicosapentaenoic acid
FA	-	Fatty acid
FAME	-	Fatty acid methyl esters
FAO	-	Food and Agriculture Organization
FDA	-	Food and Drug Administration
FFA	-	Free Fatty acid
HCl	-	Hydrochloric acid
Hg	-	Mercury

Hr	-	Hour
ICH	-	International Council for Harmonization of technical requirements for pharmaceutical for Human use
IMP	-	Indian Medicinal Plants
IMTECH	-	Institute of Microbial Technology
JAOCS	-	Japanese Association of the Official Analytical Chemists
KI	-	Potassium Iodide
KOH	-	Potassium hydroxide
LC	-	Long chain
MA	-	Maloanldehyde
MDA	-	Malonaldehyde
Mg	-	Magnesium
mg	-	Milligram
ml	-	Milliliter
MTCC	-	Microbial Technique Candida cylinderacea
MUFA	-	Monounsaturated fatty acid
Na_2CO_3	-	Sodium bi carbonate
NaOH	-	Sodium hydroxide
Ni	-	Nickel
nm	-	Nanometre
Pb	-	Lead
PL	-	Phospholipids
PUFA	-	Poly unsaturated fatty acids
PV	-	Peroxide value
RB	-	Round Bottom
rpm	-	Rotation per minute
RSA	-	Radical-scavenging activity
SFA	-	Saturated fatty acid
SPSS	-	Scientific Package of Social Science
TBA	-	Thiobarbituric acid

TBARS	-	Thiobarbituric acid Reacting Substances
TBHQ	-	Ter-butylhydroquinone
TCA	-	Trichloro acetic acid
TG	-	Triglycerol
TPC	-	Total phenolic content
U	-	Lipase unit
USFDA	-	United States Food and Drug Administration
v/v	-	Volume by volume
µl	-	Microlitre
WCOT	-	Wax coated open tubular
Zn	-	Zinc

Chapter 1

GENERAL INTRODUCTION

The difference between fish oil and other oils is mainly due to the unique variety of fatty acid it contains. Thus, marine fish oils are excellent sources of polyunsaturated fatty acids (PUFAs), mainly long-chain omega 3 fatty acids, also sometimes referred to as n-3 fatty acids. The amount and variety of the fatty acids in fish oil varies from one fish species to another, and also with the biological stage, food availability, fishing location, ocean temperature, nutritional and spawning state, etc. The major omega 3 fatty acids present are the eicosa pentaenoic acid (C20:5 omega-3, commonly called EPA) and the docosa hexaenoic acid (C22:6 omega-3, commonly entitled DHA). These Long chain omega-3 polyunsaturated fatty acids (LC *n*-3 PUFAs), have long been proposed to bestow health benefits by improving blood pressure control, alleviating symptoms of rheumatoid arthritis and depression, as well as attenuating the progression of Alzheimer's disease. Marine algae and its feeder fishes are the richest source of omega 3 fatty acids, which are essential to human metabolism. Poly unsaturated fatty acids (PUFAs) cannot be synthesized in our body, but it can be synthesized by elongase and desaturase pathway from the precursor fatty acids. This particular pathway is observed in marine planktons.

Health benefits of PUFA attribute to prevention of cardio vascular disease, reduction of the clinical onset of cancer cells, rejuvenation of the brain cells and retinal cells in infants, prevention of rheumatoid arthritis etc. The USFDA (United States Food and Drug Administration) recommends daily

requirement of PUFA as 5.0 mg/day, which is genuinely supplied from the marine sources. Hence most of the nutraceutical companies exploit fishery resources in making fish oil capsules. The best strategy to increase the intake of long chain *n*-3 PUFAs and to increase the content of long chain *n*-3 PUFAs in blood, cells and tissues is to eat oily fish regularly. An alternative is to consume fish oil capsules, which is a highly effective way of increasing long chain *n*-3 PUFA status in human blood and cells. It is apparent that foods either naturally enriched or fortified with long chain *n*-3 PUFAs will become increasingly available over the next 5 years and this will be a very good strategy to increase intake of these fatty acids, and will provide greater choice for those consumers who do not eat fish and who do not wish to take capsules.

The reliability and the stability of these PUFA enhancers is a matter of concern, since the deteriorated fish oil supplements adversely affect health. Hence studies are required emphasizing the need to exploit alternate sources of PUFA also other than fish oil. It can be a primary producer in ocean's comprising chiefly algae. Since the sustainability of fish resource is a matter of concern and based on fishery resource management data, we cannot rely fully on fish as a sole source of PUFA. There by, the utilization of fish in energy rich supply of omega 3 fatty acid need to be reduced and instead, the exploitation of marine algae need to be stabilized. Enrichment of PUFA by bio processing such as urea crystallization, super critical fluid extraction methods, lipase catalyzed reaction method, molecular distillation etc. are employed widely to meet this requirement.

There is a wide range of species used in the production of fish oils. However, the biggest part derives mostly from the fatty fishes and the liver of lean fishes. The commonly used raw materials are menhaden, sardines, anchovies, herring, capelin, mackerel, salmon, tunas, cod liver, etc. Besides

from bony fishes, fish oil and fish liver oil can be derived from cartilaginous fishes like sharks. Shark and cod are the two main fish whose liver are extracted for oil. While sharks are specifically targeted for their livers, cod livers are taken as a by-product along with the roe. Tropical marine fatty fishes which include mackerel, tuna and sardines contain high level of poly unsaturated fatty acids. Among these, considering the availability and economic cheapness, sardines could be selected as the best source of fish oil supplements for production of omega 3 fatty acids. It contributes to more than 40% of the available total lipid content from marine fishes. Seasons and ocean currents affect the life cycle pattern of all species surviving in the marine ecosystem. These overall changes affect the individual inhabitants which also influences the lipid content.

A thorough knowledge about the variation in lipid in sardines is much needed for understanding the availability of PUFA in various seasons. Many of the experts in the fishery field have determined the proximate composition as well as seasonal variation in lipid content of Sardines earlier (Gopakumar and Rajendranathan, 1975). Climate change is also likely to play an important role in the future of fishing (Lam *et al*., 2012), while ocean warming may increase productivity for some stocks, ocean acidification and warming (Voss *et al*., 2015) generally decrease the productivity of stocks.

Most of the exporting companies depend on the freezing method of storage of sardines (*Sardinella longiceps*). Even though, this method of fish preservation is effective, there can be degradation occurring, thus affecting the quality of fish on prolonged storage. The indices for deterioration show that lipid oxidation steadily increases with the storage days even in frozen fishes.

Along with the quality of fatty fishes exported, the health effect of consumers is also a serious matter of concern. Lipid oxidation produces free radicals such as super oxide hydroxyls which can be even more dangerous causing human cancers. Research data in this regard could be useful for formulating the storage period of fatty acids in industries which uses these fatty acids for manufacture of PUFA. The onset of primary oxidation products may not be ideal neither for exporting nor for human consumption as it can alter adversely the metabolism in the consumers.

The highly perishable nature of the fatty fishes leads to search for an alternative source for PUFA. The suggested source is its primary producers in ocean ecosystem ie; marine algae. Countries like Japan, Taiwan, Indonesia etc. culture, harvest, utilize and incorporate marine algae in their table served foods (Molina-Grima *et al* ., 2015). These are rich in PUFA and the synthesis of fatty acids could be dependent on culture conditions such as luminance, oxygen availability, and nutrient supply. As the culturing days progress, the bio mass increases and the utilization of nutrients increases. This will be propagated with variations in lipid content. Constant decline implies the death phase of the algae that differ widely among different species of algal culture. The growth curve of many of the algae had been determined, but lack of correlation with the lipid content made it difficult to predict the PUFA contents.

It is important to stress that there are numerous alternative methods for the concentration of omega-3 fatty acids, but only a few are suitable for large-scale production, the most widespread process being the concentration of omega-3 fatty acids by molecular distillation. Other available methods less commonly used include adsorption chromatography, enzymatic splitting, low-temperature crystallization, supercritical fluid extraction, and urea complexation. Each methodology has its own advantages and drawbacks.

Concerning enzymatic techniques, lipases are utilized. These enzymes can catalyze esterification, hydrolysis or exchange of fatty acids in esters (Marangoni and Rousseau, 1998). Accordingly, this technology may enable the performance of the trans esterification and concentration phases in a single step. The direction and efficiency of the reaction can be influenced by the choice of experimental conditions (Yadwad *et al*., 1991). PUFA Bio-enrichment by lipase enzyme is found to be eco-friendly as well as consumer friendly. The region-specificity of this enzyme will act on saturated bonds and will get cleaved, while the unsaturated double bond containing PUFA will not be cleaved due to hindrance effect. This result in the enrichment of PUFA and many studies had been carried out in fish oil samples. This information could provide nutraceutical companies in providing vegetative as well as non-vegetative PUFA capsules as enriched products replacing ordinary fish oils.

Value added food products can also be prepared using enriched PUFA. There are reports in which marine algae in powdered form are applied in biscuits, bread etc. and these can be replaced by enriched PUFA (Gouveia *et al*., 2008). Thus more specifically, health beneficial, nutritional supplements can be introduced directly into the food products.

Storage and packaging of refined fish oils are essential to preserve omega-3 PUFA from oxidation. Several studies have been published in recent years that have assessed the oxidative quality and fatty acid content of encapsulated and liquid formulations of EPA/DHA.When some studies concluded that most tested products were defective (Lee *et al*., 2016, Nichols *et al*., 2016) other studies have reported a significant proportion of tested products failing one or more quality parameters (Kleiner *et al*., 2015, Ritter *et al.,* 2013).

In addition, other strategies to improve fish oil stability and extend its shelf-life are necessary (Kamal-Eldin and Yanishlieva, 2002). For that reason, the use of antioxidants is a common method to preserve refined fish oil, preventing its oxidation. A great number of antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylidene diamine tetra acetic acid (EDTA), tocopherols, ascorbic acid, ascorbyl palmitate, propyl gallate, gallic acid, lactoferrines, and others, have been tested to prevent lipid oxidation both in bulk oil (Kamal-Eldin and Yanishlieva, 2002) and in fish oil-in-water emulsions (Jacobsen *et al*., 2008). In the last decade, the use of natural antioxidants instead of synthetic compounds has received increasing attention and several studies regarding the efficiency of plant extracts, as oregano, parsley or rosemary, on the stabilization of bulk fish oil and fish oil-in-water emulsions have been carried out (Frankel *et al*., 1996; Bhale *et al*., 2007; Jiménez-Álvarez *et al*., 2008).

The adequate concentration of antioxidant depends on the chosen substance, the storage conditions and specific applications for the omega-3 PUFA concentrates. Antioxidants are substances which are effective in preventing propagation of oxidation by terminating the progress by capturing free radicals to produce a stable product. Food and Drug Administration (FDA) has stipulated the use of synthetic antioxidants and many experts rely on natural antioxidants. There are many natural antioxidants present in plant habitat which has been used widely till now. Vitamin E is a natural antioxidant present in fruits and green leaves. The phenolic components present in various plant species are identified and applied as natural antioxidants. Herbs and spices contain rich source of antioxidants which are primarily used as an effective preservative. Moreover the medicinal value of these plants is well known and is written in ancient Ayurveda scriptures. Preservation using natural antioxidants

from the selected herbs is highlighted as an objective which makes the study very relevant for preserving products enriched with PUFA.

Objectives of the Study

The main objectives of the thesis are as follows:

- To analyze the seasonal variation of PUFA of fish oil extracted from *Sardinella longiceps*
- To identify and analyze PUFA in the marine algae such as *Isochrysis galbana*, *Chaetocerous calcitrans*, *Tetraselmis gracillus* and *Chlorella marina* as primary sources.
- To concentrate PUFA in fish oil and marine algae using microbial lipase from *Candida cylinderacea.*
- To enhance the oxidative stability of PUFA in fish oil by incorporating Indian Medicinal Plant extracts of *Aloe barbadensis*, *Boherravia diffusa* and *Osimum sanctum*.

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Chapter 2

REVIEW OF LITERATURE

Contents

2.1 Health benefits of PUFA

Long chain omega-3 polyunsaturated fatty acids (LC *n*-3 PUFAs) are those fatty acids with a long chain (20 carbons or more), with the first double bond located after the third carbon from the methyl end. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have long been proposed to bestow health benefits by improving blood pressure control, alleviating symptoms of depression and rheumatoid arthritis, and also attenuating the progression of Alzheimer's disease (Fortin *et al*., 1995, Geleijnse *et al*., 2002, Morris *et al*., 2003, Martins, 2009). It is noteworthy that some meta-analyses have reported no effect of LC *n*-3 PUFAs on incidence of fatal coronary heart disease and sudden death (Kwak *et al*., 2012, Rizos *et al*., 2012) in stark contrast to earlier trials that reported marked reductions in risk for these events (Burr *et al*., 1989, Harrison, 1999). Differences in trial design (open label against randomized, placebo controlled, double-blind), baseline clinical management (example, low *versus* high use of anti-thrombotic and anti-hypertensive

medications), and absolute risk for adverse events (example, patients with few against many risk factors, including previous myocardial infarction, with diabetes and low use of statins), have been postulated to explain the divergent findings (Kromhout, 2012). Dose is also an important determinant of patient outcome, where high doses only of LC *n*-3 PUFAs (for example, 2–4 g EPA + DHA/day) can provide cardiovascular benefit associated with serum triglyceride reduction (Laufs *et al.*, 2012). Whilst evidence for the capacity of LC *n*-3 PUFAs to lower serum triglyceride concentration is predominantly strong, which improves in patient outcome ascribed to modulation of signaling pathways involved in inflammation and oxidative stress, improvement in endothelial function, and inhibition of platelet aggregation

Although plant-derived alpha-linolenic acid (ALA) is obtained from dairy products and margarines (O'Sullivan *et al*., 2011) which are enzymatically converted to EPA and DHA found in humans, the development is inefficient (0.04%–2.84%), and restricted by high dietary intake of EPA, DHA and linoleic acid (Gibson *et al*., 2012, Burdge *et al*., 2003). Whilst low delta-6 desaturase activity in humans contributes to poor conversion of ALA to EPA and DHA (Singer *et al.,* 1986), levels of EPA and DHA can nonetheless be increased through dietary consumption of LC *n*-3PUFA-fortified foods or marine oil supplements which contains these fatty acids (Block *et al*., 2008). Major sources of LC *n*-3 PUFAs in the diet of developed countries are fish, red meat and poultry (Astorg *et al*., 2004, Howe *et al*., 2006), where combinations of these foods contribute high levels of DHA (poultry and fish), EPA (red meat and fish), and Docosapentaenoic acid (DPA; red meat, poultry and fish). Many of the aforementioned benefits of LC *n*-3 PUFAs have been credited to the consumption of DHA and/or EPA.

Countries possessing high dietary intake of fish, such as Japan, have populations that benefit from cardio protective levels of LC *n*-3 PUFAs (Yamagishi *et al*., 2008). However, many populations consume amounts of LC *n*-3 PUFAs that are regarded as adequate, avoiding problems associated with deficiency, but not enough to provide protection for health. Health authorities of these countries recommended increased intake of LC *n*-3 PUFAs to provide such benefits. For example, European health authorities recommend at least 0.45–0.50 g/day EPA + DHA to maintain good health [Scientific Advisory Committee on Nutrition, French Agency for Food, Environmental and Occupational Health & Safety, Health Council of the Netherlands]. The mean daily intake of EPA + DHA and ALA in Australian adults is 0.175 g and 1.07 g, respectively [Heart Foundation of Australia. Position Statement, 2008]. The Heart Foundation of Australia recommends a daily dietary intake of 0.5 g EPA + DHA plus 2.0 g ALA for decreasing the risk of coronary heart disease, and 1.0 g EPA + DHA plus 2.0 g ALA for patients with documented coronary heart disease, and 1.2–4.0 g EPA + DHA for patients with elevated serum triglyceride levels.

Most of these organizations have issued recommendations for combined intake of EPA and DHA, but do not provide advice for the ratio of EPA to DHA. The French Agency for Food, Environmental and Occupational Health & Safety is a notable exception, providing separate recommendations for dietary intake of EPA and DHA. The American Psychiatric Association recommended level of 1.0 g/day DHA+EPA which can be helpful in the treatment of affected disorders (Freeman *et al*., 2006, McNamara, 2009) and proposed a 2:1 ratio of EPA:DHA for optimal management of patients.

Differential association of cell membrane EPA and DHA levels and resting state electroencephalographic (EEG) activity have been reported. DHA

was associated with rapid frequency EEG activity, whereas, EPA was associated with slow frequency EEG activity in 46 adolescent boys with attention deficit hyperactivity disorder (Sumich *et al*., 2009). Health benefits of LC-*n*-3 PUFAs have frequently been ascribed to particular types of LC-*n*-3 PUFAs. For example, studies have reported blood pressure lowering effects of DHA but not EPA (Mori *et al*., 1999, Rousseau-Ralliard *et al*., 2009), while EPA has been reported to be more efficacious than DHA in reducing platelet activation (Park *et al*., 2002, Phang *et al*., 2009). The differential response to EPA and DHA suggests that an opportunity might exist to customize advice for the types or ratios of LC *n*-3 PUFAs to be used in the treatment of a particular diseases or health conditions. The application of such a strategy is certainly achievable, with a plethora of commercially available dietary marine oil supplements of defined LC *n*-3 PUFA composition (Hamilton *et al*., 2010). For example, EPAX 5510 TG/N is an EPA-rich oil (EPA/DHA ratio of 5:1), while EPAX 1050 TG/N is a DHA-rich oil (EPA/DHA ratio of 1:5) (Phang *et al.,*2012).

Salunkhe *et al*. (2011) identified a marine bacterial isolate that produces high concentrations of EPA (60% of total fatty acid content) at 30 °C, with no detectable production of DHA. The strategy can be possible by considering the dietary intake of meat and fish sources. For example, of fish high in LC *n*-3 PUFAs content, the ratio of EPA: DHA can range from ~1:2 for Atlantic Salmon and Ocean Trout to ~1:30 (Soltan *et al.,* 2008). Thus with improved understanding of the similarities and differences for EPA and DHA, an opportunity exists to modify endorsements for intake of EPA or DHA which can be tailored to the patient's condition.

2.2 Lipid Extraction

Several extraction procedures are found in books and articles aiming for improving lipid recovery from any form of organisms, cell types or tissues. After the first famous studies of Chevreul (1813) on the dissolution of lipid materials in various solvents, Franz von Soxhlet, (1879) described the first method for milk lipids based on an automatic solvent extraction (diethyl ether). A further improvisation was made to this by Bloor (1914) where a mixture of ethanol/ether in the ratio of 3:1 used for the extraction of lipids in biological samples.

Earlier chloroform was used in extracting lipids which improved the extraction of polar lipids from animal tissues when Folch (1957) described it in his classical extraction procedure. This procedure remains one of the best described and the most commonly used by lipidologists around the world.

Other procedures proposed by Bligh and Dyer (1959) also made use of the mixture of solvents of chloroform/methanol and ethanol/diethylether, respectively in a different ratio. The estimation of the fat content in foods is measured by the amount of acyl lipids. As a guide, a comparison of several methods for measuring the oil contents of oilseeds was given by Barthet *et al.* (2002). A simplified method for chloroform/methanol extraction and gravimetric estimation of total fat was tested for several foods and food stuffs by Phillips *et al.* (2008).

For extracting the bulk of oils and the fats from lipid-rich tissues, like oil seeds, direct extraction-analysis with supercritical fluids (King *et al*., 1996) is used increasingly for specific lipids or other sample, especially the fat-soluble vitamins, common in the food industry. However, the analysts usually return to the "Folch" or its variant the "Bligh & Dyer" method when they

wanted to extract all the simple and complex lipids from a tissue in a near quantitative manner,

2.3 Sardine (*Sardinella longiceps*)

Sardines are considered as fatty fishes whose lipid content may vary from 0.5 to 20% in regard to season, stage of maturity and even body size. Feeding behavior of sardine explains the PUFA content and they are accumulated in their body through ingestion of phytoplankton. They are not able to synthesize PUFA as similar to humans. So PUFA belong to the category of essential fatty acids. Considering the elemental nature of its oil content so far many scientists are concentrating on the Sardine fishery. Even though, Sardine (*Sardinella longiceps*) is a low-value fish a higher concentration of PUFAs (31.4%) particularly EPA (17.6%) in sardine oil is an added advantage. The most pervasive of the n-3 fatty acids in fish are cis-5,8,11,14,17-eicosapentaenoic acid (EPA) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), which are commonly found in triacylglycerol (TG) (Hölmer, 1989) and phospholipids (PL) (Vaskovsky, 1989). Fish oil is nearly pure triacylglycerol, which encompass more than fifty different fatty acids (Ackman, 1982). The chain length of it ranges from C14 to C24 with varying degree of unsaturation, includes saturated and polyunsaturated. Depending on the type of fish species, the polyunsaturated fatty acids (PUFA), EPA and DHA typically account in between 5 and 15% each with a total n-3 content account varying between 20 and 30%.

Phospholipids are normally much more highly supplemented with EPA and DHA, 20 to 50% of each which depended on the type of phospholipids. Based on total wet weight of fish, the total phospholipid content usually remains in between 1 and 1.5%, roughly an order of degree lower than that of

triacylglycerol (Haraldsson *et al*., 1993). Phospholipids are the major constituents present in cell membranes which play an essential role in biochemistry and physiology for the cell functioning (Mead *et al*., 1986). The n-3 polyunsaturated fatty acids apparently play a significant role in adjusting the membrane integrity and at the lower temperatures it functions to the membrane fluidity and mobility of cell which is due to their higher unsaturation.

Indian Oil Sardine

Scientific classification

Kingdom	:	*Animalia*
Phylum	:	*Chordata*
Class	:	*Actinopterygii*
Order	:	*Clupeiformes*
Family	:	*Clupeidae*
Genus	:	*Sardinella*
Species	:	*S. longiceps*

Binomial name
Sardinella longiceps
(Valenciennes, 1847)

2.4 Freezing Stability of Sardines

To retain fish properties before it is consumed or employed in other technological processes; frozen storage has been widely employed (Pigott and Tucker, 1987). During the frozen storage of fish, hydrolysis of lipid and oxidation has been shown to occur. It becomes an important factor of fish

acceptance as influencing rancidity development, protein denaturation and texture changes. Among lipids the most important concern is the oxidation of the highly unsaturated lipids and constitutes direct relation to the production of off flavours and odours (Harris and Tall, 1994, Hsieh and Kinsella, 1989). Since lipids of fatty fish contribute much to the undesirable flavor, even a slight oxidation of these may contribute to serious flavor changes. A degradation of PUFA, induced by auto oxidation during frozen storage of fish oils especially in fatty fish, causes the formation of volatiles leading to rancidity (Pazos *et al.,* 2005), and therefore, it is both lipid and PUFA content of fish that play a deciding role in its health benefits. Among fish species PUFA content constitutes varied amounts in body constitutes, little attention has been paid to and little information obtained regarding changes in fatty acids in different species during the frozen storage process and period.

In addition, frozen storage does not prevent the oxidation of the unsaturated fatty acids (Fennema, 1973, Ke *et a1.,* 1976) and these oxidised fatty acids and the further products of oxidation may react with the food proteins forming insoluble compounds. Lipids of oil sardine are known to be very rich in polyunsaturated fatty acids. Oxidation of these polyunsaturated acids during frozen storage leads to the development of rancidity. The major source of these free fatty acids is found to be the phospholipid fraction in many species of fish, especially in those with low lipid contents (Olley and Lovern, 1960, Olley *et al*., 1962).

2.5 Identification of source material for PUFA

The fatty acid composition of most common marine food fishes of the Indian coast has been reported (Gopakumar and Rajendranathan, 1975). The Indian oil Sardine enjoys a predominant position in Indian marine fisheries

and is mainly caught from the coastal waters of Kerala and Karnataka and to some extent from Goa and southern Maharashtra in the west coast. Fish oil in India is mainly extracted from the oil Sardine and is currently used as drying oils and in varnishes. The higher EPA and DHA content in marine fish oils indicates their potential for the development of value added produce in ruminants by using it in the form of protected fat (Fievez *et al*., 2003, Kitessa *et al*., 2003). Although the best source of long chain n-3 PUFA is fish and fish products, fat content and fatty acid profiles change with species, season and diet. Sardines in temperate regions show seasonal fluctuation of fatty acid composition and yield, which got influence of sea water temperature, food availability and sexual state of the animal. (Gamez-Mezaa *et al.,* 1999)

Marine Fish oil is the richest source for EPA and DHA, but may not be the ideal source of Omega 3PUFA due to its high susceptibility towards oxidation and consequent off-flavours, as well as geographical and seasonal variations in availability and quality (Belarbi *et al*., 2000, Liu and Lin, 2001). Therefore, much effort is being devoted for developing a commercially feasible technology to produce EPA and DHA directly from marine microalgae considered to be the primary producers of these w3 PUFA in food chain (Barclay *et al*., 1994, Lebeau and Robert, 2003, Molina Grima *et al*., 2003, Pulz and Gross, 2004). *Isochrysis galbana* (Ig) and *Diacronema vlkianum* (Dv) are the two marine microalgae from class Haptophyceae, recognized as a natural rich source of EPA and DHA that are accumulated as oil droplets in prominent lipid bodies in the cell (Liu and Lin, 2001). Algae are the primary producers of the oceans' ecosystems, providing the foundation of the oceanic food chain. Specifically, algae synthesize omega-3 fatty acids and then are consumed by other marine life subsequently. Algae-derived oils are vegetarian-friendly and easy to grow on a large scale due to their small size.

There is wide-ranging number of algal species that shows variability in the synthesis of EPA and DHA (Doughman *et al*., 2007, Kyle, 2001).

Nowadays marine microalgae are exploited in hatcheries as live feed and live feed component because it serves as a natural source of polyunsaturated fatty acids. Algae synthesised PUFA's are consumed by other higher organisms. Fatty acids in them are comparatively simpler than fishes and can be easily purified while possible deterioration is also less. The synthesised PUFA from algae are eco-friendly and is also vegetarian. Superfluous lipid and protein during algal growth may be used as biodiesel and biomass for oil sources and animal feed, respectively. This highlights the sustainable profits of algae and the several potential gains from creating algal bio factories (Molina-Grima *et al*., 2015).

2.5.1 *Chaetocerous calcitrans*

Chaetocerous genus is the largest genus of marine plankton diatom which is a cosmopolitan alga. It consists of about 400 or more and yet to be described. The species differentiation for this genus is a tedious task because they are very similar in nature and the tropical varieties were not fully exploited. Ehrenberg (1884) recognized first in describing this genus.

Classification:		
Empire	:	*Eukaryota*
Kingdom	:	*Chromista*
Phylum	:	*Ochrophyta*
Class	:	*Coscinodiscophyceae*
Order	:	*Chaetocerotales*
Family	:	*Chaetocerotaceae*
Genus	:	*Chaetocerous*

C. calcitrans are extensively used in aquaculture industries in the form of live feed owing to its valuable polyunsaturated fatty acid profile. They are the finest food sources for growing shellfish because it is of small size so can be able to ingest easily by larvae oysters and mussels. High portion of C16 PUFA found in this species propose that they act as effective membranes in diatoms replacing function of C18 PUFA found in higher plants. The environment condition, life cycle of algae and growth rates are the primary factors which determines its biochemical composition. To maintain in ideal conditions like light intensity, salinity, temperature etc. values to its nutritional aspects of biochemical composition. Since, they are considered difficult to grow continuously in wild, which forces many hatcheries to depend on concurrent over lapping batch culture. It has proved the bio benefits potential for *C. calcitrans* that it induces apoptosis in breast cancer cells. Diatoms are particularly recognized as for the synthesis of EPA, they also produce high amounts of palmitoleic acid (16:1n-7) and palmitic acid (16:0). In comparison with other diatoms, *C. calcitrans* has got the highest proportions of PUFA particularly 16:3 n-4 and EPA making it significant for aquaculture settings. Reports suggest that lipid content of *C. calcitrans* varies from 26.8% dry weight in grown out-doors and in under shade to 11.71% dry weight in controlled conditions.

2.5.2 *Isochrysis galbana*

Classification:		
Empire	:	*Eukaryota*
Kingdom	:	*Chromista*
Phylum	:	*Haptophyta*
Class	:	*Coccolithophyceae*
Subclass	:	*Prymnesiophycidae*
Order	:	*Isochrysidales*
Family	:	*Isochrysidaceae*
Genus	:	*Isochrysis*

Isochrysis galbana is a haptophyceae, got special interest in aquaculture, particularly to feed mollusk larvae, and also for fish and crustaceans during their early stages of growth (Wikfors and Patterson, 1994). Jeffrey *et al*. (1994) reported that it has been used as a marine culture feed because of its high content of proteins and polyunsaturated fatty acids (PUFAs). The reported characteristics of *I. galbana* are that they have up to 15-40 % dry weight of lipid content with short growth duration. They have high carbon dioxide tolerance particularly up to 2-20% concentration and also tolerable to different temperature limits. They have growth capacity at high cell concentration.

2.5.3 *Chlorella marina*

Chlorella comes under the genus of single-cell green algae, which belongs to the phylum *Chlorophyta*. The shape of which is spherical about 2 to 10 µm in diameter, having no flagella. Green photosynthetic colouring pigments chlorophyll a and b are present in its chloroplast. It multiplies rapidly

through photosynthesis which requires only carbon dioxide, sunlight, water and a little amount of minerals for its reproduction.

Classification:		
Empire	:	*Eukaryota*
Kingdom	:	*Plantae*
Phylum	:	*Chlorophyta*
Class	:	*Trebouxiophyceae*
Order	:	*Chlorellales*
Family	:	*Chlorellaceae*
Genus	:	*Chlorella*

The naming of *Chlorella* has been taken from the Greek word, *chloros*, which means green, and the Latin miniature suffix *ella*, which means small. Chlorella can be used as a potential food source due to its high content of protein and also other essential nutrients. When dried it contains about 45% protein, 20% carbohydrate, 20% fat, 10% minerals, 5% fibre and vitamins. Usage of large artificial circular ponds for its mass-production are now being used, in this system they are abundant in calories and vitamins. *Chlorella* on first harvest was optional as a low-cost protein supplement for the human diet. These can be advocated sometimes focussing on other theoretical health benefits of these algae, including claims of weight control, immune system support and cancer prevention. According to the American Cancer Society, "scientific studies available do not support its efficiency for treating or preventing cancer or any other human diseases". *Chlorella* yields oils that are rich in polyunsaturated fats under certain growing condition. *Chlorella minutissima* has generated EPA of 39.9% of total lipids.

The genus Chlorella comprises ellipsoidal or spherical non-motile green cells which produce auto spore, and they inhabit in freshwater, soil and found in marine habitats (Kómarek and Fott, 1983). The commercial potential of it has been recognised since 1960, and they are the first microalga to be cultured in masses for food, feed and also as a source of nutraceuticals (Becker, 1986, Borowitzka and Borowitzka, 1988, De Pauw and Persoone, 1988). Also these algae are considered as a good candidate for fuel production. (Li *et al*., 2007; Converti *et al*., 2009; Mata *et al*., 2010).They are rich in poly unsaturated fatty acids such as 18:2 (about 20%) and 18:3 (about 19.4%) followed by monounsaturated fatty acids such as 16:2 (about 9.5%), 16:3 (about 9%) and saturated fatty acid (14:0) (about 9%) while other fatty acids are at a minimum percentage.

2.5.4 *Tetraselmis gracilus*

Tetraselmis is a genus of phytoplankton that contains very high lipid level. They are green and motile, usually grows to about 10 µm long and 14 µm wide.

Classification:		
Empire	:	*Eukaryota*
Kingdom	:	*Plantae*
Phylum	:	*Chlorophyta*
Class	:	*Chlorodendrophyceae*
Order	:	*Chlorodendrales*
Family	:	*Chlorodendraceae*
Genus	:	*Tetraselmis*

Tetraselmis genus is one among the widely used microalga for feeding herbivores in Mariculture. They can grow under a wide range of chemical as well as physical environmental condition due to which their application is most in marine aquaculture.

All the algae classifications were obtained from Algae Base reported as a world-wide electronic publication (Guiry and Guiry, 2015).

2.6 Enrichment of PUFA

Numerous methods have been established to isolate (or concentrate) and recover specific fatty acids and their derivatives (i.e. esters, free fatty acids, triglycerols etc.) from various naturally occurring sources, but only a few are suitable for large scale production. The available methods include chromatography (adsorption and partitioning), fractional or molecular distillation, enzymatic splitting, low temperature crystallization, super critical fluid extraction and urea complexation.

2.6.1 Enzymatic Methods

Lipases may catalyse esterification, hydrolysis or exchange of fatty acids in esters. These processes can be selected by choosing appropriate substrates and reaction conditions. Lipase-catalysed processes have attracted attention because of the mild reaction conditions under which they occur and the selectivity displayed by these catalysts. In both respects they differ from typical chemical reactions. Since enzymatic reactions occur under mild temperature and pH conditions and at ambient pressure, they generally require less energy and are conducted in equipment of lower capital cost than many other chemical processes. Another advantage of enzymatic process is related

to the selectivity of many lipases which allows obtaining of products that are difficult to produce by more conventional chemical reactions.

2.6.2 Lipase-Catalysed Hydrolysis

Bottino *et al*. (1967) illustrated the mechanism of resistance of lipase towards long-chain omega3-PUFA in marine oils. Research in recent years has received much attention for using microbial lipases to produce omega3-PUFA concentrates by hydrolysis of marine oils. Tanaka *et al*., (1994 & 1998) have used six types of microbial lipases (*Aspergillus niger*, *Candida cylindracea*, *Pseudomonas spp*., *Chromobacterium viscosum*, *Rhizopus delemar*, and *Rhizopus javanicus*) to hydrolyze tuna oil and found that CC-lipase was most effective in increasing the DHA content present in the non-hydrolyzed fraction of the tested oil. The DHA content in the non-hydrolyzed fraction was increased three times that of present in the original oil; however, other lipases did not follow suit. Hoshino *et al*. (1990) also used several lipases for selective hydrolysis of cod-liver and sardine oils. The best hydrolysis results were obtained for the non-regiospecific *Candida cylinderacea* and 1, 3-specific *Aspergillus niger* lipases, whereas none of the other lipases were able to increase the EPA content of the acyl-glycerols to any great extent. However, over 50% of total omega3-PUFA was produced when these two lipases were employed.

Tocher *et al*. (1986) described the preparation of omega3-PUFA concentrates by utilizing phospholipase A_2 on cod roe phospholipids. This method was based on the assumption that the majority of the EPA and DHA of fish phospholipids were located preferentially at the sn-2 position and the specific action of phospholipase A_2 on that position. The resulting product contained 24 and 40% of EPA and DHA, respectively, with an overall EPA

and DHA recovery of approximately 60%. Shimada *et al.* (1998) reported that hydrolysis of tuna oil by *Geotrichum candidum* lipase has increased the total content of EPA and DHA from 32.1 to 57.5%. A Japanese patent describes a method based on the discrimination of lipases on EPA and DHA for preparation of omega3-PUFA concentrates. Ethyl esters from miscellaneous fish oils such as those of Sardine and mackerel were hydrolysed with lipases from *Candida cylinderacea*, *Aspergillus niger* and *Mucor miehe*. Selective hydrolysis afforded an ethyl ester concentration of up to 25% EPA and 17% DHA after separation of the hydrolysed fatty acids. Lie and Lambertsen (1986) also reported a similar discrimination of *Candida cylinderacea* lipase among fatty acids. The fraction of C14 to C18 saturated and monounsaturated fatty acids were preferentially hydrolyzed in TAG of capelin oil, whereas the long-chain monoenes of C20:1 and C22:1, and PUFA of C18:4, EPA and DHA were resistant to hydrolysis. However, lipolytic enzymes from cod guts preferentially hydrolyzed PUFA over the short chain fatty acids which showed a completely opposite fatty acid selectivity. This suggests that the fatty acid specificity of lipases is a crucial factor when considering the application of enzymes to modify marine oils.

Wanasundara (1996) and Wanasundara and Shahidi (1997 & 1998) have carried out lipase-assisted hydrolysis of seal blubber oil (SBO) and menhaden oil (MHO) in order to enrich their content of w3-PUFA in the acylglycerols, non-hydrolysed fraction.

2.7 Prevention of oxidation/rancidity

When red meat fishes such as sardine and mackerel are stored at low temperature their skin lipids undergo autoxidation at relatively higher rates than white muscle lipids (Yamaguchi *et al*., 1984). Similarly, lipids prepared

from skin are more susceptible to oxidation than those from the other tissues (Toyomizu *et al*., 1980; Ke *et al*., 1982; Yamada, 1979; Nakamura, 1984 and Yamaguchi *et al*., 1984). Oxidative stabilities of fish lipids may differ from species to species and predominantly high in red meat species, such as sardine, mackerel and saury, than in white meat species, such as dusky sole, halibut and sablefish (Koizumi *et al*., 1979). The actual cause of the differences in lipid oxidation between species, as well as between tissues, has not yet been fully elucidated.

Today, studies are concentrated mainly on the control of 'redox' status with the properties of food and food components. The resistance toward oxidative damages increases with the presence of natural antioxidants which has got a substantial impact on human health. Antioxidants present in diet such as ascorbates, tocopherols and carotenoids are well known and there is a surplus of publications related to their role in health. The following medicinal plant extracts were used in the study for their antioxidant/preservative property

2.7.1 *Aloe barbadensis* (Kattarvazha)

Aloe barbadensis Miller (Aloe vera) is a turgid lace-shaped green leaf plant present in tropical or subtropical arena.There are 360 different species or more of aloes grown in the dry regions of North America, Europe and Asia. *Aloe vera* (*Aloe barbadensis* Miller.), is a fellow of the family Liliaceae, which is a short stemmed succulent, perennial herb. The leaves are formed by a thick epidermis covered with cuticles surrounding the mesophyll, which further differentiated into chlorenchyma cells and thinner walled cells. A yellow exudate (commonly called aloe) from the inner epidermal cell layers has long been recognized as a cleansing drug by pharmacopoeias over the world (Eshun, 2004) and the phenolic compounds plentifully exist in it (Park

and Sung, 2006). In China it has been widely cultivated and used as a traditional medicine to prompt wound healing, and as an anti-viral and anti-cancerous agent (Maze *et al*., 1997).

The products of *Aloe vera* have long been used in health supplementing foods for medical and stabilizing purposes. The polysaccharides isolated or detected from the gel include tocopherols, mannan (Segal *et al*., 1968, Waller *et al*., 1978) galactan (Mandal and Gas, 1980), glucomannan (Mandal and Gas, 1980) and arabinorhamnogalactan (Mabusela *et al*., 1990). Aloe polysaccharides have been verified to have antioxidant properties and PC12 cell protective effects (Wu *et al*., 2006). Their action depends on several structural parameters such as the degree of acetylation (DSAC), the molecular weight, type of sugar, and glycosidic branching (Leung *et al*., 2004). The component and structure of polysaccharides change with the changes of environment and growing conditions. Reynolds and Dweck (1999) have studied the potential antioxidant activity of Aloe vera. A report has shown that the organic extracts from leaves of these plants possess potent *in vivo* antioxidant capacity. The organic extract of *Aloe vera* leaves provided anti-inflammatory activity in the experimental rat, and it has also been shown to be impending therapeutic agent for the treatment of sepsis and hepatotoxicity (Eshun, 2004). Although, the physiological properties of the *Aloe vera* gel are known, its antioxidant properties are not thoroughly worked out.

2.7.2 *Ocimum sanctum* (Thulasi)

Holy basil, sacred basil, or Krapao (*Ocimum sanctum* Linn. or *Ocimum tenuiflorum* Linn.) is a much branched tropical annual herb which grows up to 18 inches. Holy basil are of two types red or purple variety and white or green variety. The former has dark green leaves with reddish purple stems and a

purplish cast on the younger leaves, while the later has medium-green leaves with very light green, almost white, stems. Holy basil has a robust anise like, somewhat musky and lemony taste with a camphoraceous aroma. The principal aroma component in holy basil is eugenol. Asians use this herb as a traditional medicine for most stomach disorders, cramps, diarrhea, headaches, whooping cough and head colds (Uhl, 2000). In the last few decades the therapeutic uses of holy basil in modern medicine has been established, several Indian scientists and researchers have studied the pharmacological properties of steam distilled, petroleum ether and benzene solvent extracts of various parts of this plant and eugenol in the treatment of various ailments.

The scientific basis for the therapeutic uses of holy basil had been established by these pharmacological studies (Prakash and Gupta, 2005). Antioxidant activity of components in holy basil is one of foremost reasons of its pharmacological actions. Phenolic compounds in holy basil extracts including eugenol, isothymusin, cirsilineol, isothymonin, rosmarinic acid (Kelm *et al.*, 2000), vicenin and orientin, (Vrinda and Uma Devi, 2001) have been proved as good antioxidant compounds. However, there is an abundant deal of diversity in the composition of holy basil cultivated in different localities (Kicel *et al.*, 2005). Juntachote and Berghofer (2005) found that ethanolic extracts of holy basil showed better heat stability (80^{o}C, 1 h), and had high antioxidative stability at neutral and acidic pH, displayed strong superoxide anion scavenging, Fe2+ chelating activity and reducing power, and also acted as lipoxygenase inhibitors. Further studies revealed that *O.sanctum* decreased lipid peroxidation and increased the activity of superoxide dismutase.

2.7.3 *Boerhaavia diffusa* (Thazuthama)

Boerhaavia diffusa Linn known as punarnava in Sanskrit is a herbaceous member of the Nynctaginaceae family (Rendel, 1925). The whole plant or its particular parts (stem, leaves and roots) are known to have medicinal properties and have a long history of use by indigenous and tribal people in India and in many countries. In southwestern Nigeria, it is eaten as vegetable whereas its root is used in the treatment of dysentery and piles. In India, it is also eaten as vegetable in West Bengal while the root paste is used to treat bloody dysentery in Madhya Pradesh. The root juice is used in medication of asthma, scanty urine and inflammatory disorders, also for the treatment of leukorrhea, rheumatism and stomach ache (Awasthi and Verma, 2006 & Hiruma-Lima *et al*., 2000). People living in Indo-Nepal Himalayas use it to flush the renal system and for seminal faintness and blood pressure (Mitra and Gupta, 1997). Mostly the roots are employed for many resolutions including liver, kidney, renal, gall bladder, and urinary disorders (Gaitonde *et al*., 1974; Mudgal, 1975; Jain and Puri, 1984; Arseculeratne *et al*., 1985 & Anis and Iqbal, 1994). In Brazilian herbal medicine, it is employed as a chologogue and diuretic, for all types of liver disorder including hepatitis and jaundice, for gall bladder pains, urinary tract and renal disorders (Coimbra, 1994 & Cruz, 1995). Anti-proliferative activity was proved from extracts of B. diffusa against a variety of established tumour cell lines (Mehrotra *et al*., 2002).

The methanolic extracts of this species in posterior study found effective in dropping metastases development in some melanoma cells (Leyon *et al*., 2005). Lot of work on the characterization of the active compounds in B. diffusa has been done. In pharmacological studies, the anti-inflammatory (Bhalla *et al*., 1968); antifibrinolytic (Jain and Khanna, 1989); antistress;

antihepatotoxic (Mishra, 1980; Chandan *et al*., 1991; Rawat *et al*., 1997); anthelmintic, febrifuge, rheumatism, antileprosy, antiscabies, and anti-urethritis (Nadkarni, 1976); anticonvulsant (Adesina, 1979); antibacterial (Olukoya *et al*., 1993); antinematodal (Vijayalakshmi *et al*., 1979) and chemo preventive (Bharali *et al*., 2003) activities of this plant have been reported. Hepatoprotective activity of the whole plant as well as the root extracts has been reported (Chandan *et al*., 1991, Rawat *et al*., 1997). Most activities of the plant are localized at the midpoint on the root, whereas significant differences in the chemical composition of the root and the leaves have been reported (Pereira *et al*., 2009).

Encapsulation or microencapsulation provided with a coating material may retard lipid oxidation of fish oils and omega-3 PUFA concentrates. (Matsuno and Adachi, 1993). There are several physical and chemical methods where wall materials have been developed for this purpose to encapsulate and stabilize fish oil and concentrates (Desai and Park, 2005). Amongst these, spray-drying is the most common and cheaper method (Gharsallaoui *et al*., 2007), and other processes such as freeze drying (Heinzelmann *et al*., 2000), ultrasonic atomization (Klaypradit and Huang, 2008 & Barrow *et al*., 2009) have also been offered recently as substitute to reduce omega-3 PUFA oxidation. Meanwhile they avoid the use of high temperatures during the drying step. As functional foods, oil in water emulsion are increasingly being used for delivering fish oil. If appropriately planned, such emulsions can safeguard the fish oil from lipid oxidation before adding to foods (Day *et al*., 2007, McClements *et al*., 2007) and in some cases it may also increase the oxidative stability of the fish oil enriched food (Let *et al*., 2007).

In the last decade, the usage of natural antioxidants as an alternative of synthetic compounds has received increasing attention and many studies about the efficiency of plant extracts, as oregano, parsley or rosemary, on the stabilization of bulk fish oil and fish oil-in-water emulsions are also carried out (Frankel, 1996; Bhale *et al*., 2007& Jiménez-Álvarez *et al*., 2008). The acceptable concentration of antioxidant depends on the chosen substance, the storage conditions and the specific applications/use of the omega-3 PUFA concentrates. Among presently available drugs, synthetic drugs do have potential contrary reactions and which can be reduced to greater extent by incorporating natural compounds extracted from Indian Medicinal Plants.

2.7.4 Antioxidant capacity of Algal and Fish oil

From the microalgae total lipid extracts examined the antioxidant capacity ranged from 37 to 93 lmol Trolox eq./g oil (Ryckebosch *et al*., 2014). They showed the highest antioxidant capacity in four oils such as two Nannochloropsis species, Tetraselmis and Rhodomonas and a low value was obtained for fish oils when compared with the algal antioxidant capacity. The antioxidant capacity of the micro algal biomass (Goiris *et al*., 2012) has measured not only lipid soluble molecules like carotenoids but also hydrophilic molecules like polyphenols that occur at low concentration in a lipid extract. The antioxidant capacity of the microalgae total lipid extracts was three to four times higher than the antioxidant capacity of fish oil. The origin of the antioxidant capacity of fish oil is by the addition of Vitamin E such antioxidants. The higher antioxidant capacity of the microalgal oils will better protect oxidation of algal PUFA compared to omega 3 fatty acids in fish oil. Fish oil supplementation was investigated using several delivery methods (capsules, fish-containing meals, fortified foods, or parenteral administration) and results from two studies advised caution when supplying high dietary *n*-3

LC-PUFA levels in both type 2 diabetes mellitus patients and individuals with impaired glucose tolerance, due to risks of increased glycaemia; algal oils appeared to be well tolerated and no major negative effects have been reported (Tur *et al*., 2012).

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Chapter 3

ANALYSIS OF PUFA CONTENT IN FRESH AND FROZEN SARDINES

Contents

3.1 Introduction

Indian Oil Sardine or *Sardinella longiceps* which belongs to class Actinopterygii and order Clupeiformes is a fin fish, forms the largest fishery in Malabar upwelling region along the south west coast of India. About 10% of the total marine fish landings in India are contributed by the Indian oil sardine. The preliminary analysis includes the seasonal variation of its PUFA content, proximate composition, along with optimization of lipid recovery method. It also includes the consequences of frozen sardine consumption and its harmful effects. Clupeid fishes are chiefly known to be as a seasonal feeder. When there is scarcity in food these fishes uses reserves of energy stored, in the form of lipids which will be burnt when the energy has to be expended. Lipids are also useful in regulating stability, fluidity and permeability of cell membrane. The fat reserve and the composition of fatty acids can vary with sex, age and also seasons (Gopakumar, 1965). Roughly, these species are easily available throughout the seasons in the western coast which means the enormous supply of the essential fatty acids for human consumption. In the fishery industry this brings about great commercial implication for sardines to be a potential

nutrition supplement to pharmaceutical industries which focuss on marine PUFA.

Freezing and frozen storage have been widely employed to retain sensory and nutritional properties of fish (Erikson, 1997). Currently, in industries as well as in domestic level also freezing is one of the most widespread methods for fish preservation. From the commercial view point, loss of texture caused by frozen storage is of great concern than nutritional aspects and has led most studies of frozen fish on the oxidation centred effect of its nature and causes of these changes in fish texture. Products developed from fish and other marine species are of great economic importance in many countries and in India, a variety of these products are stored , marketed and exported in frozen state.

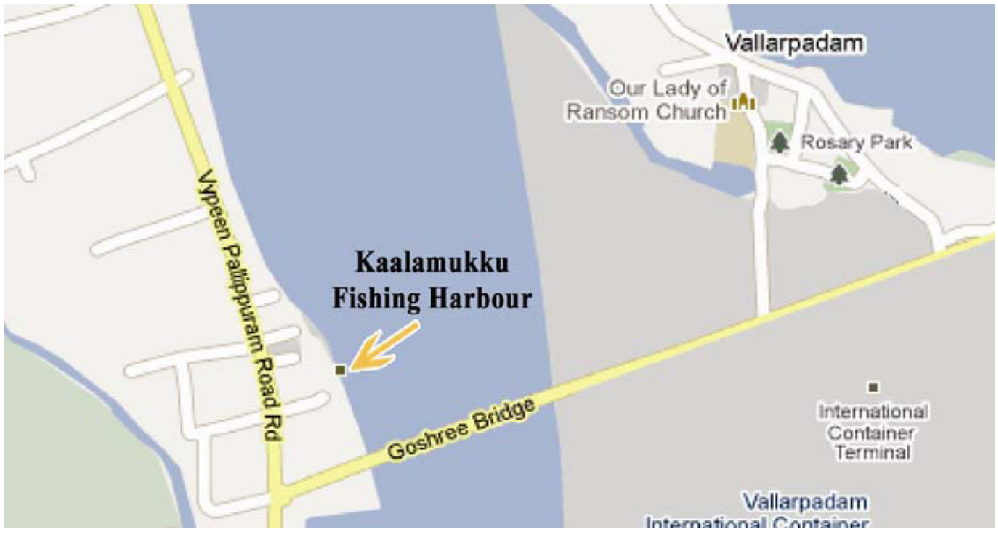
Fresh fish is susceptible to spoilage caused by both microbiological and biochemical reactions. The effects of freezing are the inhibition of microbial growth (Troller, 1980) and the reduction of enzyme activity (Connell, 1960, 1969) which makes it possible to prolong the storage life of fish muscle. The most serious concern with the storage of these fatty fish probably is the deterioration of the lipid fraction. Hydrolysis and oxidation of lipids take place during processing and preservation of fish. Hydrolysis of lipids results in release of free fatty acids and these free fatty acids cause protein denaturation. Those denatured proteins lose their characteristic properties and leads to loss of quality, especially the texture. Thus lipid hydrolysis due to lipases and consequent deterioration of quality will be less in products kept at low temperatures (–18 to –20°C). Change in lipids due to oxidation is a serious and complex problem. The presence of unsaturated fatty acids is in high degree for fish lipids and its susceptibility to oxidation is extremely high. Oxidation process is characterized by an induction period with slow rate of oxidation,

followed by an acceleration period of maximum rate, with concurrent development of hydro peroxides, which are the primary products of oxidation. Decomposition of the hydro peroxides produces secondary products like aldehydes, ketones, alcohols, carboxylic acids etc. some of which are volatile and some non-volatile. The position of the double bond being oxidized and the conditions under which the hydroperoxides are decomposed, determine the number and nature of these products. Oxidation of highly unsaturated fatty acids will lead to formation of polymerized products and under these conditions fish or oil will become totally unacceptable and even toxic. Proper utilization of this species for a long term can be achieved by adopting suitable technologies in preservation.

3.2 Materials and Methods

3.2.1Fish Samples

Sardinella longiceps (Indian Oil Sardine) is the chief source of PUFA in natural environment and was purchased from Kaalamukku fishing harbor situated near Vypeen, Kochi. This is the main landing center situated closer to Cochin University Lakeside campus. Fresh fish collected were immediately brought to lab in ice filled insulated box. One lot of the fish was washed and the cleaned fish were filleted and were used for analysis. Another lot was washed and packed in polythene bags and kept frozen at -20 ^{o}C for frozen storage studies. All sampling and estimations were done in triplicate.



Courtesy: Google map

3.2.2 Proximate Composition of Fish

3.2.2.1 Determination of moisture

The moisture content was estimated by the method of AOAC (2005) by drying 10 g sample at 103°C in a thermostatically controlled hot air oven. The samples were taken in a pre-weighed Petri dish and kept in oven. The reduction in weight was checked by repeatedly heating and weighing after cooling the sample in a desiccator, till the weight became constant. Moisture content was determined as percentage.

3.2.2.2 Determination of protein

Accurately weighed 1g of homogenized wet sample was taken in a digestion tube. About 2g of digestion mixture and 10ml of concentrated sulphuric acid were added to the sample taken in the digestion tube. The samples were digested to a clear solution in a KEL 12 PLUS EK digestion unit. The digest thus obtained was made up to 100 ml with distilled water. 5 ml of the digest was pipetted out into the Kjeldahl Micro distillation apparatus.

10 ml of 40% NaOH was added to the sample in the distillation unit to make it alkaline. The bottom end of the condenser was fitted to a delivery tube immersed in 10ml of 2% boric acid with added Tachiros indicator. The ammonia produced on steam distillation was absorbed into the boric acid solution. The distillate collected was back titrated against N/70 sulphuric acid and calculated the total nitrogen content. Crude protein content (%) in the sample was calculated by multiplying the percentage of nitrogen content by the factor of 6.25 (AOAC, 2005)

3.2.2.3 Determination of crude lipid

The fat content of the moisture free sample was determined by Soxhlet extraction method (AOAC, 2005) using a suitable solvent (Petroleum ether, boiling point: 42-62°C). About 2g of the moisture free sample was accurately weighed into an extraction thimble and was placed in the extractor. The extractor was connected to a pre-weighed dry receiving flask and water condenser. The unit was placed on a water bath and temperature was maintained at 42°C – 62°C so that solvent boiled continuously and siphoned at a rate of 5-6 times/hr. The extraction was continued till the solvent in the extractor became colorless and fat free. The solvent in the receiving flask was evaporated completely and weighed for fat content, expressed the result as percentage.

3.2.2.4 Determination of ash

According to AOAC (2005) the ash content of the sample was measured by incineration method. 2g of moisture free sample taken in a pre-weighed clean dry silica crucible was charred on low heat. It was then kept in a muffle furnace at 550 °C to get a white ash that was cooled in desiccator and weighed.

3.2.3 Methods used for lipid extraction of sardine

3.2.3.1 Folch Method

Tissue was homogenized with chloroform/methanol (2:1) to a final volume 20 times the weight of the tissue sample .After dispersion, the whole mixture was agitated for 15-20 min in an orbital shaker at room temperature (20-25 ^{o}C). The homogenate was then centrifuged to recover the liquid phase. Then the filtrate was washed with 0.2 volumes (4 ml for 20 ml) of 0.9% NaCl solution. The mixture was centrifuged at low speed (2000 rpm) to separate the two phases, after vortexing for a few seconds. The upper phase after centrifugation was siphoned out and the lower chloroform phase containing lipids was evaporated under vacuum in a rotary evaporator or after flushing nitrogen gas when the volume was under 2-3 ml.

3.2.3.2 Bligh & Dyer Method

Weighed 5-20 g fish sample (fat and water content of the sample known) into a 250 ml flask. Added water so that the total water content (added water plus water content of sample) was 16 ml. To it again added 40 ml methanol and 20 ml chloroform, homogenized for 60 seconds. Additional 20 ml chloroform was added and again homogenized for 60 seconds. To it 20 ml water was added and homogenized 30 seconds for complete extraction. Covered the flask with cap and cooled in water bath with added ice. Removed the residue by filtration and collected the solvent in a reaction tube. Removed the water/methanol phase (upper phase) by aspiration using a water aspirator and transferred the measured volume (5-20 ml) of the chloroform phase to a pre weighed dish for evaporation and when dry, weighed the dish with lipid and calculated the yield as percentage.

3.2.3.3 Soxhlet Extraction of Lipids

Lipids from the moisture free sample of tissue from sardines were extracted according to AOAC, (2005) as described in 3.2.2.3.

3.2.4 Preparation of Fatty Acid Methyl Esters (FAME)

By using a nitrogen evaporator, the lipids extracted by Bligh and Dyer method were dried completely and to it added 0.4 ml 0.5 N NaOH in methanol and placed in hot air oven at 100 ^{o}C for 5 minutes. After saponification, the tubes were cooled under running water followed by methylation with 0.4 ml 14% BF3 in methanol at 100 ^{o}C. Cooled down and methyl esters of fatty acids were dissolved in n-hexane. Added 8.5 ml distilled water and centrifuged for 5 min at 3000 rpm. Upper organic layer containing FAME was separated carefully by Pasteur pipette and stored at -30^{0}C after flushing with nitrogen gas. (Metcalf *et al*.,1966)

3.2.5 Instrumentation of Gas Chromatography (GC)

Fatty acid composition was determined by Gas chromatographic analysis of methyl esters (Official Methods of American Oil Chemist's Society, 1993) using a Varian Gas chromatograph, (CP 3800) which was equipped with a flame ionization detector and a WCOT fused silica 30M x 0.32MM coating: CP WAX 52 CB DF=0.25 (µm). The initial temperature at 160 ^{o}C was kept for 1 min, followed by temperature programmed at 250 ^{o}C at a rate of 1 ^{o}C and final isothermal program for 20 min. Temperature of the split injector was 270 ^{o}C, with a split ratio of 1:10, and the detector was at 300 ^{o}C. The carrier gas used was nitrogen at a flow rate of 1 ml/min. Identification and quantification of fatty acids were carried out using a FAME standard (Ct.No.18919-1AMP) from Sigma Aldrich (Bellefonte, PA, USA) containing

37 fatty acid methyl esters. Various peaks of fatty acids on the chromatogram were identified by comparing the retention time of the standards.

3.2.6 Determination of degree of oxidation during storage

3.2.6.1 Peroxide value (Lea, 1938)

10 g of fish muscle was ground well with anhydrous sodium sulphate to remove moisture. It was taken in a dry standard flask and extracted with small quantities of chloroform, filtered and made up to 100 ml. 10 ml of this extract was taken to determine the weight of lipid.

Another 10 ml aliquot of extract was pipetted out into a dry iodine flask, added 20 ml of glacial acetic acid and 1 ml of saturated KI solution. The mixture was kept for 30 min in dark, diluted with water and the liberated iodine was titrated against standard N/100 sodium thiosulphate using starch as indicator.

3.2.6.2 Thiobarbituric acid value (TBA) (Tarladgis *et al*., 1960)

10 g meat was blended with 50 ml distilled water for 2 min, transferred the mixture with 97.5 ml distilled water added 2.5 ml HCl solution to bring the pH to 1.5. Placed a small amount of DOW's antifoam A into the lower neck of the flask and added a few saddle stones to prevent bumping. Assembled the apparatus and heated the flask at the highest temperature obtained in a Kjeldahl distillation apparatus and collected 50 ml distillate. Pipetted 5ml of the distillate in a test tube and added 5 ml TBA reagent , mixed the contents and immersed in boiling water for 35 min. A distilled water TBA blank was also prepared and treated like sample. Read the optical density of the sample against the blank at a wave length of 538 nm. Multiplied the reading by the factor 7.8 and expressed TBA as mg malonaldehyde per 100 g of the sample.

3.2.6.3 Free Fatty Acid

Free fatty acids were estimated according the procedure JAOCS (2006). About 3-4 g of the sample oil was weighed and taken in a dry conical flask. About 25 ml of neutral alcohol and a few drops of phenolphthalein indicator were added. The flask was fitted with a reflux condenser. It was then heated on a hot plate until the solution become clear. It was then titrated against standard sodium hydroxide solution till the end point reached. Noted the titre value.

3.2.7 Statistical Analysis

All of the experiments were done in triplicate. The data were recorded as means ± standard deviations and were analysed with SPSS (version 11.0 for Windows, SPSS Inc., Chicago, IL, USA), and the statistical significance was determined at $P < 0.05$.

3.3 Results

3.3.1 Proximate composition of *Sardinella longiceps*

Table 3.1 **Proximate composition of *Sardinella longiceps*** (wet weight base) and the length at Pre-monsoon, Monsoon and Post-monsoon seasons

Seasons	Protein %	Fat %	Ash %	Moisture %	Length (mm)
Pre-Monsoon	12.12±0.007	16.23±0.077	3.12±0.007	64.24±0.063	122-183
Monsoon	10.23±0.070	18.16±0.028	2.6±0.077	68.26±0.134	110-210
Post-Monsoon	15.25±0.091	21.12±0.014	4.97±0.098	54.12±0.049	112-193

Values are expressed in mean ± standard deviation, n=3

The total length of sampled fishes ranged between 122-183 mm, 110-210 mm and 112-193 mm during pre-monsoon, monsoon and post-monsoon seasons respectively and in all seasons males dominated females. The

significance of the seasonal variation in biochemical constituents is complex. Moisture content is a major component of fish showing an average of 62.02% throughout the year. The seasonal variation of moisture content ranged from 54.12% to 68.26%. The variation of lipid content ranged between 16.23 % in pre monsoon and 21.12% in post monsoon. The average value was about 18.5 % ± 0.615 indicating that this species is a fatty fish. The protein content showed lowest value during monsoon season and it increased to post monsoon and then decreased gradually.

3.3.2 Changes in fatty acid of *Sardinella longiceps* across seasons

Table 3.2 Month-wise variation of the major fatty acids in *Sardinella longiceps* expressed as % of total fatty acids.

Month	Palmitic acid	Linoleic acid	EPA	DHA	Teradecanoic acid	Oleic acid
January	23.22±0.063	19.37±0.045	14.72±0.024	4.06±0.034	0.86±0.054	1.14±0.054
February	21.21±0.034	17.14±0.065	14.26±0.037	3.2±0.039	0.81±0.065	1.06±0.074
March	19.15±0.070	16.24±0.087	10.21±0.021	2.41±0.085	0.75±0.084	1.02±0.028
April	25.1±0.054	14.28±0.089	8.14±0.045	1.21±0.028	0.97±0.023	1.57±0.084
May	27.21±0.034	10.12±0.034	7.1±0.056	1.01±0.043	1.23±0.043	2.03±0.087
June	27.41±0.089	8.21±0.043	4.2±0.079	0.92±0.079	1.28±0.054	2.57±0.075
July	29.86±0.076	7.47±0.056	4.13±0.065	0.87±0.042	1.68±0.065	2.74±0.077
August	32.12±0.032	10.21±0.087	7.12±0.097	1.21±0.065	1.96±0.075	2.64±0.082
September	34.43±0.045	12.22±0.079	8.89±0.059	2.79±0.054	2.01±0.029	2.72±0.053
October	29.21±0.075	17.23±0.065	11.63±0.089	4.3±0.086	1.21±0.089	2.05±0.064
November	26.21±0.056	28.39±0.048	15.46±0.095	5.07±0.095	1.03±0.027	1.85±0.075
December	24.1±0.059	24.23±0.086	15.23±0.032	4.74±0.074	0.97±0.083	1.23±0.072

Values are expressed in mean ± standard deviation, n=3

Statistical analysis shows that there is no significant difference between months ($P>.05$) but there is significant difference between fatty acids ($P<.001$). Palmitic acid gives significantly higher value compared to all others, but between tetradecanoeic acid and oleic acid the difference is not significant (Annexure 3.2.).Trends in fatty acids across season are different at

various proportions of saturation and unsaturation. The occurrence of saturated fatty acids such as Palmitic acids and tetradecaenoic acid along with monounsaturated fatty acid showed a low value in the month of March reaching a maximum in September (Table 3.2). A low value of 19.15% and a maximum of 34.43% for palmitic acid were observed in the month of March and September respectively. For tetradecaenoic acid, a low value of 0.75% in March and a maximum value of 2.01% in the month of September were observed. The same trend was observed for the mono unsaturated fatty acid (Oleic acid) where there is a low value of 1.02% in March and a maximum of 2.72% in September. But the trend is different for polyunsaturated fatty acids such as EPA and DHA (Omega 3) and for the omega 6 fatty acid (Linoleic acid). The maximum value for these fatty acids are observed in the month of November which decreased slowly reaching a minimum value during the months of June-July and then increased. For DHA and Linoleic acid the lowest value obtained is in the month of July, whereas for EPA it is in June, the respective values being 0.87%, 4.2% and 7.4% for DHA, EPA and Linoleic acid, respectively. The maximum value obtained in the month of November for Linoleic acid, EPA and DHA were 28.39%, 15.46% and 5.07%, respectively.

3.3.3 Extraction methods of lipid recovery in *Sardinella longiceps*

Table 3.3 Extraction methods and lipid recovery from *Sardinella longiceps.*

Extraction methods used for lipid recovery in *Sardinella longiceps*	Lipid recovery (%)
Folch Method	4.91±0.25 %
Bligh & Dyer method	9.69±1.03%
Soxhlet Method	20.2±0.58%

Values are expressed in mean ± standard deviation, n=3

These analytical methods are usually employed for lipid extraction from tissue samples. It was meant for choosing a procedure for lipid extraction for further analysis of lipid fractions retaining the integral native quality of the fatty acids. Here, Soxhlet method had highest lipid recovery about 20.2±0.58%, followed by Bligh & Dyer and Folch methods. A lipid recovery percentage of 9.69±1.03% and 4.91±0.25 % were obtained for Bligh & Dyer and Folch methods respectively. But the lipid fractions extracted by soxhlet extraction method were found not to maintain the native structure and quality and hence not recommended for further analysis.

3.3.4 Analysis of lipids in frozen- stored Sardines

Table 3.4 Variation of different fatty acids in Sardine during frozen storage (expressed as % of total fatty acid).

Days of storage	Lipid	SFA	MUFA	PUFA
0	16.5±.357	14.1±.915	5.3±.828	6.8±.243
15	13.9±.832	11.2±.247	4.2±.265	4.3±.725
30	6.2±.182	4.6±.216	2.8±.921	1.9±.565
45	3.8±.673	ND	ND	ND

Values are expressed in mean ± standard deviation, n=3

The analysis performed in stored sardines at -20 ^{o}C showed that the sample had undergone deterioration by 45 days of storage itself (Fig 3.1). The values showed an increasing trend for PV and TBA while the fatty acid values showed decreasing trend with storage as confirmed from the above study (Table 3.4). The lipid content also decreased when storage time increased. The drop in lipid content in Sardine tissue during frozen storage is due to the lipolysis and oxidation of PUFA rich triglycerides. There is a potent positive correlation presented for lipid content and level of fatty acids. (Annexure 3.1.)

Analysis of storage index have a significant ($P<0.01$) negative correlation based on the values of Peroxide value and fatty acids. The values of SFA, MUFA and PUFA decreased as the day progressed and became almost negligible by 45th day (Table 3.4). The ratio of PUFA/Omega 6 gives its importance in artherosclerosis and other cardiovascular diseases. It is observed from the Fig 3.1 that as the storage days progress, even at -20 ^{o}C, the value of TBA and PV considerably increases after 30 days.

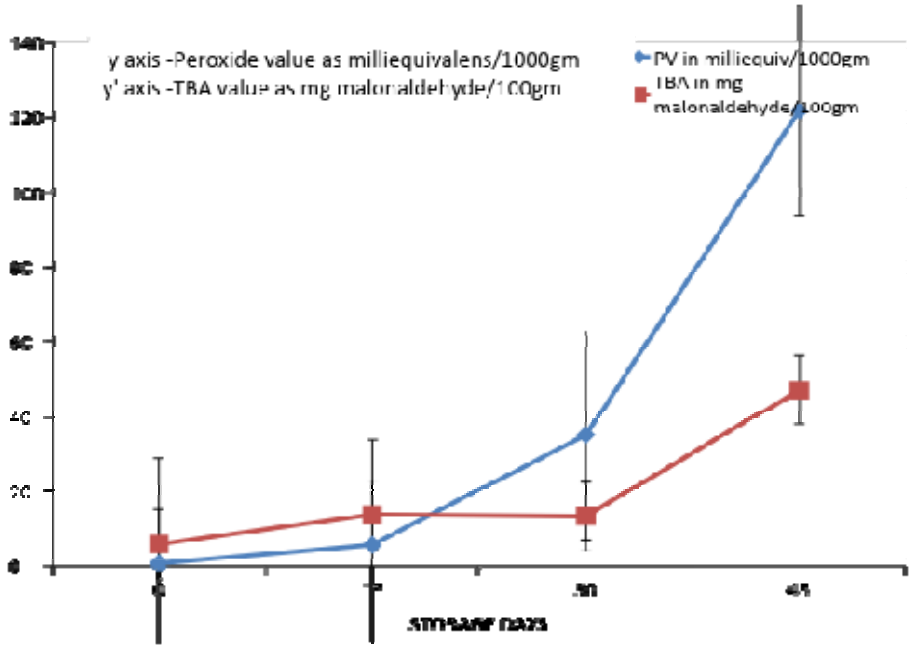


Fig 3.1 Peroxide value and TBA values of sardines stored at -20^{0}C

3.3.5 Determination of changes in fish oil during refrigerated storage

Table 3.5 Changes in Peroxide value, TBA value and FFA value of fish oil from sardines stored at refrigerated condition under Nitrogen.

Refrigerated Storage	Peroxide Value (meq/kg fat)	TBA value (mg of malonaldehyde/ml of oil)	FFA value as % oleic acid
0 (days)	3.945±0.56	0.846±0.01	0.250±0.05
15 (days)	7.836±0.65	1.38±0.03	0.403±0.05
30 (days)	22.6±0.34	1.76±0.30	0.343±0.10
45 (days)	8.493±0.56	3.086±0.06	0.658±0.08
60 (days)	4.313±0.63	3.92±0.09	0.829±0.05

Values are expressed in mean ± standard deviation, n=3

The results of study on fish oil sample stored at refrigerated condition, flushing nitrogen gas and sampled at various time intervals are shown in the Table 3.5. Peroxide oxide value showed a maximum value of 22.6 in 30 days. TBA and FFA values showed a slow rate of increase initially and a steep increase after 30 days. The results of PV showed a decrease after 30 days which shows the production of secondary oxidative compounds with decrease of primary oxidation compounds. The results of storage studies of fish oil at refrigerated condition show that fish oil is acceptable only to a maximum of 30 days. After that, there is steep increase in oxidation parameters making the fish oil unacceptable.

3.4 Discussion

The use of different methods lipid extraction resulting in different levels of lipid recovery has been demonstrated by several workers (de Boer, 1988, Randall *et al*., 1991). Here three methods of lipid extraction namely (1) Folch method (2) Bligh & Dyer method and (3) Soxhlet method were carried

out. From the results, it is confirmed that the amount of lipids varies according to extraction procedures (Baily *et al*., 1994). Since for the estimation of lipid content much of the published data for the whole fish and other samples were mainly based on Bligh & Dyer method. So also, for lipid estimation by this method was followed as a standard procedure and was of general acceptance. Table 3.2 illustrates the comparative lipid recovery for sardines by these three procedures. There is a 10% decrease in extractable lipid content in Bligh & Dyer method in comparison to the Soxhlet method. But it is accepted that the Bligh and Dyer method, is the best method for extraction of lipids (Randall *et al*., 1991, Kates, 1986) which uses chloroform/methanol, which is generally considered and commonly used for environmental samples for determining lipid contents. The soxhlet extraction procedure uses hexane/petroleum ether which is frequently employed for the analysis of lipophilic compounds and pollutants. Folch method is one of the first recognized method where the chloroform/methanol/water phase system is used and under various modifications, continues to be the classic and most consistent method for quantitative lipid extraction. While, the soxhlet extraction can be regarded as a partitioning of lipids between only two phases, the Bligh and Dyer method involves water also resulting in three phases , that of the sample and of the organic solvents. When comparing with the other two methods, soxhlet method is a gravimetric quantification method which uses solid-liquid extraction in a soxhlet apparatus.

So far the best known is the Bligh and Dyer method, which has become one of the most recommended lipid extraction procedures in biological tissues and certainly for determination of lipids in many studies of marine fish as well as for other types of samples such as milk. The primary advantage of the Bligh and Dyer method is a reduction in the solvent/sample ratio (1 part sample to 3

parts 1:2 chlorofrom/ methanol followed by chloroform (1 or 2 parts) while Folch method employs a ratio of 1 part sample to 20 parts 2:1 chloroform/methanol, followed by several washings of the crude extract with solvent. Conventionally the dual mixture of chloroform and methanol (2:1 v/v) and the use of ternary solvent systems C:M:W have been considered for the extraction and purification of lipids from biological materials (Zhu *et al*., 2002).

In the course of the studies for lipid recovery, it is found that the Bligh & Dyer method gives better recovery than Folch method, and no difference in the composition of fatty acid was observed between the two methods. Both these methodologies use chloroform and methanol as the first monophasic system for extraction of lipids followed by a biphasic system used for purification of lipids by the addition of water. This leads to the separation of polar and non-polar components separating them into upper and lower phases respectively. The lipid extraction should be performed by methods that present minimum damage to the lipid quality. Study by Brum *et al*. (2009) showed that the method of soxhlet with a single polar solvent (n-hexane) affected the quality of the lipid fraction in oat flake and chicken breast as demonstrated by the existence of peroxides and aggregation of oleic acid.

The four chief constituents present in the edible portion of fish are water, protein, lipid (fat or oil) and ash (minerals) and proximate analysis is referred quite often as the analysis of these four components of fish muscle. Biochemical composition of fish flesh vary within the same species due to changes in fishing ground, fishing season, age and sex of the individual and reproductive status. The main factors responsible for this variation are the spawning cycle of fish and its food supply (Love, 1980). The data on proximate composition is important for many investigations and applications and on these lines it had been carried out from as early as the 1880s. Stansby

(1962) observed that proximate composition is considered to be such an elementary sort of thing that it did not receive due consideration from scientists. Consequently, the knowledge of proximate composition determination of fish species has fundamental importance in the solicitation of many technological processes. The biochemical content in fishes is related to maturation of gonads and the food supply (Jacquot, 1961, Medford and Mackay, 1978).

The high content of lipid after post monsoon is due to intensity of feeding, sexual development and eventual spawning. The lowest level of lipids is observed after spawning and highest concentration occurs preceding the maturation. Thus fish utilize fat as a main source of energy sparing protein for body building. The protein content show minimum in pre monsoon then it increases rapidly to maximum in monsoon season and the highest value is reported during post-monsoon. This is supported by the findings of Phillips (1969). They found that carbohydrates were utilized for energy by trout, hence spared protein for other purposes of the body. Protein is not an efficient energy source for fish. They will be used for energy when the available energy from other sources (lipid and carbohydrates) is inadequate. The protein content of fish varies in relation to species, and also in relation to time of year in individuals of same species (Mackie *et al*., 1971) and protein content goes on increasing with the advancement in maturity. Similar observation in *Mugil cephalus* was reported by Das (1978). He suggested that high values of protein coincided with the spawning season when the gonads were ripe and it declines with post spawning period. The protein and visceral lipid resources are utilized in the pre-spawning period of *Etroplus suratensis* as noticed by Rattan (1994). Therefore, the sequence of mobilization of endogenous energy source may be the possible reason of high level of lipid content in mature spawners

(Dhulkhed, 1964).The highest protein content in pre-spawning stage may be due to its frequent supply by the liver as proposed by Van Bohemen and Lambert (1980). Medford and Mackay (1978) showed that muscle protein and lipid content of northern pike, *Esoxlucius* were high before spawning and low after spawning. Sivakami *et al. (*1986) found that protein for germ building was mobilized from the muscle of *Cyprinus carpio* especially in stages I to IV of maturity. Changes in the protein content during spawning season may occur due to changes in the endocrine system that monitors nutrients supply to gonads from all parts of body including liver and muscles (Sinha and Pal, 1990, Jyotsna *et al.*, 1995).

During January-April the percentage of lipid content showed low values which was followed by sharp increase in May - June just prior to spawning season. There shows an inverse relationship between lipid and moisture content of the fishes in the seasons studied. Fishes like other animals, store fat in their muscle for the supply of energy during starvation and reproductive phase. Intensive feeding in *Ambassis commersoni* coincides with the occurrence of high fat content in the muscle of fish (Bumb, 1992; Nair and Subrahmanyan, 1955).

Among the lower fatty acids, palmitic acid has been found to be the most abundant, recording about 34% of the total fatty acids. It has been found that the concentration of palmitic acids in lipids of many fishes remained fairly constant over the 30 to 40 per cent of total fatty acids. Among the Indian fishes the percentage of palmitic acids observed are as mackerel (*Rastrelliger kanargurta*) 34.2%, kilimeen (*Nemipteres japonicas*) 31.3%, jew fish (*Prestiper ageraca*) 37.89% and pomfret (*Pampus argenteus*) 37.3%.

The higher concentration of fatty acids in December compared to that in June and September in sardines coincides with the spawning period. A similar trend in mackerel has been accredited to fat mobilization related with gametogenesis. Also during winter, sea water temperature falls from 30-31^{o}Cto 25-26^{o}C and fishes like sardine increase their FA content, especially PUFA to survive the lower temperature. The increasing PUFA concentration in December-January allow greater consumption PUFA per sardine. For industrial production unit the yield of PUFA will be higher and more profitable during winter. A relative concentration of EPA and DHA to that of total PUFA shows variations across seasons. This complementary trend can be explained by the maturity stage prevalent in the catch, and dietary patterns as confirmed by analysis of stomach content. June-September is characterized by the presence of spawning adults of *S. longiceps*. They are exclusive phytoplankton feeders and hence have an EPA-rich diet. But the immature ones found abundantly in winter are carnivorous with varying amounts of zooplankton entering their diet. Owing to their well-formed gill rakes, adult of *S. longiceps* are capable of efficiently sieving minute phytoplankton, on the other hand the under developed gill rakes of the immature ones permit large zooplanktons. This causes increase in the DHA content in *S. longiceps* caught during December. Thus the PUFA profile of sardines are well-suited for food and pharmaceutical industries generating PUFA-enriched foods or specialized drugs, and this comprehensive knowledge will help to design processes for better yield .

Regardless of the level of anti-oxidants added to commercial fish oil supplements, peroxidation /rancidity *in vivo,* is a very significant and problematic issue. Even in the absence of exogenous oxidizing reagents,

highly unsaturated long-chain EPA is readily oxidized at room temperature; To prevent the constant intervention of heat stress, the fish commodities are kept in freezers. Oxidation of PUFA causes generation of a mixture of aldehydes, per-oxides, and also other harmful products. Importantly, *in vivo*, an increase in tissue and the plasma accumulation of fatty acid oxidation products is noted in subjects consuming fish oil even after additional anti-oxidant supplementation to the diet.

The results revealed lipid peroxidation in stored fish and fish oil as the value of TBA and PV gradually increased during the storage period. Even when they are stored at -30 ^{o}C, the results suggest that there is progressive formation of harmful hydro peroxides. These products esterified to oxidized cholesterol are found to cause inflammation leading to coronary vascular diseases. The 2011 Prostate Cancer Prevention Trial demonstrated that the high concentration of serum phospholipid of long-chain metabolites, especially of the ω-3 series fatty acids was related to a large increase in the risk of high-grade prostate cancer (Brasky *et al*., 2011). Recommendations to increase LC ω-3 PUFA intake should consider its potential risks. The index of lipid peroxidation can have a link to the human pathological disorder.

Fish oil supplements, in their "normal" even though supra-physiologic amounts, cause changes in membrane properties that impair oxygen transmission into the cells. These are frequently the result of incorporation of adulterated, non-oxygenating, or unsuitable PUFAs into the phospholipids of cell and mitochondrial membranes. Partially oxidized PUFA entities, known as trans-fat and inappropriate omega 6/omega 3 ratios (caused by marine oil supplementation), are all possible sources of unsaturated fatty acids which can

interrupt the normal membrane structure, significantly increasing the potential for cancer (King *et al.,* 2005). Parent omega 6 series support both anti-cancer membrane functionality and also cellular oxygenation. There is growing evidence supporting the chronic swelling with prostate carcinogenesis and hence, the associations of trans-fatty acids with increased inflammatory response could explain their role in the prostate cancer risk (Peskin and Carter, 2008).

Evidence is presented that the fish oil causes higher levels of both harmful malonaldehyde (MDA) and Thiobarbituric Acid Reactive Substances (TBARS) which form extremely harmful secondary and terminal stage oxidative products. Peroxide formation is likely to occur from susceptible polyunsaturated fatty acids available in the oil (Esterbauer *et al*., 1982). The evolution process of peroxide value follows an upward slope.

In the first 20 days of refrigerated storage of fish oil under nitrogen, there has been a slow increase of the peroxide, which corresponds to initial stage of the oxidation, followed by surge propagation phase in which it forms the largest amount of hydro peroxides as well as primary compounds of oxidation, reaching a highest value. In contrast to PV, TBA formation depends on oxidation accompanied by several decomposition steps (Dahle *et al*., 1962). The TBA value shows a gradual increase which imparts the formation of secondary products (Cuppet *et al*., 1989). FFA formation shows a very little upward curve, with reduced formation of FFA, during the study which may be due to the preservation conditions. Peroxide value of several fish oils remain in acceptable limit of 8 meq/kg upto 60 or 90 days and at least for 150 days at -180 ^{o}C. This fact showed that the storage temperature has important effects on the storage stability of the fish oil. It is a well-known fact that the higher the degree of unsaturation, the

higher the rate of autoxidation of oils (Stansby, 1962). Although the peroxide value is a common measurement of lipid oxidation, its use is limited to the initial stages of oxidation since peroxide are vulnerable to further decomposition, the complete oxidative history of the oil may not be revealed.

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Chapter 4

CONCENTRATION OF PUFA IN MARINE ALGAE AND FISH OIL USING MICROBIAL LIPASE

Contents

4.1 Introduction

Polyunsaturated fatty acids (PUFA) have a fundamental role in human metabolism due to their antibacterial, anti-inflammatory and antioxidant properties; and play a key role in the prevention of cardiac diseases and in the inhibition of tumor progression. Marine fish oil is the richest source for Eicosapentaenoic acid (EPA, 20:5n3) and Docosahexaenoic acid (DHA, 22:6n3) (Shahidi and Wanasundara, 1998); however, it is highly susceptible to oxidation, with subsequent production of off-flavours and toxic compounds that vary with geographical and seasonal changes. PUFA act as precursors and are involved in the synthesis of prostaglandins, leukotrienes, thromboxane and resolvins, which bind to specific protein receptors and modulate cell signaling pathways involved in inflammation, vasodilation, blood pressure, pain and fever. These play an important role in the prevention of cardiovascular diseases, type II diabetes, ocular diseases, arthritis and cystic fibrosis (Simopolous, 1997, Yagi *et al*., 2017). DHA is a key responsible factor involved in the development of cognitive functions and neurological developments. . The infants fed with breast milk (that naturally contains DHA)

performed better on tests than infants fed on formula milk with no DHA (Arteburn *et al*., 2007, Crawford, 2000). EPA were also reported to perform several vital functions including modulation of biological membranes. It also serves as a precursor for a variety of lipid regulators in cellular metabolism. These findings have led to considerable interest from various sectors for commercial PUFA production (Belarbi *et al*., 2000).

Generally, fish oils from salmon, mackerel and herring contain EPA and DHA, (Strobel *et al*., 2012). In order to protect the depleting fishery resources, it is urgent to identify an alternative source for PUFA. Currently, several sources have been explored, of which plant oils with high omega-3 content, stearidonic acid and algae oils, are dominating in usage and availability. Therefore, *n*-3 PUFA are typically associated with marine organisms, and algae, as the basis of the marine tropic chain that poses as a very promising source of PUFA. Large scale farming of marine algae has been accomplished successfully for hundreds of years. It was reported that approximately 220 algal species of marine and fresh water origin are currently cultivated and harvested all over the world for different purposes (Zemke-White and Ohno, 1999). It is mostly used as food for human consumption, particularly in Asia and other Eastern countries. Human consumption of algal foods varies by nation, with Japanese diets representing a recent (2010–2014) annual per capita consumption ranging from 9.6 (2010) to 11.0 (2014)g macroalgae day^{-1} (MHLW, 2014). Overall, the trend towards increasing nutritional demand for algal products on a global basis stems from a greater focus on health and wider use of food additives. Macroalgae also form the primary source of hydrocolloids such as agar, carrageenan and alginate, which have numerous industrial applications such as gelling agent, stabilizer, thickener or binding agents (McHugh, 2003). In addition to their nutritional

value, algae increasingly are being marketed as "functional foods" or "nutraceuticals"; these terms have no legal status in many nations but describe foods that contain bioactive compounds, or phytochemicals, that may benefit health beyond the role of basic nutrition (Bagchi, 2006; Hafting *et al*., 2012). The path from algal research to the launching of new food products or dietary supplements is strongly affected by industrial, regulatory, and nutritional considerations (Borowitzka, 2013; Finley *et al*. 2014). The next step could well be the sustainable exploitation of marine macroalgae as an alternative source of PUFA, not only in Asia, but also in the western world.

Furthermore, additional experimentation to ensure optimal growth conditions for enhancing lipid biosynthesis, ideal species selection, quality control and sufficient methods for maximizing ingestion and digestibility will aid the potential of algae oil as a major source of omega-3 fatty acids in our diet. Moreover algae-derived oils are vegetarian-friendly and easy to grow on a large scale due to their small size. Marine algae rich in n-3 PUFA being natural and readily available resource could be an alternative to fish oil derived n-3 PUFA; therefore, it could be of immense potentiality in nutraceutical and pharmaceutical industry. Lipids and protein produced during algal growth may also be used as biodiesel, biomass for oil sources, and also as animal feed (Adarme-Vega *et al*., 2014, Subhadra and Grinson-George, 2011). This highlights the sustainable benefits of algae and the many potential gains in creating algal bio-factories. Cost, extraction and purification methods are currently limiting the potential of using micro algal oils on a larger-scale.

In recent years, the use of lipase (EC *3.1.1.3)* as biocatalysts had drawn considerable attention. Lipase is an enzyme that hydrolyzes lipids, the ester bonds in triglycerides, to form fatty acids and glycerol. Currently they account for *25%* of total enzymes used in biotechnology, and this is because of the

great versatility of the enzyme in catalyzing reactions of hydrolysis (Benzonana and Esposito, 1971) and synthesis, transesterfication, (Nakamura, 1994) and interesterification. Among the lipases assayed, the enzyme from the yeast *Candida cylindracea* is of special interest as these are proved to be a nonspecific catalyst for many commercially interesting reactions such as the modification of oils and fats, reactions in organic solvents, and resolution of racemic mixtures (Mustranta, 1992). Hence the enrichment of microalgae using biolipase from the source *C. cylinderacea* is a novel attempt for establishing microalgal enriched PUFA. This will be useful for both pharmaceutical and food industries for developing PUFA enriched nutritional products.

In the present work the algal source of PUFA, *Isochrysis galbana, Chaetocerous calcitrans, Chlorella marina* and *Tetraselmis gracillus*, were subjected to enzymatic enrichment using microbial lipase. In the current chapter, an attempt is made to develop a commercially feasible technology for enzymatic (lipase) enrichment of EPA and DHA.

4.2 Materials and Methods

4.2.1 Marine algae

Algae cultures were obtained from Department of Marine Biology, Cochin University of Science and Technology. The species included *Isochrysis galbana*, *Chaetocerous calcitrans, Chlorella marina and Tetraselmis gracilus.* They were grown in separate Hoffkins flask in a room adjusted with (12:12) light/dark and 22 ^{o}C. Tubing light source of 200 lux was used. The flask was thoroughly soap washed and 7 times acid washed (0.01N HCl) for removing any contaminants. The medium for culture choice was nutrient mixture of Guillard and Ryther containing sodium nitrate, sodium

dihydrogen phosphate, disodium silicate, trace metals and vitamins (Guillard and Ryther, 1963) further updated in April 2007.

4.2.2 Lipase source

Culture of *C. cylinderacea* (MTCC No. 1908) was obtained from Institute of Microbial Technology (IMTECH), Chandigarh. It was cultured in a medium containing Yeast extract, Peptone, Dextrose, distilled water and Agar and was sub cultured to maintain the culture. A broth was prepared along with it, without any addition of agar for lipase extraction. All the cultures were kept in an orbital shaker (REMI CIS 24 plus) at 37 ^{o}C and at a speed of 300 rpm.

4.2.3 Extraction of Lipids from Algae

To 0.2 g wet-mass of algae, equivalent to 5-10 g dry-mass of sample, added 15 volumes of chloroform: methanol in the ratio 2:1 and mixed vigorously in a stoppered conical flask. An antioxidant, Butyl Hydroxy Toluene (BHT) with a resultant concentration of 0.002 mg is added along with 6 ml of water, shaken well and refrigerated overnight. The extract was passed through a muslin cloth into a separating funnel. Allowed layer separation, transferred chloroform layer (lower) into a fresh flask with anhydrous Na_2SO_4 and then passed through Na_2SO4 into an RB flask (pre weighed). Concentrated the lipid in a rotary vacuum evaporator maintained at 45 °C and determined the lipid gravimetrically and stored at 4 °C sealed with stopper under nitrogen atmosphere for further analysis.

4.2.4 Preparation of Fatty acid Methyl Esters (FAME) from Algal lipids (AOAC 2005)

To the above lipid extract of algae, 10 ml methanolic KOH (0.5N) was added and refluxed for about 10-20 min at 30-40 °C under nitrogen in a heating

mantle with a water condenser. 5 ml BF3-methanol mixture was added through the top of the condenser and continued refluxing for another 5-10 min. To the saponified sample added petroleum ether twice the sample volume and transferred the solution to a separating funnel. Shaken well and allowed layer separation. Discarded the lower layer and save the upper petroleum ether and washed twice with double distilled water. Final petroleum ether layer was collected in an RB flask after passing through anhydrous Na_2SO4. Evaporated the organic layer under vacuum and the concentrated sample was transferred into a stoppered test tube, passed nitrogen gas, sealed and stored at -20 °C for GC analysis.

4.2.5 Extraction of Fish Oil from Sardines

Freshly caught, cleaned sample of sardines were extracted for oil following the industrial method of oil extraction (FAO, 1986). The fish muscle was boiled in a steam jacketed vessel with a minimum level of water for 15 min, and withdrew the cooked sample from the vessel to a muslin cloth and screw pressed. Collected oil was then washed with hot water to remove water soluble compounds. This was then poured into a separating funnel and kept undisturbed under nitrogen atmosphere by passing nitrogen. Lower portion containing the water suspension was discarded and the oil separated was repeatedly washed with hot water. This was treated with adequate amount of anhydrous sodium sulphate and centrifuged for 10 min at 5000 rpm. Clear oil was collected from the top layer.

4.2.6 Production of Lipase from *Candida cylinderacea* (CC) (Zarev´Ucka *et al*., 2005)

For the activation of the lipase activity from the fungus, CC, a medium containing peptone (50 g), glucose (10 g), $MgSO_4 \cdot 7H_2O$ (1 g), $NaNO_3$ (1 g) and olive oil (10 g) in 1 litre was used. Medium (90 ml) was inoculated with

10 ml of prepared inoculum. After cultivation the broth was vaccum filtered. To the filterate added 70% ammonim sulphate and shaken in a cyclo mixer. It was then centrifuged at 5000 rpm for 6 min. Decanted off the upper layer and the sediments containing lipase was dissolved in 10 ml Tris buffer and refrigerated at -4 ^{o}C for further use.

4.2.7 Enzyme activity assay (Kwon and Rhee, 1986) of lipase from *Candida cylinderacea*

For the assay of lipase enzyme activity from CC, to 100 µl of a reaction mixture containing 3 mM p-nitrophenylpalmitate dissolved in 2-propanol, 1 ml of 50 mM Tris–HCl (pH 8.0) and 50 µl of crude lipase extract were added and incubated at 37 ^{o}C in a thermostat controlled waterbath. The release of yellow p-nitrophenol due to hydrolysis of p-nitrophenyl palmitate by lipase at different time intervals was monitored spectrophotometrically at 410 nm. Since substrates on auto hydrolysis produced low but significant background values at 410 nm, the absorbance in each assay against a control with substrate–buffer alone was measured. The activity of enzyme was expressed as the number of µmols of *p*-nitrophenol released per minute per ml and is defined as lipase unit (U).

4.2.8 Enrichment Process of PUFA in Algae and Fish oil

To the wet mass of algae added 0.5,1 and 1.5 ml of lipase extract (4.2.6) and incubated at 37 ^{o}C for 4, 8, 16 and 24 hrs. The hydrolysis reaction was stopped by addition of 1:1 chloroform/methanol and they were extracted for total lipids and FAME preparation. The vials were stored under nitrogen for Gas Chromatography (GC) analysis. A 5 µl sample was injected into a Varian gas chromatograph 3800 (Varian Instrument Group, Walnut Creek, CA, USA), which was equipped with a flame ionisation detector. The spilt

ratio was 1:10. This instrument used a septum-equipped programmable injector. The column was a CP-Wax 52 CB fused/ silica capillary column (30 X 0.32 X 0.45; Supelco, Bellefonte, PA, USA). The detector temperature was kept constant at 250 ^{o}C, while the injector and column were programmed with respect to the temperature. The injector temperature was first maintained at 160 ^{o}C for 1 min and was then increased at a rate of 1 ^{o}C/min to 200 ^{o}C, at which it was maintained for 80 min. These data were compared with the standard (FAME, C4:C24,Bellefonte,PA,USA) to determine the absolute amount in millimoles of the fatty acids in the sample. The GC data provided are based on triplicate measurements.

The same methodology for enrichment of PUFA was employed for fish oil with little modification (Carvalho *et al*., 2002). 500 mg sardine oil was treated with 150 U lipase and 3.5 ml of potassium phosphate buffer (0.08 M, pH 7.0) placed in a 250 ml Erlenmeyer flask. Air in the flask was replaced by nitrogen and the suspension formed was agitated in an orbital shaker at 200 rpm at 37 ^{o}C for 4, 8, 16 and 24 hrs. The reaction was terminated by the addition of 2ml of chloroform/methanol mixture (1:1). FAME preparation and GC analysis followed the same procedure as in algal sample.

4.2.9 Statistical Analysis

All of the experiments were done in triplicate. The data were recorded as means ± standard deviations and were analysed with SPSS (version 11.0 for Windows, SPSS Inc., Chicago, IL, USA), and the statistical significance was determined at $P < 0.05$.

4.3 Results

4.3.1 Lipid content in cultured Algae

Table 4.1 Total lipid content of *Isochrysis galbana*, *Chaetocerous calcitrans*, *Tetraselmis gracillus*, *Chlorella marina* in mg/gm (on wet weight basis) for successive culturing days.

Culturing days	*Isochrysis galbana*	*Chaetocerous calcitrans*	*Tetraselmis gracillus*	*Chlorella marina*
10	0.5±0.007	0.25±0.026	1.05±0.049	2.69±0.084
20	4.25±0.063	3.25±0.049	2.85±0.007	1.05±0.049
30	2.3±0.042	2.7±0.021	3.14±0.063	0.97±0.070
40	1.05±0.05	1.35±0.021	2.34±0.014	0.46±0.028

All values are expressed as mean±standard deviation,n=3b

Algae were cultured for a period of 40 days for obtaining a desirable yield of lipid for the enrichment study. *I. galbana* and *C. calcitrans* showed better yield of lipid in a period of 20 days and the observed values for *Isochrysis* spp. and *Chaetocerous spp.* were 4.25 and 3.25mg/gm of the culture respectively. *C. marina* showed good yield within a period of 10 days and *T. gracillus* after 30 days. The value of lipid for *Tetraselmis* spp. was 3.14 in a culture period of 30 days and for *Chlorella* spp., the value was 2.69 at 10 days of culture. After showing higher values for lipid, it was further reducing with culture days for all algal species

4.3.2 Lipase Activity of *Candida cylinderacea*

Extracellular lipase activity of Candida spp. in the suspension cultures with progressive time of incubation indicated a linear curve (R^2=0.935). From Fig 4.1., lipase enzyme activity was found to be 0.24 U (micromoles of paranitro phenol released per ml per min). The extracellular lipase present in Candida suspension culture showed potential linear activity with the growth of cells in lipid medium as shown by increasing incubation time. The candida

species provided by the supplier was assayed for lipase activity and was found to have a specific lipase activity of 8.0 ±0.1 U/mg protein.

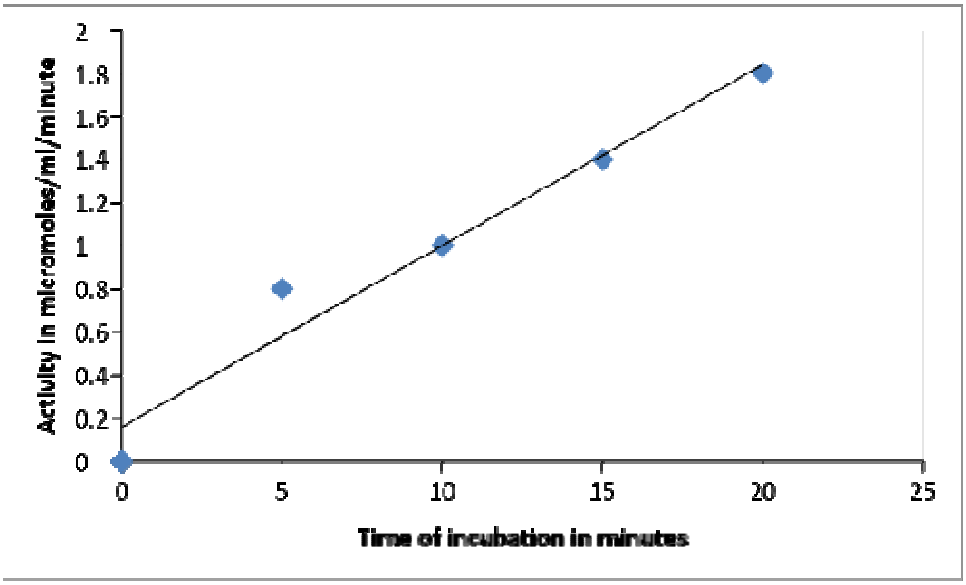


Fig 4.1. Lipase Activity of *Candida cylinderacea* at different incubation time

4.3.3 Concentration of PUFA in Fish oil

Table 4.2 Fatty Acid composition (as weight %) of untreated and lipase-treated fish oil at different time duration

Fatty Acids	Control	Time of Incubation (hr)			
		4	8	16	24
Palmitic Acid	19.3±0.072	58.81±0.486	98.71±0.386	173.21±0.386	241.24±0.386
Oleic acid	14.3±0.052	77.12±0.099	112.23±0.199	192.43±0.199	212.33±0.159
Linoleic acid	2.31±0.124	65.07±0.615	105.07±0.615	138.02±0.145	188.35±0.385
α Linoleic acid	2.07±0.023	24.88±0.924	26.78±0.724	47.35±0.624	54.39±0.512
EPA	20.4±0.031	11.05±0.231	11.25±0.261	14.15±0.581	15.35±0.581
DHA	12.2±0.054	6.71±0.064	6.93±0.154	9.62±0.144	10.34±0.234

All values are expressed as mean ± standard deviation, n=3

The percentage of hydrolysis is computed from the ratio of fatty acids released to the fatty acids in the untreated fish oil. Lipase action of *C. cylinderacea* is investigated as a function of time. It is observed that the

lipases show a significant preference to saturated fatty acids, however, the resistance to release EPA and DHA was less as the hydrolysis reaction progresses. After 24 hr the hydrolysis percentage of EPA and DHA was 15.35 and 10.34 respectively whereas the saturated fatty acids and oleic acid had a maximum value of 241.24 and 212.33 respectively (Table 4.2)

Table 4.3 Proportion of fatty acids (as percentage) in the triglyceride fraction of fish oil treated with lipase from *Candida cylinderacea*

Fatty acid	Control	Lipase treated	Enrichment
Palmitic Acid	19.3±0.072	16.2±0.354	ND*
Oleic acid	14.3±0.052	12.4±0.643	ND*
Linoleic acid	2.31±0.124	1.24±0.759	ND*
α Linoleic acid	2.07±0.023	2.94±0.823	0.87±0.36
EPA	12.20±0.072	16.0±0.023	3.8±0.051
DHA	20.47±0.052	31.47±0.031	11.0±0.021
EPA+DHA	32.67±0.124	22.5±0.054	14.8±0.072

All values are expressed in mean± standard deviation, n=3
*ND represents non-detectable or values less than that of control
Time of incubation-8hrs

EPA in fish oil treated with lipase has an increase of about 3.8% in the triglyceride fraction than that of the untreated fish oil, whereas, DHA has an increase of 11.0% in the lipase treated fish oil. The partial hydrolysis of the sardine oil by *C. cylinderacea* lipase indicates a strong discrimination by the lipase against DHA, so the DHA present in triglycerides does not get hydrolyzed, in effect get concentrated. On the other hand, this lipase has only moderate discrimination against EPA, so the concentration percentage of EPA is comparatively lower than that of DHA with a moderate enrichment. Thus, the partial hydrolysis values of the sardine oil by *C. cylinderacea* lipase indicate higher specificity of lipase towards DHA than towards EPA.

4.3.4 Enrichment of PUFA in *Isochrysis galbana* using lipase treatment

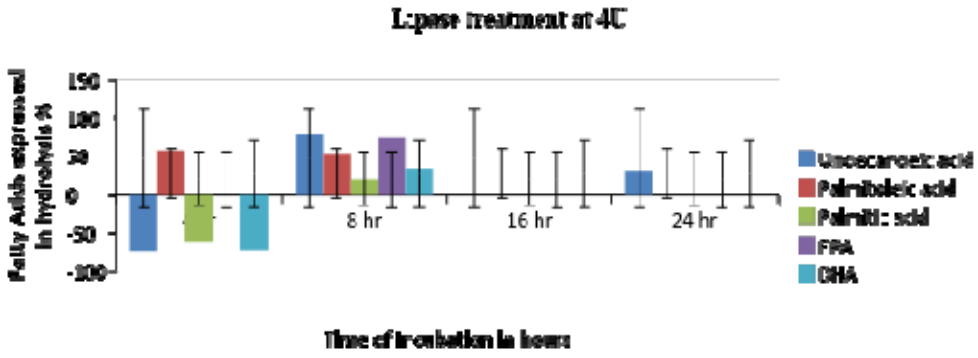
Table 4.4 Fatty Acid composition (as mg %) of untreated and lipase- treated lipids from *Isochrysis galbana* at different time durations using varying concentration of lipase from *Candida cylinderacea*.

Incubation Time in hours	Concentration of Lipase enzyme (U)	Undecanoeic acid	Myristic acid	Palmitic acid	Palmitoleic acid	EPA	DHA
	4	0.80±0.211	81.23±0.784	2.27±0.743	3.01±0.839	ND	1.78±0.634
4	8	ND	ND	ND	ND	ND	ND
	12	ND	67.63±0.854	ND	ND	ND	ND
	4	6.96±0.342	57.01±0.895	4.56±0.672	2.76±0.793	6.54±0.658	4.08±0.964
8	8	1.71±0.376	31.39±0.658	6.87±0.859	ND	ND	ND
	12	1.73±0.183	71.01±0.743	1.93±0.894	1.23±.759	1.32±0.658	1.35±0.486
	4	ND	ND	ND	ND	ND	ND
16	8	1.50±0.954	29.82±0.956	2.31±0.893	ND	ND	ND
	12	1.41±0.649	46.93±0.758	3.74±0.573	ND	1.11±0.75	2.39±0.837
	4	1.98±0.836	37.96±0.849	ND	ND	ND	ND
24	8	1.47±0.698	43.72±0.865	1.32±0.843	ND	ND	ND
	12	3.06±0.538	45.15±957	1.96±0.785	1.43±0.583	1.62±0.849	1.17±0.659
	Untreated	1.39±0.849	26.26±0.835	3.683±0.756	1.28±0.935	1.69±0.342	3.08±0.845

All values are expressed in mean± standard deviation, n=3
ND represents non-detectable or values less than that of control

In Table 4.4 higher values were obtained for myristic acid in most of the reaction conditions. The condition at which all fatty acids analysed were detected was at 8 hr of incubation with 4 U and 12 U of lipase and at 24 hours with 12 U lipase concentrations. The condition in which no fatty acids detected are at 4 hr with 8 U lipase concentration and at 16 hr with 4 U lipase. For EPA and DHA, 8 hr incubation with 4 U enzyme gave maximum values. Enzyme concentration of 12 U and 4 U also showed detectable values for these fatty acids.

a.



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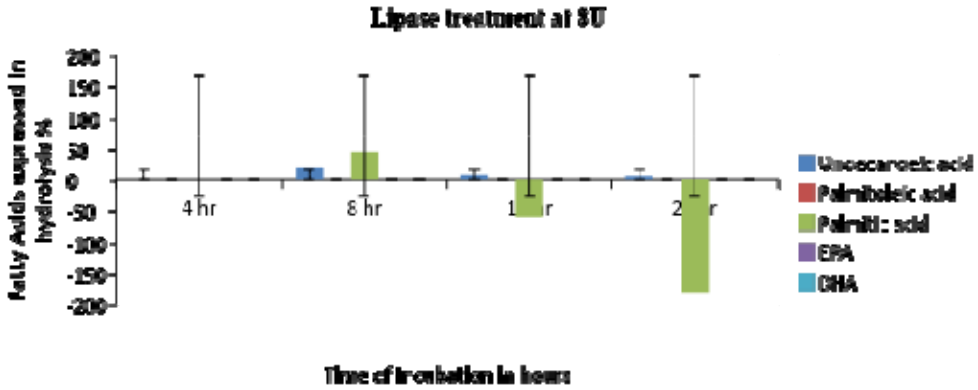

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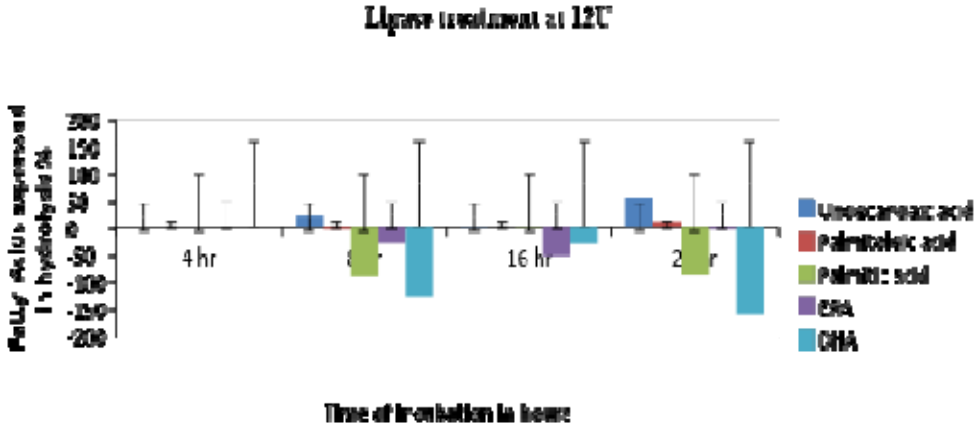


Fig. 4.2 Fatty acids content (expressed as percentage hydrolysis)on treatment with lipase at concentrations of 4 U, 8 U and 12 U against time, shown in a, b and c respectively for *Isochrysis galbana*.

The percentage of hydrolysis was computed with the ratio of fatty acids in the lipase- treated to the untreated sample. *I. galbana* when treated with lipase enzyme concentration at 4 U were found effective as compared with 8U and 12 U. The negative results shown are obtained on comparison with treated samples which have less value than that of the negative control (Fig 4.2). The zero value represents that the fatty acid is not detected because it might have got hydrolysed completely. As far as the time of incubation is concerned good results for this particular algae was obtained at 8 hr of incubation at an enzyme concentration of 4 U. The higher and good enrichment value of 74.15 and 32.46 were obtained during these conditions for EPA and DHA, respectively. There is significant difference only between columns and 8 gives significant higher value compared to the remaining (Annexure 4.1.).

4.3.5 Enrichment of PUFA in *Chaetocerous calcitrans* on treatment with lipase

Table 4.5 Fatty Acid composition (as mg %) of untreated and lipase- treated lipids from *Chaetocerous calcitrans* at different time durations using varying concentration of lipase from *Candida cylinderacea*.

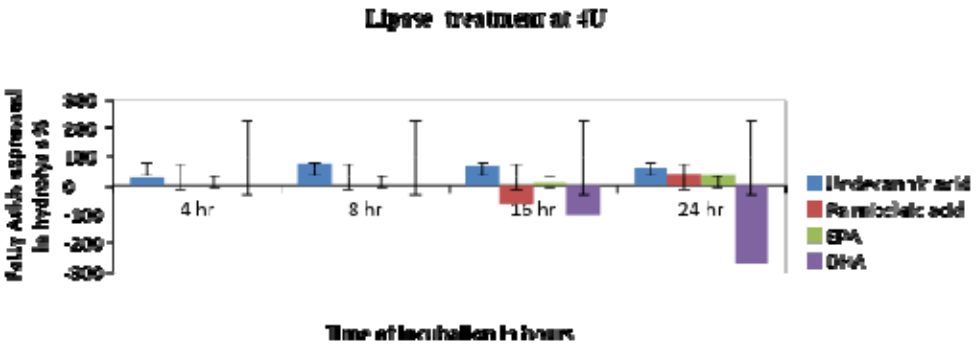
Time in Hours	Concentration of Enzyme (U)	Undecanoeic acid	Myristic acid	Palmitic acid	Palmitoleic acid	EPA	DHA
	4	4.39±0.946	236.12±0.502	ND	ND	ND	ND
4	8	ND	367.31±0.312	ND	22.97±0.239	3.51±0.674	4.72±0.138
	12	1.32±0.760	355.37±0.751	ND	9.07±0.038	3.46±0.783	1.99±0.107
	4	14.44±0.323	79.93±0.996	ND	ND	ND	ND
8	8	11.40±0.812	156.67±0.861	ND	7.45±0.044	2.45±0.524	ND
	12	3.85±0.244	97.23±0.119	ND	8.89±0.610	1.98±0.718	ND
	4	9.46±0.192	103.97±0.518	ND	6.65±0.153	2.36±0.719	0.85±0.273
16	8	10.48±0.566	115.59±0.361	0.62±0.674	8.25±0.550	2.14±0.424	2.75±0.548
	12	7.90±0.171	122.16±0.941	ND	10.76±0.524	3.17±0.133	ND
	4	8.02±0.345	110.17±0.151	ND	18.55±0.849	3.46±0.879	0.47±0.280
24	8	6.39±0.070	180.74±0.792	ND	13.46±0.673	2.00±0.329	3.31±0.222
	12	ND	74.82±0.462	ND	ND	ND	ND
	Untreated	3.30±0.044	294.56±0.571	0.40±0.497	11.13±0.908	2.14±0.717	1.76±0.132

All values are expressed in mean± standard deviation, n=3

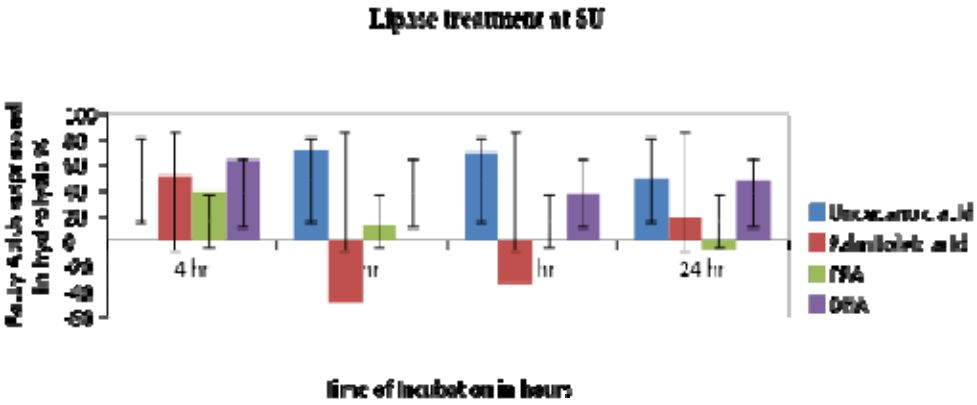
ND represents non-detectable or values less than that of control

The concentration of various fatty acids (%) in the unhydrolysed fraction and hydrolysed fraction of lipids using varying concentration of lipase from *C. cylinderacea* at different times of incubation from the algae *C. calcitrans* are presented in Table 4.5. The conditions of 16 hr incubation at 8 U lipase concentration, all the fatty acids were detected. Palmitic acid which is present in very low concentration in the untreated extract was not detected in any other conditions. Myristic acid showed high values even after hydrolysis, the minimum values being at 24 hr (12 U), 8 hr (4 U) and 8 hr (12 U). PUFA (EPA and DHA) showed maximum enrichment at 4 hr incubation with 8 U lipase.

d. 4 U



e. 8 U



f. 12 U

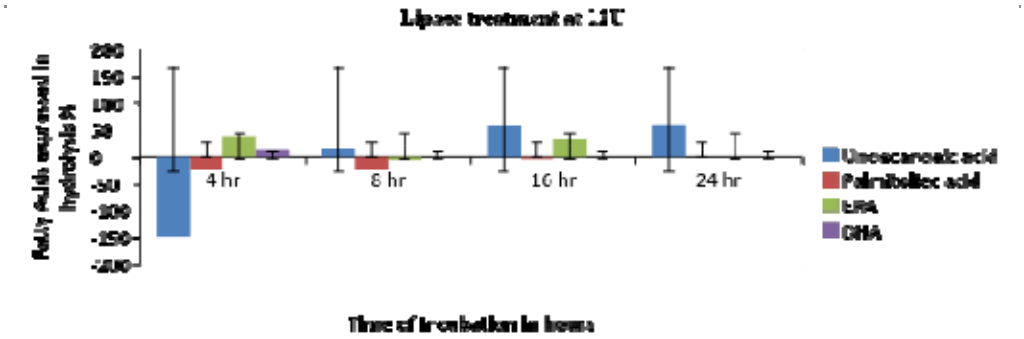


Fig: 4.3 Fatty acids content (expressed as percentage hydrolysis) on treatment with lipase at concentrations of 4 U, 8 U and 12 U against time, shown in d, e and f respectively for *Chaetocerous calcitrans*.

The percentage of hydrolysis was computed with the ratio of fatty acids to fatty acids in the original algal extract. *C. calcitrans* when treated with lipase enzyme concentration at 8 U were found effective while comparing with 4 U and 12 U. The negative results shown are obtained on comparison with treated samples which have less value than that of the negative control (Fig 4.3.). The zero value shows that the fatty acids were not detected because it might have got hydrolyzed completely. As far as the time for incubation is concerned good result for enrichment for this particular algal species was obtained at 4 hr of incubation with a lipase concentration of 8 U. The higher and good enrichment value of 51.51 and 38.94 were obtained during these conditions for DHA and EPA, respectively. ANOVA results show that between rows the difference is significant, 2 gives significant higher value compared to the remaining (Annexure 4.2.).

4.3.6 Enrichment of PUFA in *Chlorella marina* on treatment with lipase

Table 4.6 Fatty Acid composition (as mg %) of untreated and lipase- treated lipids from *Chlorella marina* at different time durations using varying concentration of lipase from *Candida cylinderacea*

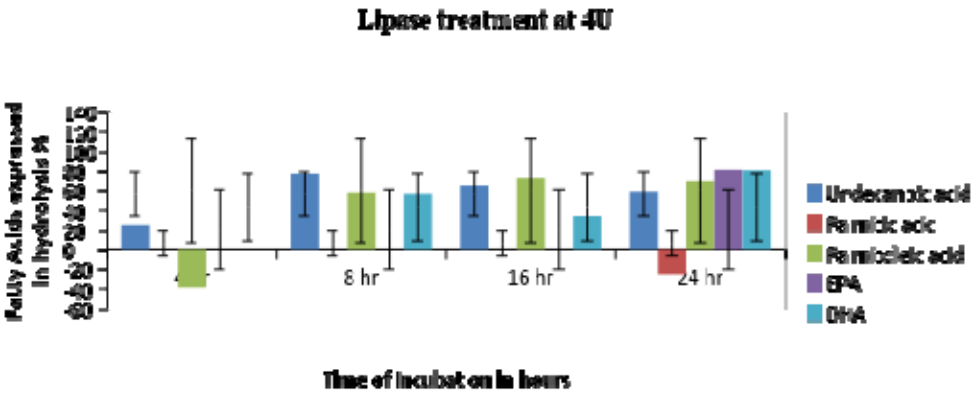
Time in hours	Concentration of Enzymes (U)	Undecanoeic acid	Myristic acid	Palmitic acid	Palmitoleic acid	EPA	DHA
	4	4.39±0.946	61.14±0.959	ND	3.85±0.979	ND	ND
4	8	ND	28.32±0.296	ND	3.47±0.917	ND	ND
	12	1.32±0.760	53.19±0.533	4.38±0.528	7.21±0.512	0.86±0.564	2.01±0.899
	4	14.44±0.324	53.45±0.991	ND	12.69±0.829	ND	2.38±0.642
8	8	11.40±0.812	84.96±0.323	ND	9.37±0.431	ND	ND
	12	3.85±0.244	232.09±0.172	ND	7.25±0.422	ND	ND
	4	9.46±0.192	197.33±0.661	ND	19.60±0.581	ND	1.56±0.697
16	8	10.48±0.567	204.17±0.182	ND	17.05±0.715	ND	3.16±0.496
	12	7.90±0.171	215.56±0.541	ND	28.54±0.852	ND	3.76±0.781
	4	8.02±0.345	100.51±0.112	4.39±0.777	17.58±0.738	2.82±0.921	5.33±0.898
24	8	6.39±0.070	167.73±0.651	4.15±0.726	11.76±0.149	ND	ND
	12	ND	43.59±0.142	ND	3.80±0.306	ND	ND
	Untreated	3.30±0.447	5.01±0.339	5.51±0.294	5.35±0.110	0.53±0.596	1.03±0.442

All values are expressed in mean± standard deviation, n=3

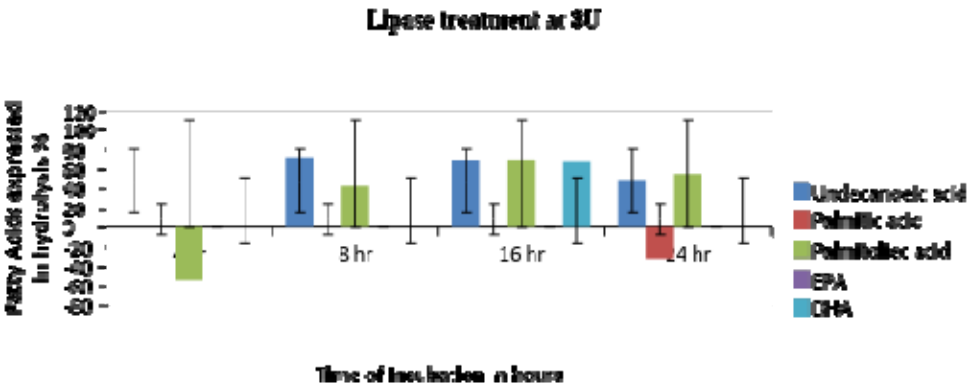
ND represents non-detectable or values less than that of control

In *C. marina* on hydrolysis using lipase , the residual lipids were found to have good content of myristic acid and palmitoleic acid in all the experimental conditions, whereas palmitic acid, EPA and DHA showed lesser content (Table 4.2). Undecanoeic acid was not detected at two conditions (4 hr at 8 U and 24 hr at 12 U). 4 hr of hyhrolysis with 12 U enzyme and 24 hr of hydrolysis with 4 U enzyme expressed maximum concentration of EPA and DHA in the algal lipids.

g. 4 U



h. 8 U



i.12 U

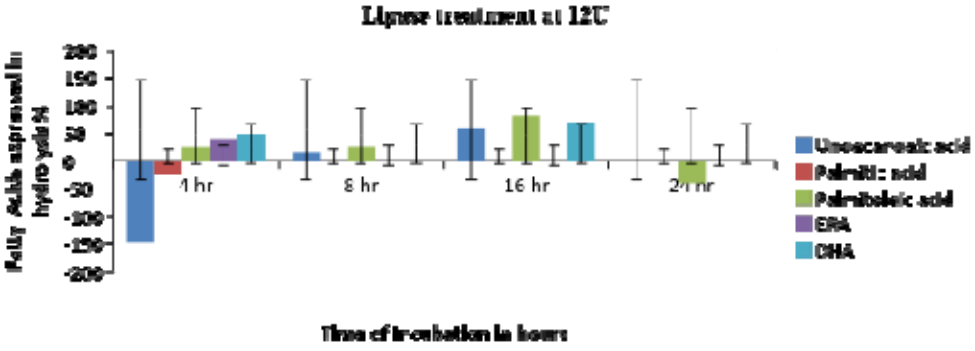


Fig: 4.4. Fatty acids content (expressed as percentage hydrolysis) on treatment with lipase at concentrations of 4 U, 8 U and 12 U against time, shown in g, h and i respectively for *Chlorella marina*.

C. marina when treated with lipase enzyme concentration at 4 U, were found effective to concentrate the PUFA content when compared to 8 U and 12 U (Fig 4.4). The percentage of hydrolysis was computed with the ratio of difference between the fatty acids in the treated and untreated fatty acids to the untreated control (as %). The negative results shown are obtained on comparison with treated samples which have lesser value than that of the control. The zero value represents that the fatty acid not detected because it might have got hydrolyzed completely. The optimum time of incubation for this particular algal species was found to be 24 hr with lipase concentration at 4 U. The maximum enrichment values of 81.05 and 80.59 were obtained at these conditions for EPA and DHA, respectively. There is statistically significant difference only between rows. Column 1 gives significantly higher value when compared to all others (Annexure 4.3.).

4.3.7 Enrichment of PUFA in *Tetraselmis gracilus* on treatment with lipase

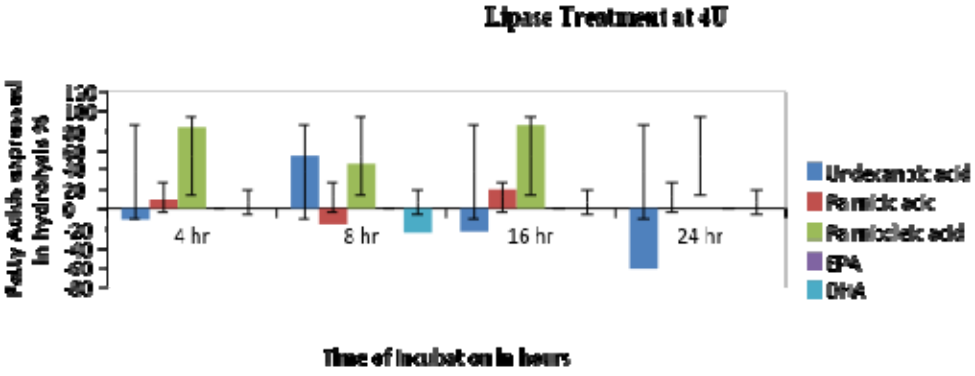
Table 4.7 Fatty Acid composition (as mg %) of untreated and lipase- treated lipids from *Tetraselmis gracilus* at different time durations using varying concentration of lipase from *Candida cylinderacea*

Time in Hours	Concentration of Enzyme (U)	Undecanoeic acid	Myristic acid	Palmitic acid	Palmitoleic acid	EPA	DHA
	4	11.80±0.369	163.35±0.141	3.21±0.128	15.31±0.878	ND	ND
4	8	20.78±0.442	359.05±0.331	1.23±0.299	5.74±0.369	ND	ND
	12	11.66±0.457	404.61±0.912	ND	2.04±0.636	1.82±0.428	4.85±0.858
	4	29.04±0.719	116.90±0.236	2.52±0.587	4.62±0.575	ND	3.55±0.837
8	8	6.14±0.844	182.96±0.421	0.59±0.429	15.60±0.365	ND	ND
	12	11.39±0.155	297.54±0.691	ND	1.87±0.169	ND	ND
	4	10.65±0.186	268.37±0.131	3.61±0.775	17.55±0.993	ND	ND
16	8	3.09±0.239	347.14±0.961	ND	14.99±0.881	ND	ND
	12	11.61±0.853	339.74±0.121	ND	16.50±0.934	1.23±0.424	ND
	4	8.18±0.135	13.33±0.132	ND	ND	ND	ND
24	8	0.90±0.328	19.76±0.327	ND	ND	ND	ND
	12	ND	32.94±0.228	ND	ND	ND	ND
	Untreated	13.13±0.704	66.53±0.249	2.93±0.144	2.55±0.343	1.79±0.571	4.42±0.754

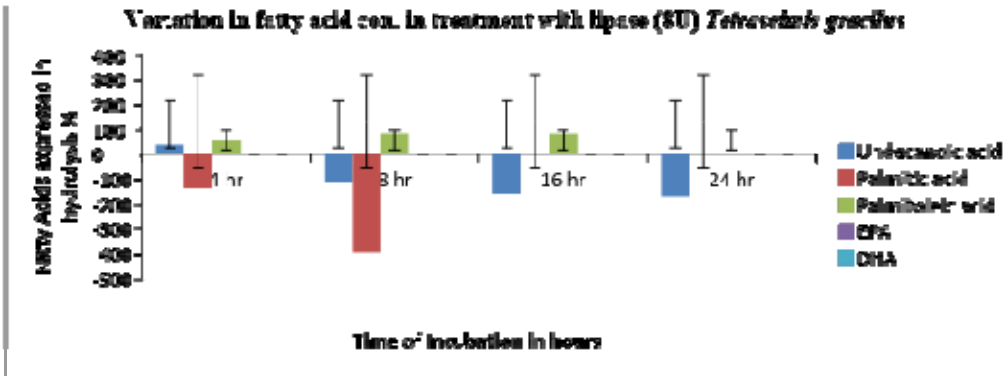
All values are expressed in mean± standard deviation, n=3
ND represents non-detectable or values less than that of control

The concentration of various fatty acids in the unhydrolysed fraction of lipids extracted from the algae *Tetraselmis gracilus* using varying concentration of lipase from *C. cylinderacea* at different times of incubation are presented in Table 4.7. All fatty acids except palmitic acid were detected after the hydrolysisof 4 hr at 12 U enzyme concentration. Myristic acid showed high values even after hydrolysis. PUFA (EPA and DHA) showed maximum enrichment at 4 hr incubation with 12 U lipase.

j. 4U



k. 8U



l. 12U

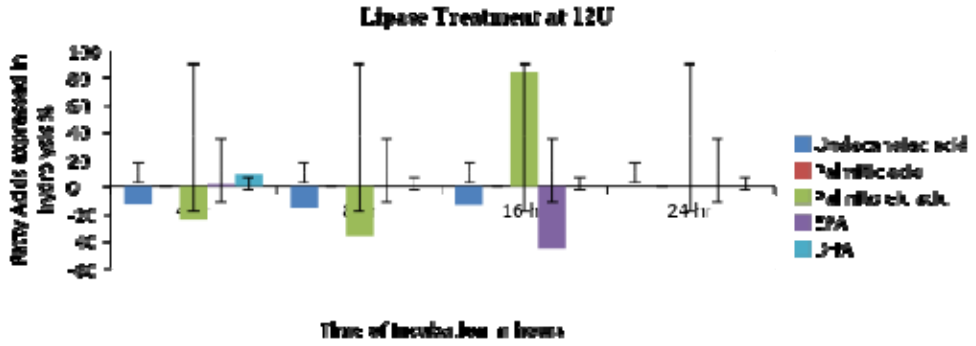


Fig. 4.5 Fatty acid content (expressed as percentage hydrolysis) on treatment with lipases at concentration of 4 U, 8 U and 12 U against time shown in j, k and l respectively of *Tetraselmis gracillus*.

The percentage of enrichment was computed with the ratio of difference between the fatty acids in the treated and untreated fatty acids to the untreated control (as %). *T. gracillus* when treated with lipase enzyme concentration at 12 U were found effective compared to 4 U and 8 U (Fig.4.5). The negative results shown are obtained on comparison with treated samples which have less value than that of the control. The zero value is got when the fatty acids are not detected because it may have got hydrolysed completely. For this particular algae 4 hr of incubation at lipase concentration of 12 U was the optimum condition. The higher and good enrichment value of 1.35 and 8.87 were obtained during these conditions for EPA and DHA, respectively. There is significant difference between rows and between column ($P<.05$). Among rows the value corresponds to 11.8 which is significantly higher than the rest and among columns 16 gives significantly higher value (Annexure 4.4.).

4.4 Discussion

The lipid content in microalgae varies as the culturing period progresses. The biochemical composition of micro algae is also influenced by their environmental conditions, growth rates and the life cycle (Richmond, 1986). The important factors used to evaluate the nutritional value of a species are growth rates, in terms of cell numbers or biomass and biochemical composition, which should be optimized in terms of vital nutrients. The documented results confirmed with other reported data that the lipid content, lipid class compositions and fatty acids of microalgae change during their growth (Alonso *et al*., 2000, Miller *et al*., 2012). Furthermore, by altering growth conditions such as light, temperature, carbon/nitrogen source and salinity, the lipid composition can be changed (Fidalgo *et al*., 1998, Guedes *et al*., 2010). This suggests that genes encoding for the lipid-modifying enzymes namely lipases, phospholipases, acyl transferases and various lipid synthases are expressed variably throughout their growth. The analysis of lipid profiles allows insight into the enzymatic control of lipid metabolism.

The results showed a difference in lipid content with growth pattern for each cultured algal species. The seawater used for the preparation of culture medium may contain wide-ranging amounts of non-conservative nutrients such as phosphate and nitrate. The composition of nutrients in the culture medium and the differences between species can also explain variations in the content of lipids. Thus, manipulation of culture conditions and the harvesting at optimum growth phases may enable the lipid and PUFA composition of microalgal cultures to be tailored for specific purposes. Table 4.1. represents the total lipid content of cultured algae at various growth stages. The variation in lipid content may be due to the depletion of fatty acid during the growing stage. 20 days of culture was suited for lipase studies since the lipid content

was comparatively high for the species *I. galbana* and *C. calcitrans*. *C. calcitrans* is widely considered difficult to grow continuously, forcing most hatcheries to rely on multiple overlapping batch culturing procedures. Diatoms which are known for their synthesis of EPA, can also produce large amounts of palmitic acid (16:0) and palmitoleic acid (16:1n-7) (Volkman *et al*., 1989, Wen and Chen, 2003). There are limited reports on how *C. calcitrans* biosynthesize oil, in particular n-3 LC PUFA, and also for the changes throughout its culture stage. Banerjee *et al*., (2011) reported that lipid content of *C. calcitrans* varies from 26.8% in dry weight grown out-doors conditions under shade to a level of 11.71% dry weight in controlled conditions. Lipid content and fatty acid profiles of *C. calcitrans* have been determined throughout the growth phase (Shamsudin, 1992, Ragg *et al*., 2010). *C. calcitrans* has been used as a single supply and can be mixed with other algae for nutrient delivery for an aquaculture system (Phatarpekar *et al*., 2000, Pettersen *et al*., 2010, Ragg *et al*., 2010). For *C. calcitrans* it is found that on the 30^{th} day there was reduction in the cell concentration indicating a declining phase. Guihéneuf *et al*. (2011) and Shah *et al*. (2014) suggested that these algae doubled their lipid content during proliferative growth days. On the other hand, at 10 days and 30 days of culture, a high content of lipid was shown for *C. marina* and *T. gracillus* respectively. Maximum density was obtained on 10^{th} day of culture, while a minimum density was observed during the initial days of culture. After 10^{th} day the declining phase was started for *C. marina* as reported by Rekha *et al*. (2011). A similar observation was found in the present study suggesting maximum lipid content at 10^{th} day of culture for this species. The variation in lipid content with culturing days may be due to the changes in growth parameters and tolerance ranges with species. The content of saturated fats present in microalgae can get improved in a culturing condition under high light.

Triacylglycerols and *p*-nitrophenyl esters were used as substrates in lipase assay of *C. cylindracea*. In the literature, differences in the abilities of microbial lipases in hydrolyzing fatty esters were found (Abramic *et al*., 1999). For example, the highest hydrolysis rates were obtained using *S. rimosus* lipase with *p*-nitrophenylcaprylate and *p*-nitrophenyllaurate, this indicated the preference of enzyme towards medium acyl chain lengths. The *p* nitrophenyl esters of palmitic and myristic acids were also appropriate substrates of the lipase, but *p*-nitrophenyl stearate was only hydrolysed at a considerably slower rate. In the present study, the activity of lipase using paranitrophenylpalmitate as substrate was found to be 8.0±0.155 U/g of protein. The use of lipases might possibly help to obtain from phospholipids a lipid fraction enriched in DHA. The broad substrate specificities of these enzymes are well known and several lipase preparations were shown to display significant phospholipolytic activity towards synthetic phosphatidylcholine and lecithin in soybean further to the main triacylglycerol hydrolase activity. Several studies on hydrolysis of triacylglycerols containing n3 PUFAs have shown that lipases discriminate against n3 PUFAs, i.e. they preferentially release saturated and monounsaturated fatty acids rather than n3-PUFAs (Ust¨un *et al*., 1997, Hoshino *et al*., 1990).The optimal growth conditions for lipase production by *C. cylindracea* were determined under different agitation speeds and aeration in a fermentor (He *et al*., 2006). Maximum lipolytic activity was observed when the microorganisms were at the beginning of the stationary growth phase.

In fish oil the results showed an enrichment of 17.05% in PUFA when it was treated with lipase. The result shows an increase of eicosapentaenoic acid (EPA) of 3.8% and the docosahexaenoic acid (DHA) of 11.0%. It has been reported that because n3 PUFA is located in the 2 nd position of triglyceride, hydrolysis of sardine oil with 1,3 specific lipase should produce PUFA rich 2-monoglycerides and 1,2 diacyl glycerides. The presence of cis carbon-carbon double-bonds in the fatty acids result in bending of the chains. Therefore, the terminal methyl group of the fatty acids lies so close with the ester bond that can cause a stearic hindrance effect on lipases. There is also a high bending effect of EPA and DHA due to the presence of 5 and 6 double-bonds, respectively, enhancing the stearic hindrance effect; therefore lipases cannot reach the ester-linkage between these fatty acids and glycerol. However, saturated and monounsaturated fatty acids of triglycerides do not present any barriers to lipases and thus may be easily hydrolyzed. Therefore, fatty acid selectivity of a lipase for EPA and DHA allows their separation and concentration from other components present in the left behind portion of marine oils. In addition to it, the lipases have been frequently used for the discrimination between EPA and DHA in concentrates containing both of these fatty acids, thus providing the possibility of producing omega 3 PUFA concentrates with dominance of either EPA or DHA.

The partial hydrolysis of the sardine oil by *C. cylinderacea* lipase indicates strong discrimination by the lipase against DHA and only moderate discrimination against EPA. This implies that the concept of positional specificity alone cannot explain the observed diversity in the hydrolysis of the sardine oil with *C. cylinderacea* lipase. This could be partly due to the fatty

acid chain length selectively, showing higher activity with C 18 or shorter fatty acids than with C 20 and C 22 fatty acids. Bottino *et al*. (1967) postulated that in the resistant acid, the location of the double bonds in the terminal methyl groups are close to carboxyl group, producing a stearic hindrance effect, which protects it against hydrolysis. According to Tanaka *et al*. (1995), this enzyme has fatty acid acyl chain specificity and appears to be unique in its resistance to DHA. The experiments in fish oil enrichment suggest the importance of its use in algae also.

Little information exists on recovering polyunsaturated fatty acids from microalgae; however, methods for concentration and purification of PUFAs from fish oil have been extensively reported. The rate of enzymatic reactions is affected by several factors, including: (i) the nature of enzyme (activity, position- and substrate-specificity, etc.); (ii) the concentrations and ratios of reactants; (iii) the composition of oils (even the glycerides structure) or fatty acid mixtures; (iv) organic solvent used and its nature; (v) the water content. Studies on *C. antartica* lipase for enrichment of PUFA in *Phaeodactylum tricornatum* and *Porphyridium cruentum* showed enrichment at 72 hr. and 96 hr. respectively with a better yield of 42.5% and 45.6 % EPA (Molina Grima *et al*., 1999). Here, hydrolysis yield of fatty acids after 4,8,16, and 24 hr. incubation of four species of algae, *I. galbana, C. calcetrans, T. gracillus* and *C. marina* with lipase from *C. cylinderacea* was examined against different concentration gradient of 4 U, 8 U and 12 U of enzyme.

The results reveal that the lipase activity has good action on the substrate *I. galbana* at a concentration of 4 U and at 8 hr incubation. As the time of incubation is a factor thus induced or reduced the activity of enzyme.

In other incubation periods and concentration the enrichment value not even detected in GC because of increased hydrolysis which leads to PUFA disintegration. While there are also results in enrichment of PUFA but less than that of control condition. It shows that enzyme concentration would also affect the enrichment process. In microalgae, polar lipids (glycolipids and phospholipids) generally contain higher amounts of n3 PUFAs than neutral lipids (Alonso *et al*., 1998). Enzymatic enrichment of the phospholipid fraction of DHA in the microalgae requires position of the ester linkage.

I. galbana is a dinoflagelate able to produce DHA, although its DHA content varies from 2 to 25% depending on strain and culture condition. The phospholipid content of algae reported two fatty acids namely C14 and DHA which were found to be as high as 25 and 50% respectively (Molina Grima *et al*., 1994). The C16 and C18:1n9 are present at levels around 10% while the other fatty acids represent in very low ratios of the total fatty acid (less than 5%) (SaoudiHelis *et al*.,1994). In *I. galbana* phospholipids, the phospholipid fraction was subjected to phospholipase A_2 hydrolysis, which selectively released fatty acids (DHA) esterified at *sn*-2 position of phospholipids. Many lipases do not tolerate the long-chain n-3 fatty acids very well as substrates. Lipases that do so usually display preference for EPA as compared to DHA. This may be the reason for the extra enrichment of EPA compared to DHA. It was reported that short-chain ester *p*-nitrophenyl butyrate was a good substrate for purified enzyme. The same behavior is also observed while using *I. galbana* phospholipids as substrate, DHA gets concentrated on to the phospholipid backbone after reaction. Hypolipidaemic activity of *I. galbana*

which induces reduction in glucose, triacylglycerol and cholesterol values (Nuńo *et al*., 2013) attribute due to the presence of n-3 PUFA.

C. calcitrans, a brown diatom belonging to the Bacillariophyceae, has recently gained attention because of its valuable polyunsaturated fatty acid profile. It is widely cultivated as live feed for bivalves and crustaceans (Tolga *et al*., 2003, Moreau *et al*., 2006). For *Chaetocerous calcitran* the pattern is different and maximum enrichment occurs at 4 hr. incubation with an enzyme concentration of 8 U. At 4 U enzyme concentration there is a trend of enrichment in 24 hr incubation time but, there is failure in enriching the DHA. But in 8 U enzyme concentration, compared with other two concentrations, most of the fatty acids are expressed, specifically PUFA and this indicates that this concentration is good for *C.calcitrans* in PUFA enrichment studies.

Chlorophytes (*Dunaliella* spp. and *Chlorella* spp.) are lacking for both C20 and C22 PUFAs, though some other species have little amounts of EPA (up to 3.2%). Because of this PUFA deficiency, chlorophytes have low nutritional value and therefore not suitable as a single species diet. The enrichment percentages of the available PUFA were maximum at 4 U lipase concentration. At 12 U there is enrichment for both EPA and DHA at 4hr. incubation time, but there is no enrichment for EPA with 8 U enzyme concentration. The calculated enrichment with 12U lipase concentration was comparatively less than that in 4U. Therefore, enrichment process at this concentration was more or less effective for commercializing PUFA enriched *C. marina*. It is important that *Chlorella* species are known for the antihypertensive activity, studies revealed that its ingestion decreased BP in human (Kim and Kang, 2011: Ko *et al*., 2012). Furthermore, *Chlorella* have

shown hypolipidaemic and anti-atherosclerotic activity (Lee *et al*., 2008 a, b) which may be due to its PUFA content.

The genus *Tetraselmis* is one among the extensively used microalga mainly as a feeder in mariculture for marine herbivores, because of its ability to grow under a wide range of physical and chemical environmental conditions. The enrichment of fatty acids in *T. gracillus* at 4 U, 8 U and 12 U enzyme concentration reveals that there is no EPA enrichment at 4 U and 8 U while DHA was enriched at 8 hr incubation with 4U enzyme concentration. From the experiment it was clear that the rate of hydrolysis was maximum at higher enzyme concentration for this species. As the time of incubation period increases the rate of enrichment decreases. Maximum enrichment was indicated at 4 hr incubation with 12 U enzyme suggesting this as an effective condition for *T. gracillus* enrichment. Microalgae are generally regarded as safe category by the US Food and Drug Administration (Chćón lee and Gonzalez-Marinó, 2010) having novel compounds with considerable potential in pharmaceuticals and nutritional supplements (Blunt *et al*., 2014; Fretias *et al*., 2012; Nikapetiya, 2012). Moreover, biological activity of microalgae and marine microorganisms were reported for as functional foods and in neutraceutical applications (Zhao *et al*., 2015).

Consumers are aware of the importance of an adequate provision of these nutrients and several properties of microalgal oils are particularly appealing, such as their sustainability, high purity and quality, “vegetarian” origin, and improved organoleptic qualities when compared to fish oils. Although the use of genetically modified microalgae by the industry is regarded as a possibility in the process of developing strains producing

tailored oils, this may, as in other sectors of food production, encounter major opposition from the public opinion. Although genetically modified crops will likely serve as n3 LC PUFA sources in the future, microalgae oils have a great potential to present purer profiles, which are highly advantageous during processing and may address differentiated purposes in the market. The antioxidant capacity of the microalgae total lipid extracts was three to four times higher than the antioxidant capacity of fish oil. The higher antioxidant capacity of the microalgae oils may mean that the omega-3 LC-PUFA in micro algal oils will be better protected against oxidation than in fish oil. The DHA in algal oil exhibited a greater degree of oxidative stability than that of fish oil and is free from the fishy odor or taste present in fish oils (Varela *et al*., 1990). Hence in the proceeding chapter, study is focused in improving the quality of fish oil by using natural antioxidants.

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Chapter 5

ANTIOXIDATIVE PROPERTY OF MEDICINAL PLANT EXTRACTS AND STORAGE STABILITY OF FISH OIL

Contents

5.1 Introduction

The health benefits of omega-3 long-chain PUFA has led to the marketing of purified fish oil supplements in health food stores. However, despite their health benefits, fish oils are highly sensitive to oxidative deterioration, which entails practical problems. Oxidation of lipids not only produces rancid odours and flavours, but can also decrease nutritional quality and safety by the formation of secondary oxidation products (Frankel, 1996). In order to solve the problem, research for safer and effective natural antioxidants are underway and several natural sources are being explored.

Oxidation of lipids not only produces rancid odours and flavours, but also can decrease nutritional quality and safety by the formation of secondary products. The products of lipid oxidation are known to be hazardous to health since they are associated with aging, membrane damage, heart disease and cancer (Suja *et al*., 2004). The consumption of such oxidised fats has been reported to cause diarrhoea, liver enlargement, growth depression and histological changes in tissues of experimental animals (Nwanguma *et al*.,

1999). The production of biologically active carbonyl compounds including acrolein, malonaldehyde (MA) and 4-hydroxyl-2-nonenal (4-HN) from lipids during oxidation has been reported by many researchers (Miyake and Shibamoto, 1996). These chemicals have been associated with human diseases such as atherosclerosis, cataracts and ageing. For example, acrolein reportedly caused several cytopathic effects that relate to multistage carcinogenesis in the human bronchial epithelium. Malonaldehyde has been implicated in ageing, mutagenesis, and carcinogenesis. The toxicity of these aldehydes is due to their ability to crosslink to proteins and bind covalently to nucleic acids. Almost all amino acids react with primary and secondary products of oxidised lipids, thereby decreasing the digestive utilization of protein, amino acids and fats, which may affect weight gain (Varela *et al*., 1995). As an example, acute food poisoning caused by deteriorated fat and oil in instant noodles was reported in Japan approximately 40 years ago (Gotoh *et al*., 2005). The degree of oxidation of the lipids in the instant noodles that induced food poisoning was at least 100 meq/kg fat in peroxide value (PV).

In order to overcome the stability problems of oil and fats, synthetic antioxidants, such as Butylatedhydroxyanisole (BHA), Butylated hydroxyl toluene (BHT), Ter-butylhydroquinone (TBHQ) etc. have been used as food additives (Iqbal and Bhanger, 2005, Suja *et al*., 2004, Krings *et al*., 2000), because they are effective and less expensive than natural antioxidants (Suja *et al*., 2004). But reports reveal that these compounds may be implicated in many health risks, including cancer and carcinogenesis (Iqbal and Bhanger, 2005). Therefore, the most powerful synthetic antioxidant (TBHQ) is not allowed for food application in Japan, Canada and Europe. Similarly, BHA and BHT have also been removed from the generally recognized as safe list of compounds (Iqbal and Bhanger 2005, Suja *et al*., 2004). Due to these safety concerns, there is an increasing trend among food scientists to replace these synthetic antioxidants with natural ones, which, in general, are supposed to be safer.

a. Aloe barbadensis

b. *Boerhavvia diffusa*

c. *Oscimum sanctum*

Plate 5.1 Photographs of the Indian medicinal plants used for the study

Research for safer and effective natural antioxidants is underway and several natural sources are being examined. A comparative, multi-method screening of antioxidant activity for a large number of Indian medicinal plants in relation to their phenolic compounds is needed to provide a better understanding of their relative importance as natural antioxidants. Here in the present study three medicinal plants namely, *Aloe barbadensis, Boerhavia diffusa* and *Oscimum sanctum* were screened for their uniqueness as natural antioxidant sources (Plate 5.1). The medicinal and antioxidant properties of Aloe vera were attributed due to the presents of components such as anthrone, chromone, aloe verasin, hydroxyalin and glycoprotein aloctin A.(Yun hu *et al*., 2003). Holy basil (*Oscimum sanctum*) contains the phenolic compounds which includes eugenol, cirsilineol, isothymusin, isothymonin, rosamarinic acid (Kelm *et al*., 2000), orienlin and vicemin (Vrinda and Uma Devi, 2001). *B. diffusa* contains a number of constituents mainly alkaloids, flavonoids, saponins and steroids (Ujowundu *et al*., 2008).

India is considered as a gold mine of traditional medicinal plants with an established record and people have a good knowledge of its use. The challenge is concerning their formulations due to its complex nature and the absence of its complete constituent evaluations. For the endurance of its quality, purity and stability a complete evaluation of the constituents is required. Stability studies provide evidence on in what way the quality of a drug or its product varies with time under influence of changing environmental factors such as humidity, temperature and light. It also establishes a retest period for the particular drug substance or its product for the recommended storage conditions. Thus, it is proved that the stability study is unavoidable for the assessment of product quality.

In general, pharmaceutical products are studied for its stability profile in an accelerated humidity and temperature, and these investigational findings can be very helpful for predicting reliable expiry date or shelf-life at room temperature by assuming certain criteria and assumptions (Cannors *et al*., 1979). Each and every product has a definite shelf-life and it depends on different physical, chemical, biological and environmental factors. Real time study is a time consuming and a long procedure. Therefore, it is difficult for the manufacturer to wait till the drug degrades naturally to about 90% of labeled amount at room temperature. Taking this into account the stability study is normally supported for assigning shelf-life of any drugs. A series of guidelines which are acceptable to multiple countries for the approval of a drug have been established and known as Quality Guidelines or ICH (International Council for Harmonization of technical requirements for pharmaceutical for Human use) guidelines. By using this method we can predict the shelf life of any drug product in a very short period of time.

In contrast to conventional preparations of medicinal plants, products incorporated with their extracts lead to a number of unique problems with regard to quality and stability. So as to ensure good reproducibility, adequate control is essential. A key part of quality control is to guarantee the chemical stability of the final product during its storage. Present study is an attempt to study accelerated stability of fish oil incorporated withthree medicinal plant extracts, which can be used for preserving it from further oxidation.

5.2 Materials and Methods

5.2.1 Indian Medicinal Plants (IMP)

The medicinal plants used for the study include *Oscimum sanctum, Boerhavvia diffusa* and *Aloe barbadensis*. Leaves of these plants were sun-

dried and were ground to get a crude powder. 200 gm. of crude powder was shaken with 70% ethanol for 24 hrs. in an orbital shaker at room temperature. Extracts were filtered through Whatman No1 filter paper using vacuum filtration unit. The filtrate was concentrated to dryness under reduced pressure using a vacuum rotary evaporator. The dried extracts were re-suspended in ethanol to attain the required concentration for each analysis (10 mg/10ml).

5.2.2 Determination of the Antioxidative activity of IMP

5.2.2.1 β Carotene linoleic acid assay (Mokbel and Hashinaga, 2006)

The inhibition of the volatile organic compounds and the conjugated diene-hydroperoxide arising from the coupled autoxidation of β-carotene and linoleic acid was measured in this assay. β carotene linoleic acid stock solution was prepared as follows: 0.5 mg of β carotene was dissolved in 1 ml chloroform, then 25 µl of linoleic acid and 200 mg of tween 40 were added to it The chloroform was subsequently evaporated using a vacuum evaporator. Then 10 ml of distilled water was added to it with vigorous shaking.

Aliquots (25 µl) of this reaction mixture were transferred to test tubes followed by 300 µl of the extracts (2 mg mL^{-1} in ethanol). The tubes were incubated for 60 min at 50 ^{o}C .The same procedure was repeated with a blank containing only 350 µl of ethanol. After the incubation period the absorbance of the mixture was measured at 490 nm in a UV Visible spectrophotometer. Antioxidant capacities of the sample were compared with the blank. The antioxidant activity (AA) is expressed as % inhibition relative to the control after incubation by using the equation:

AA= [(DRc −DRs)/DRc] ×100 where DRc is the degradation rate of the control and DRs is the degradation rate of the sample.

5.2.2.2 Reducing property

The reducing power of the ethanolic extracts of the three medicinal plants was determined according to the method of Oyasizu (1986) as described by Yen *et al*. (2000). Medicinal plant extracts, concentrated by vacuum evaporator (2.5 mg in 1ml ethanol) was mixed with 5 ml phosphate buffer (pH 6-8) and 5ml of potassium ferricyanide (1% stock solution) and the mixture was incubated at 50 ^{o}C for 20 min. 5 ml of TCA (10% stock solution) was added to the reaction mixture which was then centrifuged at 3000 g for 10 min. The upper layer of solution (5 ml) was mixed with distilled water (5 ml) and ferric chloride (1%) and the absorbance was measured at 700 nm. A higher absorbance indicates a higher reducing power.

5.2.2.3 DPPH radical scavenging assay.

The capacity to scavenge the "stable" free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) by the three medicinal plant extracts was monitored according to the method of Moure *et al*. (2000). Two ml of a 3.6 X 10^{-5} M methanolic solution of DPPH (Sigma) was added to 50 µl of ethanolic extracts (1 mg ml^{-1} dilution). The decrease in the absorbance at 515 nm was continuously recorded in a Hitachi U 2000 spectrophotometer at room temperature for 16 min. The scavenging effect (decrease of absorbance at 515 nm) was plotted against the time. Radical scavenging ability of the sample was calculated from the absorbance value at 16 min and at 0 times as follows:

The radical-scavenging activity (RSA) %= $(ABS_{t(0\ min)}-ABS_{t(16min)})/ABS_{t(0\ min)})X100$

5.2.2.4 Total Phenolic Content

Total phenolic content (TPC) of each sample was estimated using the Folin Ciocalteu colorimetric method according to Cai *et al*. (2004) and Liu *et*

al. (2002), with minor modification. From the sample extracts, 10, 20, 30, 40 and 50 µl were pipetted out and made upto 1 ml with ethanol. From it 0.1ml was taken and added 5 ml distilled water and 0.5 ml of Folin- ciocalteau reagent. After reaction of 3 min, added 1.5 ml of Na_2CO_3 (20% stock solution) and 2.9 ml of distilled water. After 2hr of incubation in dark at room temperature, the absorbance was measured in a spectrophotometer at 765 nm.The same procedure was also applied to the standard solutions of gallic acid. Total phenolic content in the sample extracts were expressed as mg of gallic acid equivalents per 100 gm sample.

5.2.3 Heavy Metal analysis of the Indian medicinal plant extracts using AAS

- ***Reagents***

All reagents used were of analytical grade. Working standards of zinc, copper, magnesium, nickel and lead were prepared by diluting concentrated standard stock solutions (Merck, Germany) of 1000 mg/l in ultra-pure water (MilliQ, Millipore- USA).

- ***Sample Preparation***

The selected portions of the samples were removed; homogenized and about 2.5 ± 0.5 g was taken for analysis. Into it added 10 ml of concentrated nitric acid–perchloric acid (10:4) mixture, covered and left overnight at room temperature. By using a microwave digester (Milestone ETHOS PLUS, Italy) the samples were digested. The completely digested samples were allowed to cool to room temperature, filtered through a glass wool, and made up to 50 ml.

- ***Chemical analysis***

Metallic elements such as cadmium (Cd), nickel (Ni)), copper (Cu), magnesium (Mg), arsenic (As), mercury (Hg), lead (Pb) and zinc (Zn) in the digested samples were determined using flame atomic absorption spectroscopy (PerkinElmer, AAnalyst 200,version 8.0, 2013). The metallic elements such as As and Hg were determined using the mercuric hydride system- atomic absorption spectroscopy. All samples were analyzed in triplicate as per standard conditions. The operating parameters for working elements were set as recommended by manufacturer. The blank and calibration standard solutions were also analyzed in the same way as for the samples.

5.2.4 Determination of oxidative changes of fish oil incorporated with IMP extracts

100 ml of freshly extracted fish oil from Sardine was taken in dark bottles were treated with the ethanolic extracts of *O. sanctum* (2 mg%), *B. diffusa* (5 mg%) and *A. barbadensis* (5 mg%). The samples were taken in triplicate and stored under nitrogen. To the control only ethanol was added. Another fish oil sample was treated with a synthetic antioxidant (BHT) at a resultant concentration of 0.002%. For storage study sampling of the fish oil was done from all the above treated samples and control on 0, 10, 20, 30 and 40 days for various analyses.

5.2.4.1 Peroxide value (Lea, 1938)

5 g of fish oil sample was weighed out into a dry conical flask and was extracted with small quantities of chloroform, filtered and made upto 100 ml. 10 ml aliquot of the extract was evaporated to dryness to determine the weight

of fat. Another 10 ml extract was pipetted out into a dry iodine flask along with 20 ml of glacial acetic acid and 1 ml of saturated KI solution. The mixture was kept for 30 min in dark and diluted with water. The liberated iodine was titrated against N/100 sodium thiosulphate solution using starch as indicator. The peroxide value is expressed as milliequivalent (meq) of peroxide/kg fat

5.2.4.2 Thiobarbituric Acid Number

Thiobarbituric acid value was estimated according to IUPAC method 2.531(1992). In a test tube, 200 mg of oil sample was taken and to it 5 ml of thiobarbituric acid reagent was added. The mixture in test tube was closed and placed in a water bath at 95 ^{o}C for 120 min. It was then cooled and the absorbance was measured (As) at 530 nm. A reagent blank was also carried out with absorbance (Ab).

Thiobarbituric acid number = 50 X (As – Ab)/weight of the sample

5.2.4.3 Variation in fatty acid profile on storage of fish oil incorporated with IMP extracts.

Sample preparation and treatment were carried out as mentioned in section 5.2.4. Sampling was done for the fatty acid analysis at 0, 10, 20, 30 and 40 days of storage. A control taken with only ethanol and a sample with a synthetic antioxidant BHT (0.002%) were also run along with the IMP treated samples. FAME was prepared and fatty acids were analysed according to the procedure given in section 3.2.4.

5.2.5 Accelerated Shelf Life study of Fish Oil

The accelerated stability study of fish oil incorporated with medicinal plant extracts was done at accelerated humidity and temperature conditions,

viz. accelerated stability study taking ICH (International Council for Harmonization of Technical requirements for pharmaceutical for Human use) guidelines as reference. Fish oil was treated with 70% ethanolic extract of IMP at an optimum concentration of 2 mg% of *O. sanctum* extracts and 5 mg% of *B. diffusa* and 5 mg% of *A. barbadensis*. All samples were prepared in dark bottles and stored under nitrogen by passing the gas into it. The samples were kept at 400 ±2 ^{o}C and 70 ± 5% humidity in a humidity chamber, and at 37±2^{0}C and normal humidity. The samples were withdrawn after one, two, three and six months in triplicate for analysis.

5.2.5.1 Detection of E. coli (API Part II, 2008)

1 ml of the fish oil sample was homogenized with 1-2 g of polysorbate 80R and heated up to 40 ^{o}C in a water bath. Added 50 ml nutrient broth into it and maintained the same temperature till emulsion formed within 30 minutes. From this took 1 gm. sample, added 50 ml nutrient broth which was kept in a sterile screw-capped container, shaken well. Allowed it to stand for 1 hr. and after shaking, loosened the cap and incubated at 37 ^{o}C for 18-24 hrs to get the enrichment culture.

By means of an inoculating loop, streaked a portion from the enrichment culture on the surface of MacConkey agar medium. Covered and inverted the dishes and incubated. Upon examination, if none of the colonies are brick-red in colour and have a surrounding zone of precipitated bile, the sample meets the requirements of the test for the absence of *Escherichia coli*.

If the colonies described above are found, transferred the suspect colonies individually to the surface of Levine eosin-methylene blue agar medium, plated on Petri dishes. Covered and inverted the plates and incubated. Upon examination, if none of the colonies exhibits both a characteristic

metallic sheen under reflected light and a blue-black appearance under transmitted light, the sample meets the requirements of the test for the absence of Escherichia coli. The presence of *Escherichia coli* was confirmed by further suitable cultural and biochemical tests.

5.2.5.2 Detection of *Pseudomonas aeruginosa* (API Part II, 2008)

1 ml of the fish oil sample was homogenized with 1-2 g of Polysorbate 80R. Then heated up to 40 ^{o}C in a water bath and mixed carefully. Inoculated homogenized sample in to 100 ml Soybean casein digest medium. Mixed thoroughly and incubated at 35-37 °C for 24-48 hr. Examined the medium for growth and if growth is present, streaked a portion of the medium on the surface of Cetrimide agar medium each plated on Petri dishes. Covered and incubated at 35-37 °C for 18-24 hr. If growth of colonies on Cetrimide agar plate are gram negative rods revealed by gram staining, usually with a greenish fluorescence occurs, then performed oxidase and pigment test.

Streaked representative of the suspected colonies from the agar surface of Cetrimide agar to the surfaces of Pseudomonas agar medium (F) for detection of fluorescein and Pseudomonas agar medium (P) for detection of pyocyanin.

Incubated for not less than 3 days at 33-37 °C. Examined the plates under UV (365 nm) for yellow and blue fluorescence respectively. If growth of suspect colonies occurs, a pinch of colony is transferred to the filter paper & placed 2-3 drops of 1 % w/v solution of N,N,N',N'- tetramethyl 4 phenylene diamine dihydrochloride. If there is no development of a pink color, changing to purple within 5-10 seconds, the sample meets the requirements of the test for the absence of *P. aeruginosa*.

Interpretation:

If there is purple color formation within 10 seconds, after adding 1% oxidase reagent on a filter paper, it is confirmed as *P. aeruginosa* and depending upon the pigment color produced on Pseudomonas agar F& P medium, it is reported as *P. flourescens* and *P. pyocyanin.*

Limit of detection:

P. aeruginosa = Presence/ Absence/gm/ml

5.2.5.3 Detection of *Staphylococcus aureus* (API Part II, 2008)

1 ml of the fish oil sample was homogenized with 1-2 g of Polysorbate 80R. Then heated up to 40 ^{o}C in a water bath and mixed carefully. Inoculated the homogenized sample in 100ml of Soybean casein digest medium. Mixed thoroughly and incubated at 35-37 °C for 48hr. Prepared a subculture on Baird Parker agar, and incubated at 35-37 °C for 24-48 hr.

Black, shiny, surrounded by clear zones of 2 to 5 mm colonies of gram positive cocci on BP agar plate and gram positive cocci in clusters revealed by gram staining, indicate the presence of *S. aureus*.

Confirmed if bacteria is present by tests such as coagulase test by using rabbit plasma. Coagulase test is carried out by transferring suspected colonies from BP agar medium to 0.5 ml of rabbit plasma with or without additives. Incubated it in water bath at 37 °C examined the tubes at every 3 hours and subsequently at suitable intervals up to 24 hr. Negative coagulation test confirms the absence of *S. aureus.*

Limit of detection:

Staphylococcus aureus = Presence/ Absence/g/ml

5.2.5.4 Detection of *Salmonella spp.* (API Part II, 2008)

1 ml of the fish oil sample was homogenized with 1-2 g of Polysorbate 80R. Then heat up to 40 ^{o}C in water bath and mixed carefully. Added 100 ml of Nutrient broth and heated up to 40 ^{o}C. This temperature was maintained for the shortest time until the formation of an emulsion within 30 min. Shaken and allowed to stand for 4 hr and shaken again.

Loosened the cap and incubated at 35 ^{o}C to 37 ^{o}C for 24 hrs.

Primary test:

Added 1 ml of the enrichment culture to a mixture of 10 ml Selenite F broth and 10 ml Tetrathionate bile brilliant green broth. Incubated the preparation at 36 ^{o}C-38 ^{o}C for 48 hrs. Sub cultured to Bismuth sulphite agar and Xylose Lysine Deoxycholate agar. Incubated at 36 ^{o}C to 38 ^{o}C for 18-24 hrs.

If no growth of colonies on the agar plates, Salmonella spp. is absent. If growth is present on BSA with black or green colonies and Red colonies with or without black centers on XLD, done the secondary test.

Secondary test:

Sub cultured from the agar plates to TSI by first inoculating the surface of the slope and then making a stab culture with the same inoculating needle, and at the same time inoculated a tube of urea broth. This was incubated at 36 ^{o}C to 38 ^{o}C for 18-24 hrs. The formation of acid and gas in the stab culture (with or without concomitant blackening) and the absence of acidity from the surface growth in the triple sugar iron agar, together with the absence of a red color in the urea broth, indicated the presence of Salmonella spp. If acid but no gas is produced in the stab culture, the identity of the organisms should be confirmed by agglutination tests.

Agglutination test procedure:

From the test culture on nonselective media, transferred a loopful of growth to a drop of sterile 0.85% saline on clean slide and emulsified the organism. Rotated the slide for 1 minuteand then observed for agglutination. If agglutination (auto agglutination) occurs, the culture is rough and cannot be tested. Sub cultured to non- selective agar, incubated and tested the organism again as described in step 1 and 2. If no agglutination occurs, it was proceeded with testing. Carried out the control test in parallel by repeating the primary and secondary tests using 1 ml of the enrichment culture and a volume of broth containing 10 -50 Salmonella abony (NCIM 2257) organisms prepared from a 24 hr culture nutrient broth, for the inoculation of the tubes (a) and (b). The test is not valid unless the results indicate that the control contains Salmonella.

Limit of detection

Salmonella spp =Present/Absent/gm./ml

5.2.5.5 Method of Analysis for Total Yeast and Mold Count (API Part II, 2008)

10 ml of the fish oil sample was homogenized with 5 g of Polysorbate 80R. Then heated it not more than 40 ^{o}C in a water bath and mixed carefully. Added 85 ml of buffered sodium chloride peptone solution (pH-7) and heated up to 40 ^{o}C. Maintain this temperature for the shortest time till the formation of an emulsion within 30 min. Adjusted the pH to 7. From the pretreated suspension, serial dilution was carried out till 10-5 dilution. Poured 1 ml each of the homogenized samplesto labeled petri platestaken in duplicate. 15-20 ml of Sabouraud dextrose agar was added with Chloramphenicol cooled to 45 ^{o}C, into petri dishes. Immediately mixed the sample homogenate/dilutions and

agar thoroughly by alternate rotation and rocking to and fro of the plates on a level surface. Allowed the agar to solidify. The solidified agar plates were incubated in the dark at 20-25 °C for 3-5 days. A negative control was carried out by using sterile distilled water in the place of sample homogenate.

Counting of plates

Reported results in colony forming units based on average count of duplicate set, if no growth of colonies on all plates is less than 1 for the corresponding lowest dilution used.

Limit of detection:

TPC = >1 CFU /ml

Permissible Limit: >10 CFU/gm.

5.2.5.6 Method of Analysis for Total Aerobic Plate Count (API Part II, 2008)

10 ml of the fish oil sample was homogenized with 5 g of polysorbate 80R. Then heated it not more than 40 ^{o}C in water bath and mixed carefully. Added 85 ml of buffered sodium chloride peptone solution (pH-7) and heated up to 40 ^{o}C.This temperature was maintained for the shortest time until the formation of an emulsion within 30 min. Adjusted the pH to 7. From the pretreated suspension, serial dilution was carried out till 10-5 dilution. Dispensed 1 ml each of sample homogenate into petri plates. Added 15-20 ml of Soybean Casein Digest agar, cooled to 45 ^{o}C, into petri dishes. Immediately rotated the plates in a back and forth motion. The agar plates were kept for 5-10 min. The agar plates were incubated at 35 °C for 5 days. A negative control was conducted by using sterile distilled water in the place of sample homogenate.

Counting of colonies

The colonies on the plate was counted with highest number of colonies but not more than 300 per plate as the maximum consistent with good evaluation, by using colony counter. For plates with 300 colonies, reported results in colony forming units based on average count of duplicate set.

Limit of detection:

TPC = >1 CFU /ml

Permissible Limit:105 CFU/gm

5.2.6 Statistical analysis

All of the experiments were done in triplicate. The data were recorded as means ± standard deviations and were analysed with SPSS (version 11.0 for Windows, SPSS Inc., Chicago, IL, USA), and the statistical significance was determined at $P < 0.05$.

5.3 Results

5.3.1 Determination of antioxidant activity of the selected Indian Medicinal Plant extracts

Table 5.1 Total antioxidative activity and phenolic content of ethanolic extracts of the Indian Medicinal Plants.

SAMPLE	DPPH (% of radical scavenging activity)	Reducing property(Abs 700nm)	β-Carotene assay (%of inhibition)	Total Phenolic Content (gallic acid equivalent in mg/100gm)
Aloe barbadensis	7.35±.004	0.222±.083	64±.063	94±.072
Boerhaavia diffusa	16.38±.054	0.389±.073	72±.025	238±.093
Oscimum sanctum	34.25±.047	0.653±.035	78±.078	256±.012

All values are expressed in mean± standard deviation, n=3

Antioxidant activity of the medicinal plant extracts was measured in terms of DPPH assay, % reducing property, Total Phenolic content and β Carotene assay (Table 5.1). In DPPH assay the percentage of radical scavenging activity showed highest value for *O. sanctum* (34.25) followed by *B diffusa* (16.38) and *A. barbadensis* (7.35). The same trend was observed in other parameters viz, reducing property, % of inhibition as shown by β Carotene assay and Total phenolic content. The values of these assays reveal that antioxidant property is highest in *O. santum* compared with the other two,whereas, *B. diffusa* showed higher antioxidant activity than *A. barbadensis*. The experimental values of antioxidant activity of the medicinal plants selected for the study followed the order, *A. barbdensis*< *B. diffusa*< *O. sanctum*.

5.3.2 Determination of heavy metals in IMP extracts

Table 5.2 Heavy metals (μg/mg wet weight) present in the Indian Medicinal Plants

Scientific Name	Cu	Zn	Mg	Ni	Pb	Hg	Cd	As
Aloe barbadensis	0.005 ±0.10	0.110 ±.067	0.019 ±.091	0.315 ±.074	0.001 ±.002	0.0005 ±.038	0.003 ±.021	0.004 ±.063
Boerhaavia diffusa	0.003 ±.049	0.072 ±.014	0.018 ±.033	0.216 ±.032	ND	0.0015 ±.082	0.0026 ±.042	0.002 ±.073
Oscimum sanctum	0.0002 ±.002	0.084 ±.091	0.056 ±.057	0.118 ±.010	ND	0.0002 ±.092	0.0012 ±.038	0.0018 ±.063

All values are expressed in mean± standard deviation, n=3
ND –Not detected

Analysis of metals and heavy metals in IMP performed for Cu, Zn, Mg, Ni, Pb, Cd, As and Hg. Nickel showed high values of 0.315, 0.216 and 0.118 for *A. barbadensis, B. diffusa* and *O. sanctum* followed by Zinc and Magnesium. Mercury and lead showed negligible concentration in these medicinal plant extracts. The values represented in Table 5.2 are well within

acceptable limits as per the guidelines of heavy metals in products. The results showed that heavy metals are present in least amounts in *O. sanctum* when compared with *B. diffusa* and *A. barbadensis*.

5.3.3 Storage stability of fish oil incorporated with medicinal plant extracts

Table 5.3 TBA value of fish oil treated with *O. sanctum* extracts expressed as OD±SD at 532 nm

Storage Days	Control	Concentration of extracts added			BHT (0.002%)
		2mg%	5mg%	10mg%	
0	0.530±.006	0.288±.008	1.241±.099	1.736±.093	0.030±.004
10	1.745±.086	0.653±.085	1.494±.045	1.934±.004	0.601±.001
20	2.815±.004	1.348±.003	2.707±.004	2.708±.002	0.958±.092
30	3.0±.002	1.708±.005	3.0±.009	3.0±.043	1.704±.033
40	3.0±.092	2.342±.083	3.0±.084	3.0±.007	2.235±.003

All values are expressed in mean± standard deviation, n=3

For storage stability study, fish oil incorporated with various concentrations of the three medicinal plant extracts along with a control and BHT treated sample were stored under controlled conditions and samples withdrawn at 0, 10, 20, 30 and 40 days of storage. The results of Thiobarbituric acid value/ Thiobarbituric acid Reacting Substances (TBA/TBARS) as an index of secondary oxidation, showed that *O. sanctum* treated samples (Table 5.3) were acceptable for 20 days. But on 30 days and 40 days, the values for 2 mg% treatment and BHT treatment showed much lower value compared to 5 mg% and 10 mg% concentration. (Annexure 5.1.)

Table 5.4 TBA value of fish oil treated with *B. diffusa* extracts expressed as OD±SD at 532 nm

Storage Days	Control	Concentration of extracts added			BHT
		2mg%	5mg%	10mg%	
0	0.530±.090	0.861±.004	0.460±.093	0.778±.004	0.030±.084
10	1.745±.004	1.399±.094	1.309±.085	1.733±.090	0.601±.093
20	2.158±.032	1.812±.003	1.581±.002	2.979±.033	0.958±.006
30	3.0±.053	2.094±.070	1.794±.020	2.744±.006	1.704±.050
40	3.0±.093	3.0±.092	2.845±.003	3.0±.050	2.235±.085

All values are expressed in mean± standard deviation, n=3

In Table 5.4., fish oil incorporated with 5 mg% of *B. diffusa* extracts and with BHT, theTBA value is shown below 2.0 on 30 days of storage. But the observed values for 2 mg% and 10 mg% concentration on 30th day were 2.094 and 2.744, respectively. On 40 days of storage the absorbance value for 5 mg% treatment was kept low (2.845) and was similar to BHT treated sample (2.235), while other treatments and control crossed the peak value of 3.0 at this stage.

Table 5.5 TBA value of fish oil treated with *A. barbadensis* extracts expressed as OD±SD at 532 nm

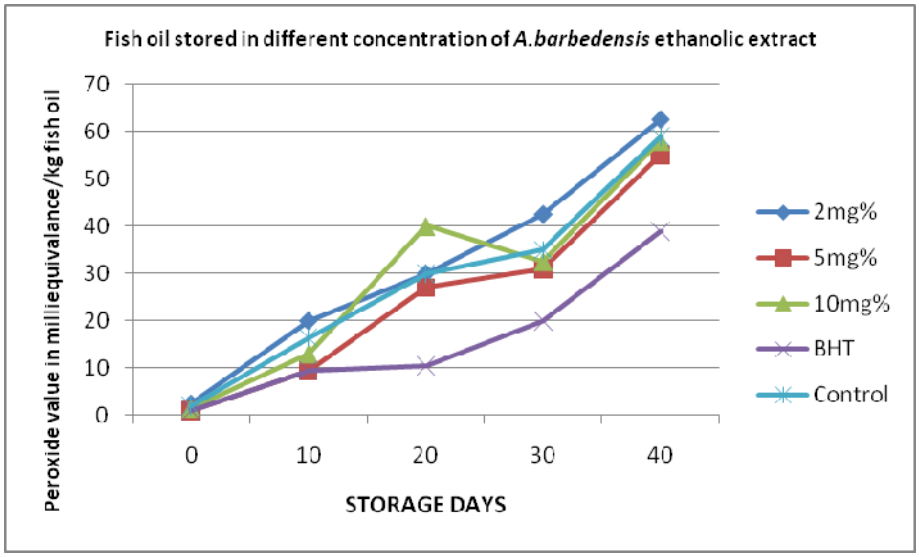
Storage Days	Control	Concentration of extracts added			BHT
		2mg%	5mg%	10mg%	
0	0.530±.006	0.490±.070	0.358±.006	0.515±.005	0.030±.002
10	1.745±.043	1.533±.084	1.445±.040	1.803±.020	0.601±.058
20	2.158±.074	1.793±.003	1.528±.008	1.944±.083	0.958±.038
30	3.0±.092	3.0±.098	2.443±.033	3.0±.043	1.704±.093
40	3.0±.004	3.0±.003	2.935±.074	3.0±.094	2.235±.004

All values are expressed in mean± standard deviation, n=3

Table 5.5. shows the results of absorbance of TBA in fish oil treated with *A. barbadensis* extracts along with control and BHT. The absorbance reached a peak value of 3.0 on 30 days in control, 2mg% and 10 mg% treated

samples. In sample with 5mg% of *A. barbadensis* treated fish oil, the absorbance was 2.443 and 2.995 on 30 days and 40 days respectively.

a.



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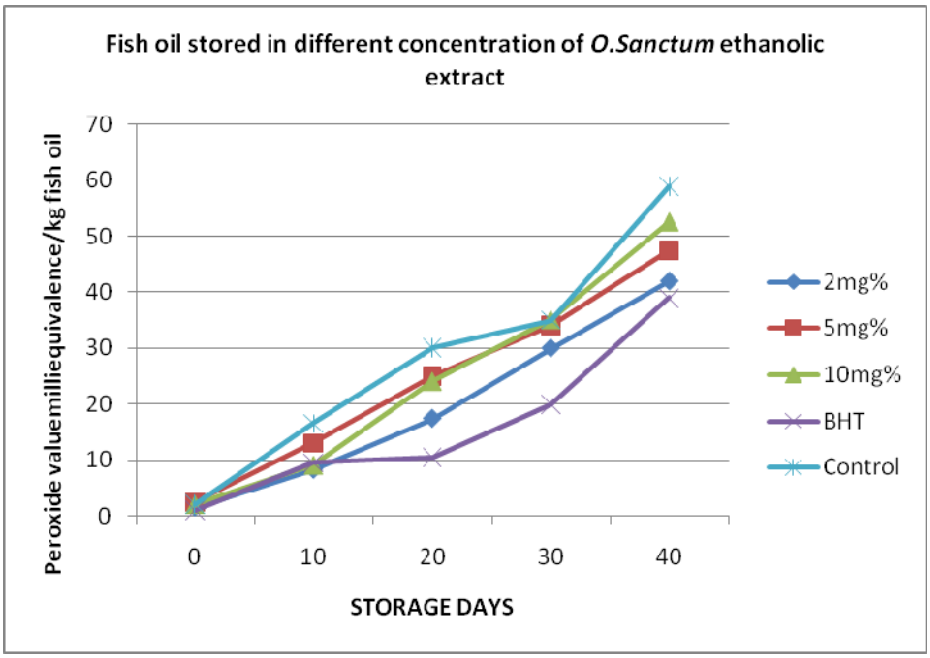

c.

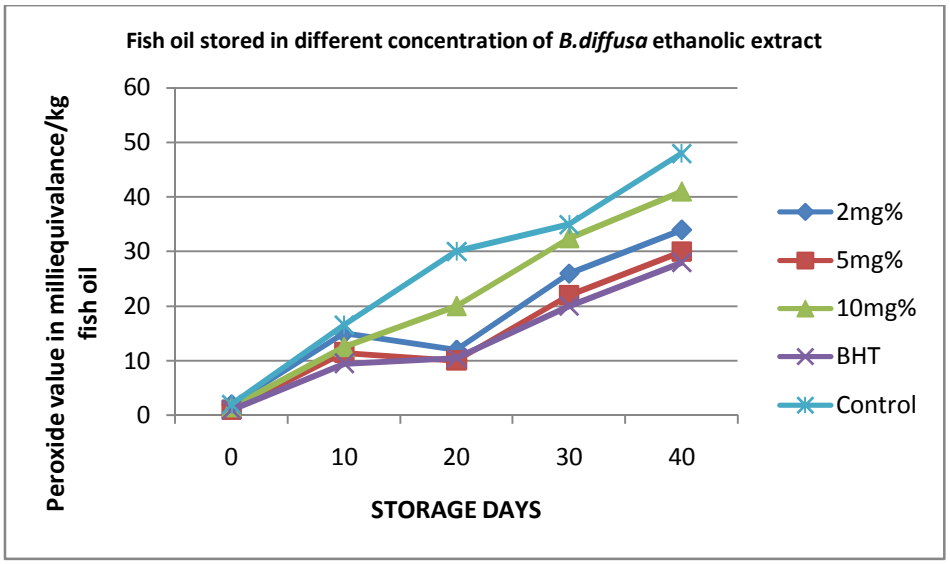

Fig 5.1 Peroxide Value of fish oil treated with ethanolic extracts of *A. barbadensis* (a) *O. sanctum* (b) and *B. diffusa* (c) at various concentration and storage days

Fig 5.1 a, b and c depict the variations in peroxide value of fish oil incorporated with three concentrations of the medicinal plant extracts on storage for 45 days. The treated samples were compared with oil sample incorporated with a synthetic antioxidant, BHT and a control sample of fish oil. The entire samples showed gradual increase in the peroxide value with number of storage days, but with varying gradation in the increase. In *A. barbadensis* treated sample, PV of 30 meq/kg was obtained with 5 mg% treated sample on 30th day (Fig 5.1(a)). 5 mg% concentration was found to be the optimum level of the extract to control the progress of lipid oxidation as compared with 2 and 10 mg% (Annexure 5.3.).The peroxide value of fish oil in Fig 5.1 (b) shows slow acceleration for BHT and 2 mg% O. sanctum extract treated samples. But for control sample, the PV reached 60 meq/kg in 40 days. Here also the TBA value and PV index were found to be at a lower rate of acceleration as the days progressed when compared with 5mg% and 10mg%

concentrations. But for BHT treated and 2mg% *O. sanctum* treated oil, the value was below 40 meq/kg in 40 days. In two factor ANOVA there is significant difference between rows as well as columns (Annexure 5.1). The results of experiment with ethanol extracts of *B. diffusa* 5.1 (c) was also comparable with that of *A. barbadensis* treated oil with 5mg% of extract concentration as the optimum level. At this concentration, the value of TBA and PV showed slow increase while for the other two there was sudden increase of value (Annexure 5.2.).

5.3.4 Fatty acid profile of fish oil preserved with IMP extracts

Table 5.6 Variations in fatty acid content of treated fish oil on zero day against control

Fatty Acid	*O. sanctum* (2 mg%)	*B. diffusa* (5mg%)	*A. barbadensis* (5mg%)	Control	BHT
Myrstic acid	8.23±.093	8.23±.089	8.24±.004	8.24±.001	8.23±.058
Palmitic acid	16.23±.005	16.21±.020	16.23±.030	16.24±.038	16.23±.093
ϒ Linolenic acid	7.02±.092	7.02±.043	7.01±.074	7.02±.033	7.02±.057
EPA	19.28±.008	19.27±.092	19.28±.093	19.27±.050	19.28±.043
DHA	37.31±.030	37.31±.094	37.32±.073	37.30±.083	37.31±.030

All values are expressed in mean± standard deviation, n=3

Table 5.7 Variations in fatty acid content of treated fish oil on storage for 15 days against control

Fatty Acid	*O. sanctum* (2mg%)	*B. diffusa* (5mg%)	*A. barbadensis* (5mg%)	Control	BHT
Myristic acid	8.23±.049	8.21±.020	8.14±.038	8.12±.029	8.23±.038
Palmitic acid	16.21±.095	16.20±.054	16.21±.092	16.20±.039	16.23±.005
ϒ Linolenic acid	7.01±.036	7.01±.098	7.01±.028	6.98±.054	7.01±.083
EPA	19.26±.006	19.25±.048	19.24±.094	19.21±.060	19.27±.089
DHA	37.29±.039	37.28±.023	37.26±.028	37.24±.039	37.29±.030

All values are expressed in mean± standard deviation, n=3

Table 5.8 Variations in fatty acid content of treated fish oil on storage for 30 days against control

Fatty Acid	*O. sanctum* (2mg%)	*B. diffusa* (5mg%)	*A. barbadensis* (5mg%)	Control	BHT
Myristic acid	8.20±.040	8.19±.038	8.12±.023	3.12±.003	8.20±.089
Palmitic acid	16.19±.029	16.18±.029	16.16±.084	10.17±.055	16.20±.028
ϒ Linolenic acid	6.98±.049	6.96±.054	6.94±.003	3.98±.074	6.98±.073
EPA	19.24±.090	19.23±.005	19.21±.064	6.98±.082	19.25±.039
DHA	37.25±.063	37.22±.090	37.19±.026	7.24±.007	37.29±.082

All values are expressed in mean± standard deviation, n=3

Table 5.9 Variations in fatty acid content of treated fish oil on storage for 45 days against control

Fatty Acid	*O.sanctum* (2mg%)	*B. diffusa* (5mg%)	*A.barbadensis* (5mg%)	Control	BHT
Myristic acid	8.01±.023	7.98±.053	7.07±.043	ND	8.21±.083
Palmitic acid	15.86±.053	14.97±.039	14.54±.092	2.67±.004	16.07±.091
ϒ Linolenic acid	5.98±.020	5.34±.012	5.02±.003	ND	6.78±.040
EPA	18.67±.005	18.45±.093	18.21±.043	ND	18.84±.032
DHA	36.56±.029	36.21±.002	35.87±.020	3.21±.030	36.84±.090

All values are expressed in mean± standard deviation, n=3

Changes in fatty acid content of fish oil treated with ethanolic extracts of O. sanctum (2mg%), B. diffusa (5mg%) and A. barbadensis (5mg%) were compared with the fish oil treated with BHT (0.002%) and control fish oil sample with added equivalent volume of ethanol only. Table.5.6. shows the initial values of fatty acids analyzed. Values of fatty acids did not vary much on 15th day which is represented in Table 5.7. As the storage period increases, as on 30th day the fatty acids in control decreased as shown in Table 5.8. The value of EPA on 30 days of storage was 6.98 for control and for treated samples the values were 19.24, 19.23 and 19.21 for *O. sanctum*, *B. diffusa* and *A. barbadensis*, respectively. In BHT treated sample also the values were

comparable (19.25). On 30th day degradation in fatty acid DHA was shown in control with a value of 7.24 which is significantly lower than the treated samples (Annexure 5.4.). On 40thday, in control myristic acid, ϒ Linolenic acid and EPA were not detected (Table 5.9). The retaining capacity of fatty acid was observed maximum for *O. sanctum* with values on 0th day for myrisitc acid, palmitic acid, ϒ Linolenic acid, EPA and DHA as 8.23, 16.21, 7.01, 19.26 and 37.29 respectively while that on 45th day the values were 8.01, 15.86, 5.98, 18.67 and 36.56 respectively for these fatty acids. Thus the oxidative changes in fatty acids are found to be reduced significantly in these treated samples. A period of 45 days was chosen for storage studies, as above that period, the fatty acids in control samples were degraded beyond detection. The retaining capacity of native fatty acids in IMP treated fish oil is in the order as *O. Sanctum> B. diffusa> A. barbadensis*.

5.3.5 Accelerated shelf-life study of Fish oil incorporated with medicinal plant extracts

Table 5.10 Changes in microbial and fungal parameters and rancidity index during accelerated shelf life study of fish oil with added *Oscimum sanctum* extracts (2mg%).

Parameters	0 (initial)	1st month	2nd month	3rd month	6th month
Salmonella Spp/g	Absent	Absent	Absent	Absent	Absent
E coli/g	Absent	Absent	Absent	Absent	Absent
P. aeruginosa/g	Absent	Absent	Absent	Absent	Absent
TPC	1250000cfu/gm	16300 cfu/gm	110 cfu/gm	<10 cfu/gm	<05 cfu/gm
Fungus & Yeast	<10 cfu/gm	900 cfu/gm	1000 cfu/gm	200 cfu/gm	110 cfu/gm
RancidityIndex	Absent	Absent	Absent	Absent	Absent

Table 5.11 Changes in microbial and fungal parameters and rancidity index during accelerated shelf life study of fish oil with added *Aloe barbadensis* extracts (5mg %).

Parameters	0 (initial)	1st month	2nd month	3rd month	6th month
Salmonella Spp/g	Absent	Absent	Absent	Absent	Absent
E coli/g	Absent	Absent	Absent	Absent	Absent
P. aeruginosa/g	Absent	Absent	Absent	Absent	Absent
TPC	150000cfu/gm	13900 cfu/gm	210 cfu/gm	<10 cfu/gm	<05 cfu/gm
Fungus & Yeast	<10 cfu/gm	<10 cfu/gm	200 cfu/gm	1700 cfu/gm	2110 cfu/gm
RancidityIndex	Absent	Absent	Absent	Absent	Absent

Table 5.12 Changes in microbial and fungal parameters and rancidity indexduring accelerated shelf life study of fish oil with added *B. diffusa* extracts (5mg%).

Parameters	0 (initial)	1st month	2nd month	3rd month	6th month
Salmonella Spp/g	Absent	Absent	Absent	Absent	Absent
E coli/g	Absent	Absent	Absent	Absent	Absent
P. aeruginosa/g	Absent	Absent	Absent	Absent	Absent
TPC	1050000cfu/gm	1590 cfu/gm	90 cfu/gm	<10 cfu/gm	<05 cfu/gm
Fungus & Yeast	<10 cfu/gm	400 cfu/gm	800 cfu/gm	3900 cfu/gm	4710 cfu/gm
RancidityIndex	Absent	Absent	Absent	Absent	Absent

Table 5.10. shows the results of accelerated shelf life studies of fish oil treated with *O. sanctum* extracts (2mg%). It is found that *E. coli*, *Salmonella* and *P. aeruginosa* were absent throughout the storage period of 6 months in the treated sample. Though TPC was found high at initial stage (1250000cfu/gm.), by 6 months the value was reduced to <05 cfu/gm. Fungus and yeast contents with a high value during 2nd month (1000cfu/gm) decreased to a value of 110 cfu/gm in 6 months. Rancidity index showed absent in all the days.

The results of accelerated shelf life study of fish oil treated with *A. barbadensis* extracts (5mg %) and for *B. diffusa* extracts (5mg %) are shown in Table 5.11 and Table 5.12 respectively. Contrary to *O. sanctum* treated fish oil, the total fungus and yeast content which was minimum at the initial period (<10 cfu/ml) increased to a maximum value of 2110 cfu/gm in *A. barbadensis* and *B. diffusa* treated samples. Rancidty was absent throughout the storage period in both the treatments. Thus total fungus and yeast content was significantly lowered in *O. sanctum* treated fish oil when compared with *A. barbadensis* and *B. diffusa* treated fish oil.

5.4 Discussion

Research for safer and effective natural antioxidants is underway and several natural sources are being examined. A comparative, multi-method screening of antioxidant activity for a large number of Indian medicinal plants in relation to their phenolic compounds is needed to provide a better understanding of their relative importance as natural antioxidants. Here in the present study three medicinal plants namely, *A. barbadensis, B. diffusa* and *O. sanctum* were screened for their uniqueness as natural antioxidant sources. The medicinal and antioxidant properties of Aloe vera were attributed due to the presence of components such as anthrone, chromone, aloe verasin, hydroxyalin and glycoprotein aloctin A(Pereira *et al*., 2009; Jordan, 2002). Holy basil (*O. sanctum*) contains the phenolic compounds which includes eugenol, cirsilineol, isothymusin, isothymonin, rosamarinicacid, orienlin and vicemin (Kelm *et al*., 2000, Devi *et al.*, 2000). *B. diffusa* contains a number of constituents mainly alkaloids, flavonoids, saponins and steroids (Pratt and Hudson, 1990).

The antioxidative activity of the selected medicinal plant extracts was assessed. Total Phenolic content (TPC) was expressed in mg/100 gm of gallic acid equivalents and there was variation for phenolic content among the plants.The variation in the concentration of principal components of phenolics results in changes in total phenolic content of *A. barbadensis, B. diffusa* and *O. sanctum* in experimental trials. In particular, flavonoids and catechins are important antioxidants and super oxide scavengers as reported in recent studies and their scavenging efficiency depend on the concentration of phenol and the numbers and locations of the hydroxyl groups (Benavente-Garia *et al*., 1997). Shahidi *et al*. (1992) reported that the phenolic antioxidants function as free radical terminators or metal chelators.

The decrease in absorbance of the DPPH is due to the free radical scavenging capability of the ethanol extract of the medicinal plants. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability. The inhibition percent for *A. barbadensis* in previous studies was compared with the present investigation (Yun *et al*., 2003). The present value of decrease in absorbance at 16 minute for *A. barbadensis*, *B. diffusa* and *O. sanctum* were low compared with previous studies, probably due to lesser concentration of samples taken. The EC50 of red holy basil in this study (333 µg/ml) was close to the value of 300 µg/ml reported by Juntachote and Berghofer (2005). In a similar study conducted for *B. diffusa*, the absorbance value at 16 min was shown as 14.39% which was very close to the present study (Singh *et al*.,2011).

The presence of metals and heavy metals in the medicinal plants viz; Cu, Zn, Mg, Ni and Pb, As and Hg were also assessed in conformity to food

safety regulations, using atomic absorption spectroscopy. The results showed that the occurrence of heavy metals was less in *O. sanctum* compared to *B. diffusa* and *A. barbadensis* and all the values were within permissible limits of the heavy metals as stipulated by national and international standards. The reports of Adesuyi *et al*. (2012) for *A. barbadensis* showed values of 0.002, 0.007, 0.033, 0.00017 ppm for Copper, Zinc, Magnesium and lead respectively which are similar to the present findings. In the studies of Nwaogu *et al*. (2006), zinc and copper were not detected in *B. diffusa*, may be due to the particular soil pattern in which the plant was cultivated. The absorption of minerals by plants may vary depending on the moisture content of the soil and climatic changes. Among the species selected *O. sanctum* showed less value for all minerals and metals except for zinc, when compared with *B. diffusa* and *A. barbadensis*.

Variations in fatty acids profile of fish oil incorporated with ethanolic extracts of *O. sanctum*, *B. diffusa* and *A. barbadensis* was studied for 45 days of storage and compared with a control and another sample incorporated with a synthetic antioxidant (BHT). The retaining capacity of fatty acids in fish oil using medicinal plant extracts is in the order *O. sanctum*> *B. diffusa*> *A. barbadensis*. By 45th day of storage some fatty acids were totally oxidized in the control samples where no extracts or antioxidants were added, but those incorporated with medicinal plant extracts still retained the fatty acids including PUFA.

Antioxidative property of the plant extracts is significantly important which implies its effective role in reducing lipid peroxidation. The use of synthetic antioxidants being strictly banned, alternative sources like Indian

medicinal plants with their other valuable propertiesin addition to preventing lipid deterioration, need to be explored. Today, studies also emphasize on the control of 'redox' status of food and food components rich in PUFA. The resistance towards oxidative damages increases with the presence of natural antioxidants and thus has got a substantial impact on human health. Antioxidants present in diet such as ascorbates, tocopherols and caroteneoids are known for their role in health. Indian herbs and spices, consisting of their dried leaves, flowers, buds, fruits, seeds, barks or rhizomes, have proved to be good sources of active ingredients with anti-oxidative and antimicrobial activities.

The oxidative changes in freshly extracted oil from Indian sardines were investigated after incorporating medicinal plant extracts at concentration of 2, 5 and 10 mg%. Oxidative deterioration was determined from the results of Peroxide value and TBA assay. The antioxidant activity of holy basil reached a maximum at 0.5 mg /ml concentration (Juntachote and Berghofer, 2005). Similarly Duh *et al*.(1997), observed that antioxidant activity of mug bean hull extract increased with increased concentration upto 100 ppm and no significant differences in antioxidant activity was observed with concentration range from 100 to 500 ppm ($P<0.05$). It is evident from the present study that the fish oil sample stored after 40 days (control) had undergone oxidative deterioration since the OD was above 3.0. In fish oil stored in 2mg% concentration of *O. sanctum*, the increase in TBA was very slow when compared with 5mg% and 10mg% concentration of extracts and thus *O. sanctum* can be effective in reducing the oxidative stress at 2mg % concentration. An activity of 72.24% was reported at this concentration and at

higher concentration the activity in ethanol extract was reduced to 55.03% (Singh *et al*., 2011). Antioxidant activity of holy basil extracts increased with increasing concentration range from 0.10 to 0.75 mg/ml and then reached a plateau at concentrations range from 0.75 to 1.0 mg/ml (Olaleye and Roch, 2008). It was also reported that leaves of this plant has got more antioxidant activity than roots. Ethanol extracts of holy basil have strong superoxide anion scavenging activity, Fe^{2+} chelating activity and reducing power in a concentration dependent manner and additionally act as radical scavengers and lipogenase inhibitors. Primary oxidation products (hydroperoxides) were determined by peroxide value measurement

The percentage scavenging of hydroxyl ions by using *B. diffusa* leaves extract shows that 5mg/ml was the optimum level and it decreased when concentration increased. An activity of 72.24% was reported at this concentration and at higher concentration the activity in ethanolic extract was reduced to 55.03% (Singh *et al*., 2011). It was also reported that leaves of this plant has got more antioxidant activity than roots. The extract with a concentration of 5mg% was found effective in the fish oil treatment. The antioxidant constituents present in the extracts might have been responsible for their ability to reduce the acetaminophen-induced lipid peroxidation (Olaleye and Roch, 2008).

It is suggested that *A. vera* of various development stages contain different active compounds and possess antioxidant activity to different degrees. In the previous studies it was observed that *A. vera* extracts provided equivalent or higher antioxidant activity as compared to BHT and α-tocopherol, which led to screening of antioxidants from *A. vera* for foods, cosmetics and medicines (Yun *et al*., 2003). In this study *A. vera* is found to have optimum antioxidant

activity at 5mg/ ml of the ethanolic extracts. Also studies confirm that the methanolic extracts of *A. vera* showed maximum antioxidant activity at a concentration of 500 µg/microlitre (Saritha *et al*., 2010).

Storage stability studies provide evidence on in what way the quality of a drug or its product varies with time under influence of changing environmental factors such as humidity, temperature and light.In general, pharmaceutical products are studied for its stability profile in an accelerated humidity and temperature, and these investigational findings can be very helpful for predicting reliable expiry date or shelf-life at room temperature by assuming certain criteria and assumptions. It also establishes a retest period for the particular drug substance or its product for the recommended storage conditions. Thus, it is proved that the stability study is unavoidable for the assessment of product quality. In contrast to conventional preparations of medicinal plants, products incorporated with their extracts lead to a number of unique problems with regard to quality and stability. A key part of quality control is to ensure good reproducibility and guarantee chemical stability of the final product during its storage.

In this study, results are reported based on accelerated stability study of fish oil incorporated with the three medicinal plant extracts.As per the results of the previous studies, *Ocimum* extract has shown antimicrobial properties against both gram negative bacteria (*Salmonella enteritica, Vibrio parahaemolyticus, E. coli*) and gram positive bacteria (*Listeria monocytogenes*) (Mishra and Mishra, 2011) with higher antibacterial activity against gram negative bacteria compared to gram positive bacteria. These results are in agreement with the results of present study. Tulsi extract has also been shown to be effective against

filamentous fungi such as *Aspergillus niger*, *Aspergillus fumigatus* (Dharmagadda *et al*., 2005; Bansod Rai, 2008), *Aspergillus flavus* (Kumar *et al*., 2010) *Rhizopus stolonifera* and *Penicillium digitatum* (Grover and Rao, 1977). Other clinically important filamentous fungi like *Fusarium solani*, *Penicillium funiculosum*, *Rhizom ucortauricus* and *Trichoderm areesi* are also susceptible to Tulsi extract (Dharmagadda *et al*., 2005). The leaf extract has also been effective against fungi such as Rizopous, Cladosporium, Curvularia and Lunata. These effects against fungus got similar action in the present study on fish oil storage. Ibrahim *et al*. (2017), investigated the phytoconstituents and antimicrobial activity of aqueous, ethanol and acetone extracts of *A. vera* gel against some human and plant pathogens by disc diffusion method. According to his reports the ethanol and acetone extracts recorded significant antimicrobial activity against many of the pathogens. In general, according to the results of the study, leaves of *Ocimum sanctum* were found to be containing chemical compounds which can be used as antimicrobial compounds against food borne microbial pathogens (Mann *et al*., 2000). According to Girish and Satish, (2008), in experiments of *B. diffusa* extracts against certain bacteria, maximum inhibition was observed in S. aureus followed by megaterium and bacilu cereus, respectively at 50 microlitre concentration. Therefore, the use of *B. diffusa* was experimentally trailed in fish oil, and the results found to be interestingly good in increasing shelf life of fish oil.

Thus *Oscimum* extract showed high antibacterial activity against gram negative bacteria compared to gram positive bacteria, so also the other medicinal plant extracts. The medicinal plant extracts were also found effective in fungal inhibition when incorporated with stored fish oil. This

approach will unquestionably build an innovative way for applying different herbal extracts in maintaining the quality, consistency, safety as well as stability of various food products. These efforts can ensure uniform therapeutic functionality and stability of products. Above studies indicate that the fish oil preserved in medicinal plant extracts were stable at room temperature for more than 2 years. According to "ICH Guidelines, when data show little or no variation with time, then statistical analysis are not required, and proposed shelf life is twice of real time data, but not more than 12 months. By using this method the shelf life of any drug preparation can be predicted in a very short period of time.This approach will unquestionably build an innovative way for maintaining the quality, consistency, safety as well as stability of different products.

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Chapter 6

SUMMARY AND CONCLUSION

Epidemiological studies have related the high habitual intake of fatty fish with low incidence of ischemic heart disease in the Eskimo and Japanese population. Many studies have investigated the effects of supplementation with marine *n*-3 polyunsaturated fatty acids (PUFA) especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on human plasma lipids by lowering of plasma triglycerides and other metabolic changes. Many of the available sources of PUFA in market are fish oil and other EPA and DHA enriched food. Supplementation of PUFA in health drinks has also got predominant application. Marine algae, considered as the primary source of PUFA are presently less utilized for its extraction for PUFA. The non-vegetative source i.e. the marine fish stocks is widely used for PUFA extraction. Due to potential fish sustainability concerns, microalgae have the potential to be a viable alternative for obtaining oils rich in *n*-3 fatty acids. Moreover, fish oil is highly susceptible to oxidative deterioration and needs to be stabilized by adding antioxidants preferably of natural origin. In this respect a coordinated study on various aspects of PUFA enrichment in fish oil and marine micro algae was undertaken. The content of the thesis has been divided into six chapters.

Chapter 1, entitled "General Introduction**"** outlines a brief introduction with the scope and significance of the study. It is apparent that foods either naturally enriched or fortified with long chain *n*-3 PUFAs will become increasingly available over the next 5 years. This will increase intake of these

fatty acids and will provide greater choice for those consumers who do not eat fish and who do not wish to take capsules. The reliability and the stability of these PUFA enhancers is a matter of concern, since the deteriorated fish oil supplements adversely affect health. It can lead to formation of oxidized PUFA (deteriorated fatty acids) which will affect the human metabolism negatively. Bio-enrichment of PUFA using lipase enzyme is found to be eco-friendly as well as consumer friendly. Value added food products can also be prepared using enriched PUFA. There are reports in which marine algae in powdered form is applied in biscuits, bread etc. and these can be replaced by PUFA enriched algae .One of the major drawbacks of fish oil rich in omega-3 PUFA is their high susceptibility to oxidation, leading to the formation of toxic substances such as peroxides or volatile secondary oxidation compounds. Therefore, storage and packaging of PUFA enriched fish oils are essential to preserve omega 3 PUFA from oxidation. Under this perspective, the objectives and scope of the study were discussed.

In Review of Literature (**Chapter 2)** the health benefits of PUFA, lipid extraction methods, bio enrichment methods, prevention of oxidation/rancidity studies and a brief survey of the samples used in the study are reviewed. EPA and DHA, have long been proposed to bestow health benefits by controlling blood pressure ,alleviating symptoms of depression and rheumatoid arthritis, and also attenuating the progression of Alzheimer's disease. Although the best source of long chain n 3 PUFA is marine fishes, fat content and fatty acid profile are found to change with species, season and diet. Sardines in temperate regions show seasonal fluctuation of fatty acid composition and yield, influenced by sea water temperature, food availability and breeding state of the animal. Algae are the primary producers of the oceans' ecosystems, providing the foundation of the oceanic food chain. Specifically, algae

synthesize omega-3 fatty acids and then are consumed by other marine life subsequently. Algae-derived oils are vegetarian-friendly and algae are easy to grow on a large scale due to their small size.

Analysis of different lipid extraction methods from sardine, proximate composition of sardine and its month wise-variation in fatty acid profile are given in detail **Chapter 3**. The changes of fresh sardines and extracted fish oil were performed with emphasis on deterioration of PUFA during storage. Bligh & Dyer method for lipid extraction was found to be the best method for recovery of native lipids and for subsequent analysis of the lipid components. Data for proximate composition of sardine during various months of the year is also reported in this chapter. High content of lipid was obtained during post monsoon, mainly due to intense feeding, sexual development and eventual spawning. The maximum decrease in lipid content was observed after spawning and an increase occurred preceding the sexual maturation. The observations of the study confirm that the fish utilizes fat as a main source of energy and spare protein for body building. The protein content was minimum during post monsoon months, which eventually increased to a maximum value during monsoon season. The high content of fatty acids and specifically PUFA during December-January months allows higher dietary intake of PUFA per unit weight of sardine. Relative concentrations of EPA and DHA to that of total PUFA also showed seasonal variations across the year. Among the lower fatty acids, palmitic acid has been found to be the most abundant fatty acid recording about 34% of the total fatty acids. Lipid peroxidation in stored sardine was assessed by analyzing TBA and PV which showed an increase during the storage period.

Omega-3 PUFAs are typically associated with marine organisms and algae, as the basis of the marine tropic chain and pose as a promising source

of PUFA. Marine algae rich in *n*-3 PUFA being natural and a readily available resource, could be an alternative to fish oil derived *n*-3 PUFA. The use of lipase (EC *3.1.1.3)* as biocatalysts has drawn considerable attention to concentrate PUFA content. Lipase is an enzyme that hydrolyzes lipids, the ester bonds in triglycerides, to form fatty acids and glycerol. Among the lipases assayed, the enzyme from the yeast *C. cylindracea* is of special interest as these are proved to be a nonspecific catalyst for many commercially important reactions such as the modification of oils and fats, reactions in organic solvents, and resolution of racemic mixtures . Hence the enrichment of microalgae using bio-lipase from *C. cylinderacea* has proved to be a novel method for producing PUFA enriched micro algae which will further be useful for both pharmaceutical and food industries for developing PUFA enriched nutritional products. In the present work the algal source of PUFA from *I. galbana, C. calcitrans, C. marina* and *T. gracillus* were subjected to enzymatic enrichment using lipase. In **Chapter 4,** an attempt is made to develop a commercially feasible technology for enzymatic (lipase) enrichment of EPA and DHA in fish oil and the selected marine algae.

The assay of lipase enzyme activity from CC was conducted at 25 ^{o}C at different time intervals and the enrichment of PUFA in fish oil and marine algae used time and concentration of enzyme as variants. For *I. galbana* and *C. calcitrans*, culturing days of 20 was optimum for lipase studies since the lipid content was comparatively high at this stage, while 10 days and 30 daysof culture produced high levels of lipid for *C. marina* and *T. gracillus* respectively. The lipid content in algal species varied with culturing days due to specific growth parameters and tolerance levels. The content of saturated fats present in microalgae improved in a culturing condition under high light.

Thus, manipulation of culture conditions and the harvesting at optimum growth phases may enable the lipid and PUFA composition of micro algal cultures to be tailored for specific purposes. The specific activity of lipase enzyme was found to be 8.0±0.155 U/g of protein. In fish oil, the lipase treatment resulted in an increase of 3.8% for EPA and 11.0% for DHA. It has been reported that because *n*-3 PUFA is located in the 2nd position of triglyceride, hydrolysis of sardine oil with 1,3 specific lipase produced PUFA rich in 2-monoglycerides and 1,2 diacyl glycerides.

In this chapter, hydrolysis yield obtained after 4,8,16, and 24 hr incubation of *I. galbana, C. calcetrans, T. gracillus* and *Chlorella* was examined against a concentration gradient of lipase enzyme. The results revealed that the lipase activity was maximum for the lipids of *I. galbana* at a concentration of 4U and at 8 hr incubation. For *C. calcitran* the pattern was different and maximum enrichment occurred at 4 hr incubation at an enzyme concentration of 8 U. The enrichment percentages of the available PUFA were maximum at 4 U lipase concentration for Chlorella and 12 U enzyme concentration for *T. gracillus*. Already established methodology utilizing lipase from *C. cylinderacea* for the enrichment of PUFA in fish oil is effectively applied in algal PUFA enrichment also.

Thus PUFA can be supplied throughout the seasons if cultured on regular basis both in batch culture and *in vivo*. Lipid biosynthesis can be achieved by regular monitoring of culture days where the maximum accumulation depends on the type of species, nutrient supply and even light source. This novel study will further improve the industrial procedure for preparing PUFA enriched products. The results reported in this study can be effectively applied in pharmaceutical industry for maximizing PUFA enriched products. The development of feasible technology for natural selective

hydrolysis can be an opportunistic way in synthesizing PUFA. As there is a scarcity of marine fish and fish lipids, marine algae, a primary autotroph synthesizing PUFA, can be used as an alternative ideal source. Further enrichment of PUFA by lipases from *C. cylinderacea* can be an effective method suited for industrial processes. Due to the variation in substrate specificity of the enzyme, the pattern of enrichment differed for each alga. The time of action of enzyme for enrichment also differed in each species indicating that enzyme activity also depended on incubation time. Brown algae also should be investigated for PUFA enrichment, to be used as a functional food because of its richness in other bioactive compounds such as carotenoids, phenolic compounds, and sulphated polysaccharide and for their antioxidant activity.

Research for safer and effective natural antioxidants is underway and several natural sources are being examined. A comparative, multi-method screening of antioxidant activity for a large number of Indian medicinal plants in relation to their phenolic compounds is needed to provide a better understanding of their relative importance as natural antioxidants. Here in the present study (**Chapter 5**) three medicinal plants namely, *A. barbadensis, B. diffusa* and *O. sanctum* were screened for their uniqueness as natural antioxidant sources. The medicinal and antioxidant properties of Aloe vera were attributed to the presence of components such as anthrone, chromone, aloe verasin, hydroxyalin and glycoprotein aloctin A. Holy basil (*O. sanctum*) contains the phenolic compounds which includes eugenol, cirsilineol, isothymusin, isothymonin, rosamarinic acid, orienlin and vicemin. *B. diffusa* contains a number of constituents mainly alkaloids, flavonoids, saponins and steroids.

Oxidative deterioration occurred mainly in fish oil compared to algal oil, and hence the study was focused on stability of fish oil. Antioxidative property of medicinal plant extracts was measured in terms of four experiments such as DPPH radical scavenging assay, reducing property assay, total Phenolic content and β carotene assay. The results confirmed highest antioxidative activity in *O. santum* followed by B. *diffusa* and *A. barbadensis* in the order *A. barbdensis*< *B. diffusa*< *O. sanctum*. The presence of metals and heavy metals in the medicinal plants viz; Cu, Zn, Mg, Ni and Pb, As and Hg were also assessed in conformity to food safety regulations, using atomic absorption spectroscopy. The results showed that the occurrence of heavy metals was less in *O. sanctum* compared to *B. diffusa* and *A. barbadensis* and all the values were within permissible limits of the heavy metals as stipulated by national and international standards.

Variations in fatty acids profile of fish oil incorporated with ethanolic extracts of *O. sanctum*, *B. diffusa* and *A. barbadensis* was studied for 45 days of storage and compared with a control and another sample incorporated with a synthetic antioxidant (BHT).The retaining capacity of fatty acids in fish oil using medicinal plant extracts is in the order *O. sanctum*> *B. diffusa*> *A. barbadensis*. By 45th day of storage some fatty acids were totally oxidized in the control samples where no extracts or antioxidants were added, but those incorporated with medicinal plant extracts still retained the fatty acids including PUFA. Antioxidative property of the plant extracts is significantly important which implies its effective role in reducing lipid peroxidation. The use of synthetic antioxidants being strictly banned, alternative sources like Indian medicinal plants with their other valuable properties in addition to preventing lipid deterioration, need to be explored. Today, studies also emphasize on the control of 'redox' status of food and food components rich

in PUFA. The resistance towards oxidative damages increases with the presence of natural antioxidants and thus has got a substantial impact on human health. Antioxidants present in diet such as ascorbates, tocopherols and caroteneoids are known for their role in health. Indian herbs and spices, consisting of their dried leaves, flowers, buds, fruits, seeds, barks or rhizomes, have proved to be good sources of active ingredients with anti-oxidative and antimicrobial activities.

Storage stability studies provide evidence on in what way the quality of a drug or its product varies with time under influence of changing environmental factors such as humidity, temperature and light. In general, pharmaceutical products are studied for its stability profile in an accelerated humidity and temperature, and these investigational findings can be very helpful for predicting reliable expiry date or shelf-life at room temperature by assuming certain criteria and assumptions. It also establishes a retest period for the particular drug substance or its product for the recommended storage conditions. Thus, it is proved that the stability study is unavoidable for the assessment of product quality. In contrast to conventional preparations of medicinal plants, products incorporated with their extracts lead to a number of unique problems with regard to quality and stability. A key part of quality control is to ensure good reproducibility and guarantee chemical stability of the final product during its storage. In this Chapter results are reported based on accelerated stability study of fish oil incorporated with the three medicinal plant extracts. *Ocimum* extract showed high antibacterial activity against gram negative bacteria compared to gram positive bacteria, so also the other medicinal plant extracts. The extracts were also found effective in fungal inhibition when incorporated with stored fish oil. Above studies indicate that the fish oil preserved in medicinal plant extracts were stable at room

temperature for more than 2 years. According to "ICH Guidelines, when data show little or no variation with time then statistical analysis are not required, and proposed shelf life is twice of real time data, but not more than 12 months. By using this method the shelf life of any drug preparation can be predicted in a very short period of time. This approach will unquestionably build an innovative way for maintaining the quality, consistency, safety as well as stability of different PUFA based products. These efforts can ensure uniform therapeutic functionality and stability of these products.

Future perspectives:

- To commercialize capsules of bio- enriched (PUFA) marine algae using the microbial lipase esterification technology.
- To explore the extraction and use of active compounds from other medicinal plants, herbs and spices so as to enhance oxidative and microbial stability of marine fish oil and PUFA enriched foods.
- To investigate the feasibility of providing normal manufactured foods, enriched with omega-2 PUFA and incorporated with medicinal plant/herbal extracts.

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ANNEXURE A

3.1.Correlation test showing storage Index of Frozen Sardine

PARAMETERS	Peroxide value	TBA value	Lipid	SFA	MUFA	PUFA	EPA/DHA	ω-6	PUFA/ω 6
Peroxide value	1								
TBA value	0.57	1							
Lipid	-0.86[a]	-0.40	1						
SFA	-0.48	-0.69[b]	0.71[b]	1					
MUFA	-0.97[a]	-0.62[b]	0.93[a]	0.65[b]	1				
PUFA	-0.88[a]	-0.58[b]	0.98[a]	0.80[a]	0.96[a]	1			
EPA/DHA	-0.69[a]	0.00	0.39	-0.30	0.53	0.32	1		
ω-6	-0.48	-0.69[b]	0.71[b]	1.00[a]	0.65[b]	0.80[a]	-0.30	1	
PUFA/ω 6	-0.71[a]	-0.67[b]	0.88[a]	0.95[a]	0.84[a]	0.94[a]	0.00	0.95[a]	1

[a]Correlation is significant at the 0.01 level (2-tailed).
[b]Correlation is significant at the 0.05 level (2-tailed).

3.2.Two factor ANNOVA for Annual Variation of Fatty Acids

Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
January	6	63.35	10.55833	95.95354
February	6	57.68	9.613333	80.90279
March	6	49.77	8.295	65.81379
April	6	51.27	8.545	93.39115
May	6	48.7	8.116667	100.9161
June	6	44.59	7.431667	102.8128
July	6	46.75	7.791667	122.2608
August	6	55.26	9.21	137.9927
September	6	63.06	10.51	154.0495
October	6	65.63	10.93833	118.3255
November	6	77.91	12.985	148.9654
December	6	70.5	11.75	119.2804
Palmitic acid	12	319.21	26.60083	19.54672
Linoleic acid	12	185.01	15.4175	40.70206
EPA	12	121.08	10.09	17.24393
DHA	12	31.79	2.649167	2.573899
Teradecanoic acid	12	14.76	1.23	0.187782
Oleic acid	12	22.62	1.885	0.457191

ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Rows	196.4037	11	17.85489	1.420285	0.190293	1.967547
Columns	6011.899	5	1202.38	95.64454	7.34E-26	2.382823
Error	691.4236	55	12.57134			
Total	6899.726	71				

4.1. Two factor ANNOVA for Enrichment of PUFA in *Isochrysis galbana*

Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
1	5	10.51	2.102	4.34922
	5	7.05	1.41	2.0939
	5	8.23	1.646	8.02028
	5	8.94	1.788	3.32912
2	5	14.18	2.836	6.90083
	5	1.28	0.256	0.32768
	5	1.69	0.338	0.57122
	5	3.08	0.616	1.89728
3	5	11.31	2.262	2.37832
	5	3.94	0.788	0.52287
	5	5.74	1.148	0.46637
	5	7.99	1.598	1.40547
4	12	7.06	0.588333	1.202524
8	12	30.64	2.553333	5.963152
16	12	9.55	0.795833	1.697063
24	12	7.5	0.625	0.630427
Control	12	29.19	2.4325	1.051384

ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Rows	35.1879	11	3.1989	1.741929	0.09532	2.014046
Columns	48.24809	4	12.06202	6.568253	0.00031	2.583667
Error	80.80215	44	1.836413			
Total	164.2381	59				

4.2. Two factor ANNOVA for Enrichment of PUFA in *Chaetocerous Calcitrans*

Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
1	5	0.4	0.08	0.032
	5	36.33	7.266	62.05703
	5	7.96	1.592	2.36212
	5	3.08	0.616	0.53593
2	5	1.02	0.204	0.08408
	5	63.24	12.648	38.86412
	5	12.24	2.448	0.37957
	5	12.54	2.508	3.11107
3	5	0.4	0.08	0.032
	5	39.85	7.97	20.83675
	5	10.75	2.15	1.852
	5	3.75	0.75	1.0613
4	12	45.7	3.808333	44.13243
8	12	20.77	1.730833	9.864372
16	12	37.55	3.129167	12.67544
24	12	41.25	3.4375	37.32962
Control	12	46.29	3.8575	19.68897

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	872.0311	11	79.27555	7.139484	8.78E-07	2.014046
Columns	36.26377	4	9.065943	0.816471	0.521588	2.583667
Error	488.5681	44	11.10382			
Total	1396.863	59				

4.3. Two factor ANNOVA for Enrichment of PUFA in *Chlorella marina*

SUMMARY	Count	Sum	Average	Variance
1	5	9.9	1.98	7.50755
	5	59.09	11.818	49.88157
	5	3.36	0.672	1.50797
	5	10.28	2.056	4.06553
2	5	9.66	1.932	7.22987
	5	47	9.4	28.9151
	5	0.53	0.106	0.05618
	5	4.18	0.836	1.87223
3	5	9.89	1.978	7.49552
	5	48.36	9.672	120.1334
	5	1.4	0.28	0.16145
	5	6.86	1.372	2.52637
4	12	21.86	1.821667	5.76047
8	12	31.67	2.639167	20.30752
16	12	73.69	6.140833	96.73157
24	12	46.03	3.835833	31.23604
Control	12	37.26	3.105	5.934627

ANOVA						
Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	963.8649	11	87.62408	4.844713	7.32E-05	2.014046
Columns	129.6034	4	32.40086	1.791435	0.147636	2.583667
Error	795.8076	44	18.08654			
Total	1889.276	59				

4.4. Two factor ANNOVA for Enrichment of PUFA in *Tetraselmis gracillus*

Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
11.8	4	61	15.25	88.60113
15.31	4	24.72	6.18	61.0266
0	4	1.79	0.4475	0.801025
0	4	7.97	1.9925	5.419558
20.78	4	23.26	5.815	28.39923
5.74	4	33.14	8.285	66.6659
0	4	1.79	0.4475	0.801025
0	4	4.42	1.105	4.8841
11.66	4	36.13	9.0325	36.85896
2.04	4	20.92	5.23	57.6126
1.82	4	3.02	0.755	0.8123
4.85	4	4.42	1.105	4.8841
8	12	72.21	6.0175	77.91213
16	12	75.62	6.301667	53.42692
24	12	9.08	0.756667	5.531988
Control	12	65.67	5.4725	22.32188

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	926.0058	11	84.18234	3.366819	0.003343	2.093254
Columns	245.1833	3	81.72777	3.26865	0.033387	2.891564
Error	825.1163	33	25.00352			
Total	1996.305	47				

5.1. Two factor ANNOVA for Fish oil storage in *Oscimum sanctum*

Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
10	5	55.97	11.194	11.85613
20	5	106.64	21.328	58.51067
30	5	154.09	30.818	40.73412
40	5	239.76	47.952	64.18497
1.4	4	97.61	24.4025	217.9154
2.7	4	119.44	29.86	210.7407
2.01	4	120.35	30.0875	333.0068
1.01	4	78.62	19.655	189.828
2.01	4	140.44	35.11	315.9241

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	3664.277	3	1221.426	106.2351	6.59E-09	3.490295
Columns	563.175	4	140.7937	12.24572	0.000338	3.259167
Error	137.9686	12	11.49738			
Total	4365.42	19				

5.2. Two factor ANNOVA for Fish oil storage in *Boerhavia diffusa*

Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
10	5	64.56	12.912	8.66327
20	5	82.86	16.572	72.60607
30	5	135.16	27.032	41.22082
40	5	181.18	36.236	67.99298
2.04	4	87.17	21.7925	103.4727
1.02	4	73.65	18.4125	88.36642
1.02	4	105.07	26.2675	164.1584
1.05	4	68.04	17.01	76.56687
2.01	4	129.83	32.4575	167.6336

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	1671.971	3	557.3238	51.99614	3.78E-07	3.490295
Columns	633.3098	4	158.3275	14.77133	0.000139	3.259167
Error	128.6227	12	10.71856			
Total	2433.904	19				

5.3. Two factor ANNOVA for Fish oil storage in *Aloe Bardabensis*

Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
10	5	67.75	13.55	21.5098
20	5	137.43	27.486	116.2894
30	5	160.2	32.04	63.85085
40	5	273.12	54.624	82.43923
2.7	4	154.12	38.53	326.9206
1.02	4	122.55	30.6375	353.0815
1.6	4	143.09	35.7725	347.7452
1.07	4	78.42	19.605	191.2187
2.08	4	140.32	35.08	318.3939

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	4363.016	3	1454.339	70.07066	7.1E-08	3.490295
Columns	887.2934	4	221.8234	10.68754	0.00063	3.259167
Error	249.0638	12	20.75532			
Total	5499.373	19				

5.4. Two factor ANNOVA for Fatty Acid storage variation

Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
16.23	4	64.91	16.2275	0.000158
7.02	4	28.07	7.0175	2.5E-05
19.28	4	77.1	19.275	3.33E-05
37.31	4	149.24	37.31	6.67E-05
8.23	4	32.7	8.175	0.002833
16.21	4	64.84	16.21	0.0002
7.01	4	28.01	7.0025	0.000225
19.26	4	76.97	19.2425	0.000625
37.29	4	149.07	37.2675	0.000492
8.2	4	27.63	6.9075	6.376892
16.19	4	58.71	14.6775	9.030292
6.98	4	24.86	6.215	2.220367
19.24	4	64.67	16.1675	37.51589
37.25	4	118.94	29.735	224.9018
8.01	4	23.26	5.815	15.27083
15.86	4	48.25	12.0625	39.62356
5.98	4	17.14	4.285	8.7465
18.67	4	55.5	13.875	85.6299
36.56	4	112.13	28.0325	274.0088
8.23	19	338.49	17.81526	126.9314
8.24	19	336.03	17.68579	127.9427
8.24	19	204.95	10.78684	124.7438
8.23	19	342.53	18.02789	125.9318

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	7700.534	18	427.8075	16.50869	7.26E-16	1.798236
Columns	710.6287	3	236.8762	9.140834	5.46E-05	2.775762
Error	1399.36	54	25.91407			
Total	9810.523	75				

ANNEXURE B

LIST OF PUBLICATION

- Jithu Paul Jacob and Saleena Mathew, 2016. Increase of oxidative stability in fish oil using potent natural antioxidants present in three Indian Medicinal Plants (IMP), International Journal of Nutrition and Agriculture Research, Vol 2, issue 1, 25-34
- Jithu Paul Jacob and Saleena Mathew, 2016. Effect of Lipases from *Candida cylinderacea* on enrichment of PUFA in marine microalgae, Journal of Food Processing and Preservation ISSN 1745-4549.

ANNEXURE C

CURRICULUM VITAE

JITHU PAUL JACOB

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Objective

To work in an organization, which provides a challenging work atmosphere and allows me to grow both professionally and as an individual for the betterment of science and society.

Professional Expertise

Asst. Professor

From June 2018 till date, working as Asst. Professor in St. Alberts College, Ernakulam in the Dept. of Fisheries and Aquaculture.

Key Responsibilities

Provide academic instruction to students through lecture and lab courses. Create assignments and activities in one-on-one, classroom, and distance mediated instructional settings for development of cognitive abilities, communication skills, and higher order thinking skills among students.

Observe and evaluate student performance in meeting course objectives and student learning outcomes through assignments, projects, discussions, and

examinations; provide feedback in a timely manner to student inquiries in class, online, or during established consultation office hours.

Maintain current, accurate records of course enrollment, attendance, student academic progress, course curriculum; prepare and submit grades, data, and reports related to course and student progress in a timely manner.

Provide a course syllabus to students in each class within the first week of the class, as outlined in the faculty handbook.

Adhere to the responsibilities related to teaching, advising, service, and scholarly, professional and creative activities, as outlined in the faculty handbook.

Attend and participate on committees and in department, division, campus, and University meetings.

Performs related duties as assigned.

Research Scholar

From Dec 2011 till date, working as research scholar in School of Industrial Fisheries, CUSAT in the topic "Enrichment of Poly unsaturated fatty acids (PUFA) in marine algae and fish oil and their oxidative stability using medicinal plant extracts"

MPEDA : Worked as Technical Consultant in Ornamental fish division for a period 16/03/2016 -31-07-2017.

My Key responsibilities:

- Arranging financial subsidies to entrepreneurs
- Taking trainings and awareness programme for the production of Ornamental fish sector

Pathologist

National Prawn Company, Saudi Arabia: Worked as Pathologist in the lab for a period 11/4/2008-07/08/2009

My Key responsibilities:

- Collection of samples inside company and nearby areas
- Isolation of DNA/RNA from shrimps, fishes and crabs collected
- Amplification of samples by PCR techniques
- Analysis by electrophoresis and gel-doc
- Reporting and Documentation

Biochemist

Worked in Hospital as Biochemist from 1/1/2005 - 30/6/2005, analyzed clinical biochemical test of blood, stool and urine samples

My key responsibilities:

- Collection of blood samples
- Routine biochemical analysis of samples using sophisticated instruments
- Reporting and documentation

Educational Qualification

- **PhD Thesis under submission**
- **Master of Philosophy in Fisheries Science(2011)**

  School of Industrial Fisheries, Kochi

  Cochin University of Science and Technology

  With an aggregate of GPA 8.00

- **Master in Marine Biology, Micorbiology & Biochemistry (2007)**

  School of Marine Science, Kochi

  Cochin University of Science and Technology

  With an aggregate of GPA 7.08

- **Bachelor in Medical Bio-Chemistry (2004)**

  School Of Health Science, Calicut

  University Of Calicut, with an aggregate of 62%

Dissertations

- MPhil Thesis-Studies on fractionation of lipids from fish oil and the enrichment of poly unsaturated fatty acids (PUFA) by lipase hydrolysis
- MSc Thesis-Studies on meiobenthos of Cochin backwaters

Publications

- Jithu Paul Jacob and Saleena Mathew, 2016. Increase of oxidative stability in fish oil using potent natural antioxidants present in three Indian Medicinal Plants (IMP), International Journal of nutrition and agriculture research, Vol 2, issue 1, 25-34
- Jithu Paul Jacob and Saleena Mathew, 2016. Effect of Lipases from *Candida cylinderacea* on enrichment of PUFA in marine microalgae, Journal of food Processing and Preservation ISSN 1745-4549.
- Jithu Paul Jacob and Saleena Mathew, Enrichment of PUFA in *Isochrysis galbana* and *Chaetocerous calcitrans* using lipases from *Candida cylinderacea* presented poster in World Ocean Science Congress, 2015.

Professional training

1. International Course on Biology and Pathology of Shrimp 6-17 Oct, 2008, Faculty of Science, Mahidol University, Bangkok, Thailand
2. PCR detection of WSSV, general health assessment shrimp, histopathology,haematology and analysis of water quality parameters
3. Training programme on Ornamental Fish Breeding and Farming conducted by MPEDA in association with Department of Aquaculture,SH College during the period from 19-22 July, 2016.
4. Training in Biochemistry Laboratory for a period of 60 days from 23.07.2002 to 30.09.2002 at National Hospital, Calicut.
5. Training in Biochemisrty department for a period of 60 days from 21.07.2003 to 21.09.2003 at Jeeva Speciality Laboratory, Trissur.

6. Training in Clinical Chemisrty Laboratory from 1.06.2004 to 30.06.2004 at AMritha Institute of Medical Sciences and Research Center, Kochi.

Workshops and seminars attended

- National seminar on biodiversity held on 17.12.2010 organized by Swadeshi Science movement, Kerala and School of Marine Science, CUSAT, Kochi.
- Workshop on Profining Reseaarch Publications for Quality Research held on 3.01.2012 organized by the University Library, CUSAT, Kochi.
- Colloquium on Indian Aquaculture-Challenges in the new millennium held on 20-21, October, 2005 organized by PG Department of Aquacultre & Biochemistry, St. Albert's College, Kochi.
- National seminar on Modern Statistical Tools in Fisheries (MoSTiF) held from 7-9, March, 2013 organized by School of Industrial Fisheries, CUSAT, Kochi.
- National training workshop on Modern Statistical Tools in Fisheries Applications of SYSTAT-13 Held on 26-27, February,2015 organized by School of Industrial Fisheries, CUSAT.
- Seminar on Solid Waste Management, Issues and Options held on 15.03.2012 at School of Environmental Studies, CUSAT, Kochi.
- UGC Sponsored National Seminar on Marine Biology-Advance & Prospects held on 10.9.2006 at Department of Marine Biology, Microbiology and Biochemistry, School of Marine Science, Cusat, Kochi

- Seminar and workshop session on Automation in histopathology lab, Leptospirosis, Karyotyping and Role of anitoxidants & Exercise in health and disease conducted by the Dept. of BSc. MLT, School of Medical Eduaction, Kottayam on 9-10, December, 2002.
- Seminar and workshop session on Quality Control in Microbiology, HLA –in Health and Disease, Automation in Haematology conducted by BSc MLT Depatrment, School of Medical Education, MG University on 14-15, November, 2000.

Specialisation

- Exposure to topics like algology, planktonology, marine microbiology, biochemistry, marine pollution, marine ecology and fish pathology.
- Experienced in handling microbiology, biochemical and molecular biology techniques. Have got experiencing in using Gas chromatography, HPLC, Texture analyser, other chormotography techniques, electrophoretic, PCR techniques.
- On board sample collection in Arabian estuary for the project of Post graduation.

Interested areas

- Laboratory analysis
- Teaching and Reading
- Queer Scientific Innovations
- Cruises and explorations in sea

Skills & Potentials

- Research activities
- Conducting Lectures & Workshops
- Computer Applications
- Dance choreographing and singing

Linguistic skills

- English, Hindi, Malayalam (Speak, Write & Read)

Personal Information

Age	:	35 Years
Date of Birth	:	26-07-1982
Sex	:	Male
Marital Status	:	Married
Nationality	:	Indian
Mother Tongue	:	Malayalam
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References:

1. Prof.(Dr.) Saleena Mathew
   Professor (Retd.)School of Industrial fisheries
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2. Prof. (Dr.) Bijoy Nandan
   Dept.of Marine biology, Microbiology & Biochemistry
   Cochin University of Science and Technology
   Kochi-16

3. Mr. Lal Paul
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   CUSAT, S.Kalamaserry
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   Cell: 91-9995215064

I hereby declare that the above mentioned information's are true to my knowledge

Kochi **JITHU PAUL JACOB**

20-03-2018

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