EXTRACTION OF OMEGA-3 FATTY ACIDS FROM ATLANTIC HERRING (*Clupea harengus*)

by

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DEDICATION

TO:

My parents

Mohanarangan. B.V and Maduram. N

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ABSTRACT

The fish processing industry faces problems due to the negative environmental impact of its wastes. Fish wastes can be used to produce oil, protein, calcium, bio-diesel and numerous bioactive compounds. In particular, fish oil is rich in omega-3 fatty acids which can aid prevention and treatment of various diseases. The challenge is to develop environmentally friendly and economically feasible techniques for extracting omega-3 fatty acids. In this study, extraction of fish oil and omega-3 fatty acids from Atlantic herring (*Clupea harengus*) by enzymatic hydrolysis was studied. The effects of temperature, pH and enzyme loads on the extraction of fish oil using enzyme alcalase were evaluated; the effect of mixing on the extraction of omega-3 fatty acids was also investigated. Fatty acids were categorized into three groups: saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Increasing the reaction temperature decreased the recovery yield (RY), and increased peroxide value (PV), acid value (AV), *p*-anisidine value (*p*-AV), SFA, PUFA and MUFA. Increasing the pH up to 7.5 decreased PV and increased AV, *p*-AV, RY, SFA, MUFA and PUFA. Generally, increasing enzyme load increased RY, PV, AV, *p*-AV. However, few samples showed increased SFA, MUFA and PUFA with increased enzyme load. The optimum conditions of enzymatic extraction were at 55°C, pH of 7.5 and enzyme load of 2.0%. Concentration of omega-3 fatty acid should be carried out with immobilized enzymes and a higher centrifugal speed should be used to separate the oil after hydrolysis. Also, antioxidants should be used to avoid oil oxidation. A constant fast stirring (200 rpm) gave the highest omega-3 fatty acid content. The results showed that solvent extraction was better (RY of 97.5%, PV of 12.04 Meq/g, AV of 5.11 mg KOH/g and *p*-AV of 9.33) compared to enzymatic extraction (RY of 20.2%, PV of 31.44 Meq/g, AV of 11.13 mg KOH/g and *p*-AV of 58.27). Extraction of fish oil using solvent system can be used but choloroform-methanol should be replaced with hexane-isopropanol.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Ab	Absorbance
ALA	Alpha-linolenic acid
AOCS	American oil chemists' society
ARA	Arachidonoic acid
AV	Acid value
BHT	Butylated hydroxytoluene
CC	*Candida cylindracea*
CO_2	Carbon dioxide
CR	*Candida rugosa*
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionization detector
FM	Fish meal
FWM	Fish waste meal
GC	Gas chromatography
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
LFW	Liquid fish waste
MHO	Menhaden oil
PUFA	Polyunsaturated fatty acid
PV	peroxide value
RA	Rheumatoid Arthritis
SBO	Seal blubber oil
SFW	Solid fish waste
Spp	Species

TAG	Triacylglycerol
TOTOX	Total oxidation value
VLDL	Very low density lipoprotein
WHO	World health organization

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CHAPTER 1. INTRODUCTION

Fish is considered to be one of the most perishable human foods throughout the world. It has an excellent nutritional value, providing high quality protein and a wide variety of vitamins (including vitamins A and D) and minerals including phosphorus, magnesium, selenium, as well as iodine in marine fish (FAO, 2005).

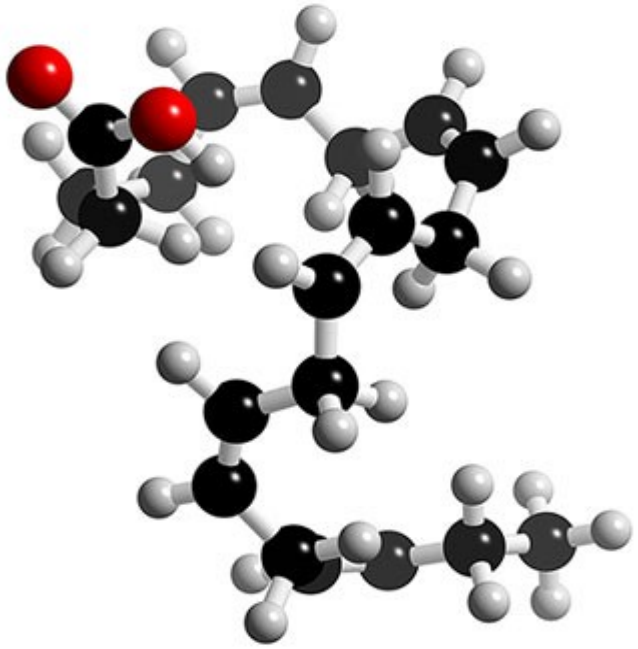
Canada has the world's most important commercial fishing and the capture industries which operate in three broad regions namely the Atlantic Ocean, Pacific Ocean, and freshwater bodies (CFS, 2006). Canada was ranked 19th in terms of total volume of fish landings in 2005 (FAO, 2005). Freshwater commercial fishing in Canada includes the Lakes of Winnipeg, Cedar, Manitoba and Winnipegosis in the Province of Manitoba as well as the Great Slave Lake in the Northwest Territories (FAO, 2005). In the year 2001, the fisheries capture in Atlantic and Pacific coasts reached 958,744 tones (AMEC, 2003). Fish processing generates wastes of up to 50% of the body weight of the processed fish (Babbit, 1990). Fish processing involves scaling, cutting, filleting, cooking, salting and canning. Large amounts of processing waste are discarded every year, including the fins, frames, trimmings, heads, skin and viscera. The waste produced from fish processing can be utilized for the production of fish meal and fish oil (Babbit, 1990).

Herring is the most widely processed fish species of which there are two types: Atlantic herring (*Clupea harengus*) with 9%-18% fat and Pacific herring (*Clupea harengus pallasi valenciennes*) with 14%-17% fat (Hilakivi-Clarke et al., 2002; Foodlexicon, 2008). In the year 2007, the total Canadian catch (by volume) of Atlantic herring was about 55% (James, 2009). According to British Columbia seafood production (2010), the harvest of herring for the years 2008, 2009 and 2010 were 11.4, 12.3 and 10.1 thousand tonnes, respectively. Herring waste from roe harvesting is about 95% of the total landings and it is particularly oily (Mathur, 1994). Fish lipids have gained great importance because of the presence of health beneficial omega-3 fatty acids (polyunsaturated fatty acids) in them which include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Holub and

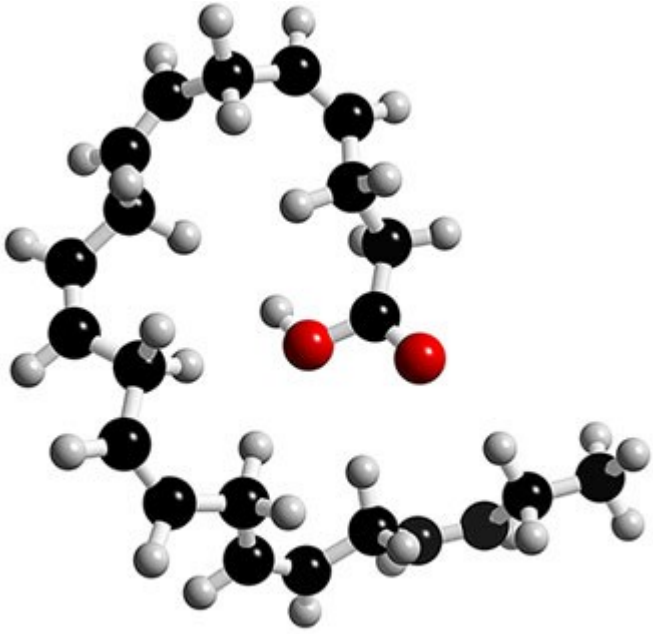
Holub, 2004). The importance of polyunsaturated fatty acids in the prevention of disease and in human nutrition was scientifically recognized three decades ago (Bang et al., 1976).

Omega-3 fatty acids are long-chain polyunsaturated FA commonly having 18, 20, or 22 carbon atoms in chain length with the first of the 3-6 double bonds adjacent to the third carbon atom when counting from the methyl carbon end of the fatty acid molecule (Darren and Bruce, 2004). Figure 1.1 represents the structure of EPA and DHA. Long chain omega-3 fatty acids are mostly obtained from seafood or dietary alpha-linolenic acid, whereas EPA and DHA are synthesized in marine plants such as phytoplankton and micro algae. These are transferred through the food web into lipids in aquatic species like fish and marine mammals (Cho et al., 1987). Increased intake of marine lipid will in turn increase omega-3 fatty acids in the human diet. The health benefits of the omega-3 fatty acids include a major role in the prevention of atherosclerosis, heart attack, depression, stroke, diabetes, obesity, premature aging, hyper tension and cancer as well as improving vision and memory (Chin and Dart, 1995; Connor, 2000).

Concentration of omega-3 polyunsaturated FA from fish oil has been carried out by urea complexation (Gamez et al., 2003; Tor and Yi, 2001), supercritical fluid extraction (Letisse et al., 2006; Mishra et al., 1993) and enzymatic hydrolysis (Zuta, 2003; Wanasundara and Shahidi, 1998; Hoshino et al., 1990). Gas chromatography has been used to analyze and quantify FA composition (Razak et al., 2001). The aim of this study was to investigate the potential of developing environmentally friendly and cost effective enzymatic techniques for the extraction of fish oil from Atlantic herring and separation of omega-3 FA from fish oil.



(a) EPA



(b) DHA

Figure 1.1. Structure of EPA and DHA (Darren and Bruce, 2004).

CHAPTER 2. OBJECTIVES

The aim of this study was to extract fish oil with high omega-3 content from Atlantic herring (*Clupea harengus*) by enzymatic hydrolysis and to concentrate omega-3 fatty acids from the fish oil by enzymatic hydrolysis. The specific objectives of this study were:

1. To optimize the recovery process of fish oil from Atlantic herring with enzyme alcalase and to study the effects of the following parameters on the oil yield
   (a) pH (7.0, 7.5 and 8.0)
   (b) Enzyme load (0.5, 1 and 2% by weight)
   (c) Temperature (55 and 70°C)

2. To determine the quality of oil by measuring the acid value, *p*-anisidine value, peroxide value, fatty acid composition and lipid yield.

3. To compare the effectiveness of the optimized enzymatic hydrolysis method with the the solvent extraction method with respect to oil yield and quality.

4. To determine the effect of stirring of oil samples (extracted by chemical method) with the enzyme *Candida rugosa* (constant-fast stirring, constant-slow stirring, intermediate stirring, and no stirring) on the enrichment of omega-3 fatty acids in the oil at the optimum condition (20 h, 35°C and enzyme load of 800 Units).

CHAPTER 3. LITERATURE REVIEW

3.1. Canadian Fish Production

The total freshwater fishery landing in Canada for the year 2007 was 32,000 tonnes valued at $63M. In the year 2009, commercial sea fisheries had total landings 924,756 tonnes which include top four species: herring 166, shrimp 141, snow crab 97 and hake 69 thousand tonnes which was valued at $1.7 billion. Figure 3.1 shows the total Canadian value by species groups in 2009. Aquaculture production was 155 thousand tonnes, representing 14% of total marine production. The salmon aquaculture represents 65% of the total aquaculture production and 75% of the total aquaculture value. In 2010, Canada's fish and seafood exports were valued at $3.9 billion (Fisheries and Ocean Canada, 2011). Table 3.1 describes the commercial sea fisheries landings by species groups and region in 2008 and 2009.

3.1.1. Atlantic region

There was a decline in the catch of fish in northwest Atlantic during the past few years due to environmental variations, fishing pressure and predation. Herring stocks, the most important pelagic fisheries in northwest Atlantic, showed a decline in the recent years but mackerel stocks remained abundant. The Canadian fishing efforts were dependent on market conditions. The most important species in Atlantic Canada is Atlantic salmon. Bluefin tuna was highly migratory species that is managed by International Commission for the Conservation of Atlantic Tunas (FRBSS, 1996).

3.1.2. Pacific region

The major fisheries in Pacific Canada are divided in groups of 12 species that represent about 80–90% of the total catch. The population dynamics of these species, climate and ocean environment had a major impact on their productivity (Beamish et al., 2004). The Pacific salmons have five major species in Canada's Pacific coast (sockeye,

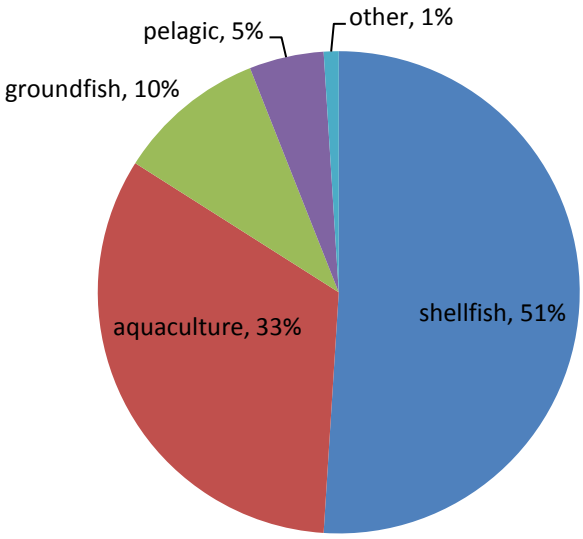


Figure 3.1. Total Canadian landed fish value by species groups 2009.

Table 3.1. Canadian commercial sea fisheries landings by species groups and region, 2008 and 2009 (DFO, 2009; DFO, 2010).

Landings (tonnes)	Atlantic		Pacific		Canada	
	2008	2009	2008	2009	2008	2009
Total	772,574	767,573	148,799	157,183	921,373	924,756
Ground fish	111,811	105,459	109,940	98,189	221,751	203,648
Pelagic	213,219	238,472	25,457	45,648	238,676	284,120
Shellfish	432,478	407,029	13,395	13,185	445,873	420,214
Other	15,065	16,613	6	161	15,071	16,774

pink, chum, chinook and coho) which were fished commercially (Groot & Margolis, 1991). The Pacific halibut fishery is the oldest and successful fisheries in Canada's pacific coast used for commercial purposes (Clark and Hare, 2002). The Pacific cod are a relatively fast growing species that reaches maturity by age 2–3 years (DFO, 1999). The Canadian fishery for albacore tuna started in the north Pacific with troll vessel in mid 1930s. By mid 1980s albacore tuna fishery began in the south Pacific (Shaw, 2001). According to Benson et al. (2002) it was found that there were inverse relationship between Pacific hake distribution and upwelling. The link between upwelling and Pacific hake was the biomass of euphausiids, the key prey item. Ware (1991) showed that climate related changes in the ocean environment strongly affected the trends in abundance of Pacific herring off Canada's west coast and these species were also strongly affected by predators.

3.2. Fish Processing Waste

The fish processing industry is wide spread worldwide and varies in its operations, scale of production and outputs. The industry produces fresh fish, frozen fish, marinated fillets and canned fish (UNEP, 2000). The wastes obtained from filleting operations include heads, tails, bones, skin and viscera which are rich in omega-3 fatty acids (Junker et al., 2006). Fish processing wastes are of two types: (a) solid fish waste (SFW) which consists mainly of scales, bones, fins and tails and (b) liquid waste which contains soluble proteins, enzymes, pigments, blood and lipids. Varying amounts of solid matter including offal, skin, and bone are present in untreated effluents from fish processing plants (AMEC, 2003).

Table 3.2 describes the maximum possible waste produced by different Atlantic Province in Canada. The by-products from filleting, gutting and other fish processing operations serve as good raw materials for fish meal and fish oil production. These by-products are rich in valuable minerals, enzymes, pigments and flavors that are required by many

Table 3.2. Amount of fish waste produced in Atlantic Provinces in 2001 (AMEC, 2003).

Province	Total Landing (Metric Tonnes)	Product Weight (Metric Tonnes)	Possible Waste (Metric Tonnes)
NewBrunswick	113588	89012	24576
New Foundland	267959	120999	146960
Nova Scotia	366381	146708	219673
PrinceEdward Island	66046	39000	27046
TOTAL	813974	395719	418255

industries including food, agriculture, aquaculture and pharmaceuticals (Archer et al., 2001).

3.3. Utilization of Fish Processing Waste

The utilization of by-products is considered an important clean production opportunity for the fish processing industry as it potentially generates additional revenue, reduces disposal costs of these materials and eliminates or reduces the environmental and health impacts of waste disposal (Ioannis and Aikaterini, 2008). Utilization of fish processing waste includes animal feed, fish meal, chitin/chitosan, enzymes and biodiesel.

3.3.1. Animal feed

Offal from the fish processing industry is used as an animal feed ingredient, as a good source of high-quality protein and energy (New, 1996; Gabrielsen and Austreng, 1998). Carotenoids which are extracted from shrimp waste (head and body covering) are used in the formulation of aquaculture feed (Sachindra et al., 2001; Sachindra et al., 2006).

Faid et al. (1997) evaluated chopped pilchard waste (heads, tails and viscera), combined with 25% molasses and inoculated with bacteria (*Saccharomyces* sp. and *Lactobacillus plantarum*) for their nutritional quality and biochemical properties (pH, dry matter, ash, total and volatile nitrogen, lipids and trimethylamine) during 15 days of fermentation period at 22°C. The results indicated that the pH and the total nitrogen decreased whereas non-protein nitrogen and volatile nitrogen increased significantly.

Rahmi et al. (2008) fermented fish waste (viscera, heads, tails and skins) that was grounded with 10% molasses and inoculated with culture (*Lactobacillus plantarum*) at 25°C for 10 days. The results showed that pH and total nitrogen decreased but non-protein nitrogen and volatile nitrogen increased. The trial feeding of fish silage to young sheep showed increases in weight above controls and good improvements of meat characteristics and carcass shape.

3.3.2. Fish meal

Fish meal has been used as livestock feed for many years and has high nutritional value as it is a good source of vitamin, phosphorous, calcium, iron and other trace minerals, but is low in fiber (Hall, 1992). Vannuccini (2004) reported that the production of fish meal from fish processing waste is a better utilization because it reduces waste and the fish meal price is high based on the protein content. However, the meal produced from waste with high lipid content and low protein is rated low quality. Large scale productions of fish meal from fish processing wastes are available (FAO, 1986).

Composition of the raw material is expressed in terms of fat, dry matter and protein. The recovered dry matter has 20% higher yield of protein which depends upon the freshness and nature of the raw material. Generally, wet pressing methods (physical method) are adapted to produce fish meal and oil. The pressing method yields two different phases: the solid phase (press cake) which contains 60-80% of the oil-free dry matter (protein, bones) and oil; the liquid phase (press liquor) which contains water and the rest of the solids like oil, dissolved and suspended protein, vitamins and minerals.

Olomu and Nwachukwu (1977) reported on the production of fish meal which includes result from anchovy, herring, menhaden and white fish. Sotolu (2009) compared dietary fish waste meal (FWM) and imported fishmeal (FM) at 10 and 15% inclusion level of catfish and found no significant difference between FWM and FM based diets. Honczaryk and Maeda (1998) studied the fish meal production of arapaima (Arapaima gigas), a very important freshwater carnivorous fish from the Amazon area, and concluded that they presented a higher level of ingestion. Abiola and Onunkwor (2004) compared the hatchery waste meal (HWM) with fish meal in layer diets at 0, 33, 66 and 100% levels and found the protein content of HWM diets to be superior.

3.3.3. Chitin/chitosan

One of the most abundant natural polysaccharides produced by many living organisms is chitin. It is usually found as a component of crustacean shell and this polymer consists of

linear chains (Rinaudo, 2006). Chitin is a nitrogen containing polysaccharide, related chemically to cellulose (Patil and Satam, 2002). Chitin and chitosan are nontoxic and biodegradable (Rashidova et al., 2004). Three different polymers of chitin are found in nature: alpha chitin, beta chitin and gamma chitin. Alpha-chitin, the most abundant in nature with anti-parallel chains structure, is found in crabs, shrimp and lobsters. Beta-chitin found in squid has intrasheet hydrogen bonding by parallel chains. Gamma-chitin is a combination of alpha and beta chitin which has a mixture of parallel and antiparallel chains (Jang et al., 2004).

Abdou et al. (2008) studied the extraction of chitin from six different local sources in Egypt. The chitin obtained was converted to useful soluble chitosan by steeping into solutions of NaOH (various concentrations) for long time. The obtained alkali chitin was heated in an auto clave which significantly reduced the time of deacetylation. The chitin and chitosan were characterized by X-ray diffraction, spectral analysis and thermo gravimetric analysis.

Manni et al. (2010) used protease from *Bacillus cereus* SV1 for chitin extraction from shrimp waste material. Using crude protease from *B. cereus*, chitin was converted to chitosan by N-deacetylation, and the antibacterial activity of chitosan solution against different bacteria was investigated. Results showed that chitosan obtained inhibited the growth of most Gram-negative and Gram-positive bacteria tested.

Chitin and chitosan are used in biomedical applications including drug delivery systems, implantable and injectable systems (Berger et al., 2004). The positive charge of chitosan allows it to interact with negative molecules (DNA). This phenomenon allowed for chitosan to be used in preparing a non-viral vector for a gene delivery system (Mumper et al., 1995). Studies on regenerative tissue engineering recommend the use of chitosan scaffolds to support damaged tissue. Chitosan scaffolds are promising materials for the design of tissue engineered systems due to their low immunogenic activity, porous structure and controlled biodegradability (Ho et al., 2005). Diabetic ulcers showed immediate response to chitosan (Batista et al., 2006). Chitosan has also been used in the

food industry as preservatives, for the formation of biodegradable films and recovery of material from food processing waste (Aranaz et al., 2009). According to Chien and Chou (2006), chitin and chitosan were used as supports for enzyme and cell immobilization due to their characteristics (density, rigidity and mechanical stability).

3.3.4. Enzymes

Fish processing waste can be used to produce enzymes such as pepsin and chymotrypsin. Pepsin is an enzyme secreted in the gastric juice of the stomach lumen and it helps in the digestion of proteins in animals (Haard and Simpson, 2000; Effront et al., 2007). Pepsinogen is a proenzyme of pepsin. The major role of pepsin in protein proteolysis is to cleave aromatic amino acids (phenylalanine and tyrosine) from the N-terminus of proteins (Raufman, 2004). Chymotrypsin is a digestive enzyme found in pancreatic tissues of vertebrates and inverbrates which is secreted into the duodenum (Geiger, 1985). Chymotrypsin was classified into three types: chymotrypsin (A, B, C) found in mammals but only two types of chymotrypsin (A and B) have been found in fish (Yang et al., 2009).

Benhabiles et al. (2012) studied the optimizationof fish protein hydrolysate (FPH) production by enzymatic hydrolysis of sardine solid waste using crude pepsin. The crude pepsin prepared by autolysis of the mucous membranes of a sheep stomach at optimal conditions (i.e. pH = 1.5‑2 and incubation time of 6 h) was satisfactory and used for the enzymatic hydrolysis of fish solid waste. The optimal conditions for enzymatic reaction were: temperature 48°C and pH 1.5.

Hinsui et al. (2006) studied the extraction of tryptic and chymotryptic activities from spleen, liver, stomach, intestine and mixed viscera of Nile tilapia (*Oreochromis niloticus Linneaus*). The results showed that the intestine was the best source for trypsin and chymotrypsin and they extracted using 30-70% saturated ammonium sulfate precipitation, followed by dialyzed, acetone precipitation and separated by SBTI affinity chromatography column. The obtained specific activities of trypsin and chymotrypsin

were 0.529 and 0.380 unit/mg protein, respectively. Also it was noted that trypsin fraction was stable at 0-60°C and chymotrypsin was stable at 0-50°C for 30 min at pH 8.0.

Espósito et al. (2010) studied the purification of trypsin from the viscera of lane snapper (*Lutjanus synagris)* by heat treatment, fractionation with ammonium sulfate and affinity chromatography. The maximal activity was exhibited by the enzyme at pH 9.0 and 45 °C. After incubation at the optimal temperature for 30 min, 100% of the activity was retained. With the presence of different surfactants (Tween 20, Tween 80 and sodium choleate), the enzyme retained more than 80% activity after 60 min. They also showed the compatibility with commercial detergents (7 mg/mL), such as Bem-te-vi, Surf and Ala, which retained more than 50% of initial activity after 60 min at 25 °C and 30 min at 40 °C.

3.3.5. Biodiesel

Biodiesel is an organic fuel for diesel engines which is made from organic oils, such as vegetable or animal oils, combined with an alcohol and catalyst (sodium hydroxide). Biodiesel has similar characteristics to petroleum diesel fuel (petro-diesel) and it does not require modification to existing fuel storage, delivery and engine systems (Canakci, 2007). It helps in reducing particulate emissions and providing longer engine life due to increased lubricity (Tickell, 2000).

Biodiesel produced from fish processing waste is a biodegradable, renewable and non toxic fuel that can be used in current diesel engines. It is environmentally friendly because it reduces the air toxins, CO_2, particulates, black smoke and other hydrocarbons (Piccolo, 2008). Various techniques have been used for oil production, followed by transesterification which is used to convert oil into biodiesel, where triglycerides react with alcohol in the presence of a catalyst to produce mono alkyl esters (Meher et al., 2006).

Kato et al. (2004) studied ozone-treated fish waste oil to produce biodiesel fuel and evaluated its properties as an alternative to diesel fuel. The result showed that the flicker point of produced fuel was lowered due to ozone treatment which helps in easy

combustibility. Also, the ion chromatograms obtained from gas chromatography showed that the fatty acids in the fish waste oil decomposed to produce hydrocarbons. Lin and Li (2009) obtained crude oil from soap-stock of the fish which was then refined and transesterified to produce biodiesel. El-Mashad et al. (2008) studied the production of biodiesel by transesterification in a two step process (sulphuric acid-catalysed and KOH-catalysed) using by-product of salmon processing. Fukuda et al. (2001) reported the transesterification of oils for the production of biodiesel.

Recently, enzymatic transesterification using lipase has become more attractive for biodiesel fuel production. By-products such as glycerol produced can be recovered easily. Cost of lipase makes the production difficult to commercialize and by means of reducing the cost, the use of whole cell immobilized biocatalysts has significant advantage. Immobilization can be achieved spontaneously during batch cultivation, and does not require purification.

3.4. Fish Lipids

Marine lipids from the flesh of fatty fish (herring, capelin, sardine, mackerel, anchovy, sand eel, menhaden, sprat and blue whiting), blubber from marine mammals and livers of lean fish (haddock, halibut, perch, sole, cod, flounder, pike and red snapper) contain triacylglycerols (TAG). Lean fish are those that are low in fat and fatty fish are those which are high in fat. Both fish oil and marine mammal oil are rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The amount of docosapentaenoic acid (DPA) is higher in marine mammal oil than in fish oil (Shahidi and Miraliakbari, 2006). Lipids are primarily located in subcutaneous tissues such as belly flaps, livers, muscle tissues and heads (Ackman, 1994). The amounts of lipid in dark muscles are greater than that of white muscles (Zhou and Ackman, 1995). Fish skin contains more lipids than the muscle tissues. In the skin of lean fish, the lipid content averages 0.2-3.9% w/w whereas the lipid content of fatty fish are higher than 50% w/w. In cod, the average lipid content in roe is 0.3-1.5% w/w and is up to 70% w/w in liver. Lipid content of non-edible offal is higher than in fillet (Kolakowska et al., 2003). Table 3.3 shows different fish species and their lipid contents.

Table 3.3. Comparison of different fish species and their lipid content.

Fish Species	Fish Part	Lipid Content (%) *	Reference
Fatty fish			
Herring	Waste	>8	Ackman (1994)
Mackerel	Skin	38	Ki-Teak and Akoh (1998)
Mackerel	Viscera	9	Ki-Teak and Akoh (1998)
Mackerel	Muscle	9	Ki-Teak and Akoh (1998)
Sardine	Liver	5.8	Khoddami et al. (2009)
Wild eel	Muscle	11.6	Oku et al. (2009)
Wild eel	Liver	4.7	Oku et al. (2009)
Cultured eel	Muscle	13.1	Oku et al. (2009)
Cultured eel	Liver	7.6	Oku et al. (2009)
Lean fish			
Cod	Roe	0.3-1.5	Kolakowska et al. (2003)
Cod	Liver	70	Kolakowska et al. (2003)
Wild catfish	Liver	3.5	Shirai et al. (2001)
Cultured catfish	Liver	3.3	Shirai et al. (2001)

*by weight

The oils from lean fish are of high quality and are used as medicinal fish oil (Hjaltason, 1992). Fatty fish have small livers and the bulk of fatty oil is present in their fatty flesh (Bimbo, 1998; Haraldsson and Hjaltason, 2001). Pink salmon heads have high lipid content but the viscera have the lowest oil content (Wanasundara et al., 1998; Aidos et al., 2002). Catfish processing by-products consist of heads, frames, skin and viscera. Extraction of oil from viscera added value to the catfish viscera and increased the total unsaturated fatty acids. However, the combined n-3 fatty acids of purified catfish viscera oil were only 4.6 mg/g of oil (Sathivel et al., 2003).

3.5. Omega-3 Fatty Acids

Naturally occurring polyunsaturated fatty acids (PUFA) are divided in four major families: omega-3 (alpha-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid), omega-6 (arachidonic acid and linoleic acid), omega-7 (palmitoleic acid and vaccenic acid) and omega-9 (oleic acid and erucic acid). Figure 3.2 shows PUFA formation pathway. The designation Omega (ω) is related to the position of the first double bond from the methyl end of fatty acid. Omega-3 fatty acids have been known as an essential nutrient to normal growth in human being. However, there is a solid evidence that they may also favorably modulate many diseases (Connor, 2000; Covington, 2004). Omega 3 fatty acids are essential nutrients for human body but cannot be manufactured in the body and must be obtained from the external source (Cunnane et al., 2007). Alpha-linolenic acid (ALA) is the primary omega 3 fatty acids and is converted into the fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the body. Unfortunately, the percent of the conversion (less than 1%) of ALA into EPA and DHA in human body is inefficient (McKeigue and Sevak, 1994; Covington, 2004).

3.5.1. Alpha-linolenic acid (ALA)

Alpha-linolenic acid (ALA 18:3n-3) is a polyunsaturated fatty acid which has 18 carbon atoms and 3 double bonds (Holub and Holub, 2004). Only this omega-3 fatty acid is found in vegetable products. It is produced *de vivo* in plants by the Delta 12 and Delta 15

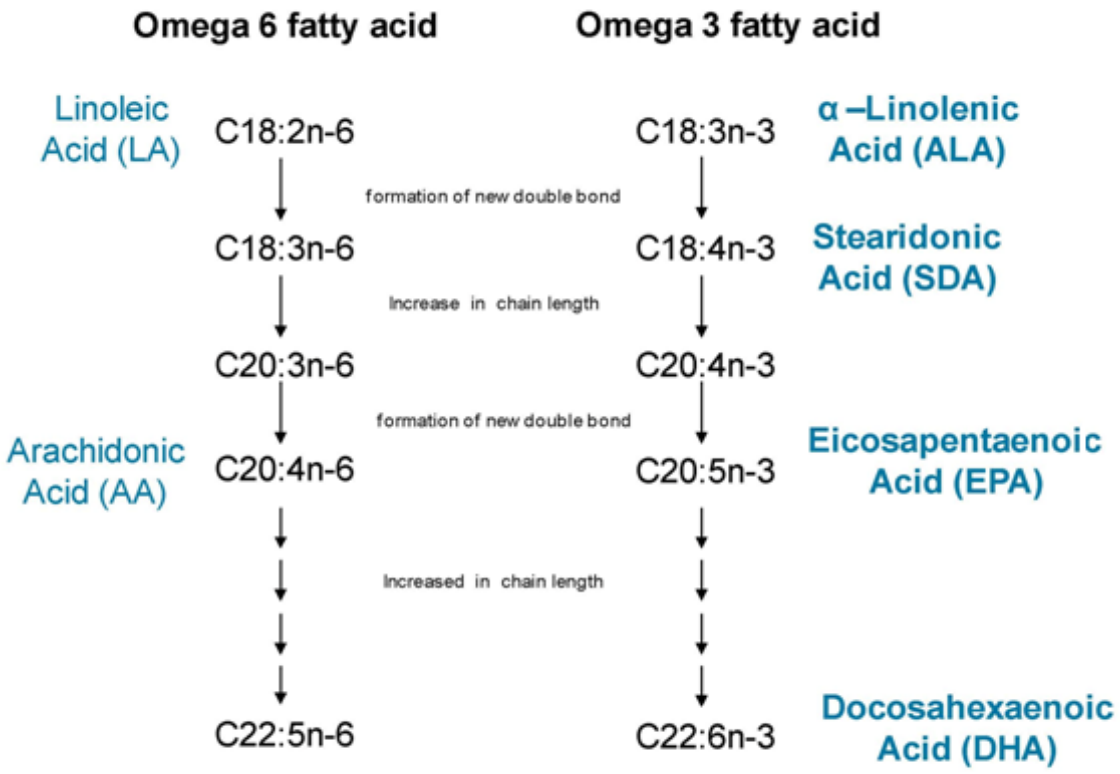


Figure 3.2. PUFA formation pathway (Holub and Holub, 2004).

desaturation of oleic acid in plants. This fatty acid is a metabolic precursor for the production of omega-3 fatty acids in animals (Charles, 2009). According to Simopoulos (2004) edible wild plants provide ALA and higher amounts of vitamin E and C than cultivated plants. ALA intake is related to the inhibitory effects on the clotting activity of platelets. In clinical studies, ALA was used in lowering blood pressure and an epidemiological study showed that ALA was inversely related to the risk of coronary heart disease in men (Simopoulos, 2004). The advantage of consuming ALA over ω-3 fatty acids from fish is that the problem of insufficient vitamin E intake does not exist with high intake of ALA from plant source.

Jager et al. (2008) studied the isolation of linoleic and alpha-linolenic acids as COX-1 and 2-inhibitors in rose hip. It was carried out by extraction with petroleum ether fractioned by VLC on silica, on C-18 column and by HPLC. Every step was COX-1/2 activity-guided. The results showed that the COX-2/COX-1 ratio was 0.007 for linoleic acid and 0.2 for alpha-linolenic acid.

Ghafoorunissa and Pangrekar (1993) studied the vegetable sources of alpha-linolenic acid in Indian diet. Total lipids were extracted from vegetable, dry beans and fenugreek seeds and their fatty acid compositions were determined. From the results, it was observed that fenugreek seeds contained 2% alpha-linolenic acid and green leafy vegetables provided about seven times more alpha-linolenic acid than fresh beans and other vegetables. Cereal pulse-based a lacto vegetarian diets which are rich in alpha-linolenic acid made important contributions to the intake of n-3 fatty acids.

Vijaimohan et al. (2006) investigated the effect of flaxseed oil (FO) rich in alpha-linolenic acid on growth parameters and lipid metabolism of rats fed with high fat diet. A high fat diet increased the body weight and had a negative effect on lipoprotein metabolism. But FO supplementation lowered the body weight gain, liver weight, plasma cholesterol, free fatty acid, HDL, LDL in the high fat diet fed rat and also reduced the hepatic and plasma lipid level. There was no adverse effect of FO on growth parameters and plasma lipids in rats fed with the fat free diet.

3.5.2. Eicosapentaenoic acid (EPA)

Eicosapentaenoic acids (EPA: 20:5n-3) are derived from long-chain fatty acids with a long un-branched aliphatic tail (chain) of saturated or unsaturated carboxylic acid (Bruinsma and Taren, 2000). The differences in geometry among the various types of unsaturated fatty acids and between saturated and unsaturated fatty acids play an important role in biological processes (Harrison, 2007). EPA is found in fish oil, sea weeds, algae and sea food.

Pettinello et al. (2000) studied the preparative supercritical fluid chromatography process to extract fractions of eicosapentaenoic ethyl ester (EPA-EE). EPA-EE is a stabilized ethyl ester form of ω-3 C20:5 PUFA and is shown in Figure 3.3. The starting mixture with 68% of EPA-EE was fractionated by a silica adsorption column (CO_2 was used as the supercritical solvent). Samples eluted were collected and analyzed by capillary and packed column gas chromatography. The results showed that the bench scale equipment gave 95% EPA-EE fraction and the pilot plant gave maximum purity of 93%.

3.5.3. Docosahexaenoic acid (DHA)

Docosahexaenoic acids (DHA: 22:6n-3) are long-chain fatty acids with a long unbranched aliphatic tail (chain) of saturated or unsaturated carboxylic acid (Bruinsma and Taren, 2000). DHA are found in fish oil, sea weeds, algae and sea food.

Tang et al. (2011) studied the isolation of the lipids from microalgae by supercritical CO_2 (SC-CO_2) extraction followed by a further enrichment of crude lipids to produce high-purity docosahexaenoic acid (DHA) by the urea complexation method. It was observed that 33.9% of lipid yield and 27.5% of DHA content were achieved. The urea complexation method for DHA enrichment considerably increased the DHA purity from 29.7% to 60.4% with an enrichment ratio of 60.6%.

Mendes et al. (2007) studied a simple procedure involving saponification and methylation in wet biomass. Winterization and urea complexation was used to concentrate

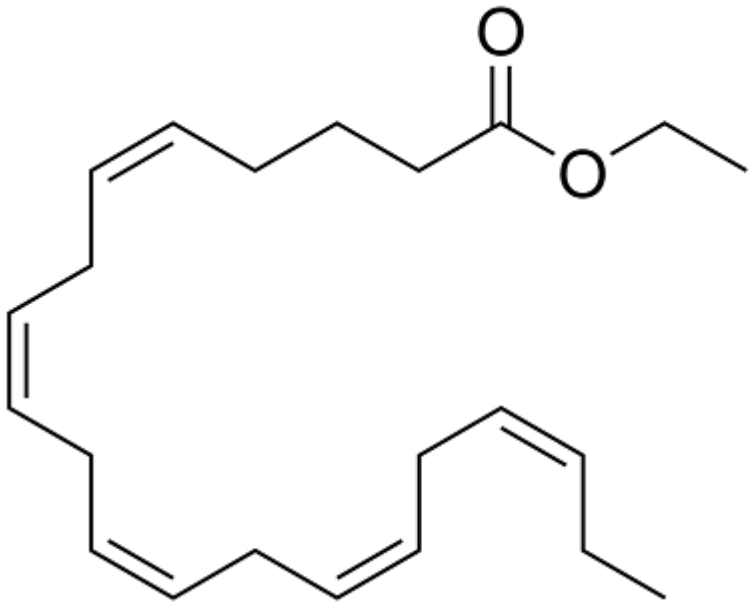


Figure 3.3. Structure of eicosapentaenoic ethyl ester (Pettinello et al., 2000)

docosahexaenoic acid (DHA) from *Crypthecodinium cohnii* CCMP 316 biomass. The results showed that the temperature had the most significant effect on the DHA concentration. The highest DHA fraction (99.2% of total fatty acids) was found at the urea/fatty acid ratio of 3.5 at the crystallization temperatures of 4-8 °C. The highest DHA recovery (49.9 %) was observed at 24°C at the urea/fatty acid ratio of 4.0, corresponding to 89.4 % DHA of total fatty acids.

3.6. Dietary Sources of Polyunsaturated Fatty Acids

Daily dose of EPA and DHA combination per day is 0.3-0.5 grams and 0.8-1.1 grams for alpha-linolenic acid (FAO, 1994). Omega-3 fatty acids can be obtained in human diets through marine or plant sources. The marine source (fatty fish) provides EPA and DHA and the plant sources (flax, walnuts, canola oil) provide ALA (Eckert et al., 2010).

3.6.1. Marine Sources

Primary sources of the omega 3 fatty acids are of marine origin: algae, fungi and phytoplankton. They are integrated into phospholipids and fat deposits in fish and other marine animals (seal and whale) via bioaccumulation. Higher levels of EPA are found in the algae *Nitzschia* spp., *Nannochloropsis* spp., *Navicula* spp., *Phaeodactylum* spp., *Porphyridium* spp., *Crypthecodinium cohnii* and *Schizochytrium* spp. were known for their higher level of DHA (Ward and Singh, 2005). Fungi species like *Mucor circinelloides. Mortierella*, *Pythium*, *Thraustochytrium*, and *Entomophthora* were used for the production of arachidonic acid (ARA) and eicosapentaenoic acid (EPA) (Sneha, 2008).

Table 3.4 shows omega-3 polyunsaturated fatty acids content from various sources. Table 3.5 shows the content of omega-3 fatty acids, EPA and DHA in various fish. Table 3.6 shows omega-3 fatty acid content in various fish species. Cold water fatty fish including salmon, tuna (bluefin tuna has five times more DHA than other types of tuna), menhaden mackerel, sardines, shellfish and herring are rich sources of omega-3 fatty acids (Singh,

Table 3.4. Omega-3 polyunsaturated fatty acids content from various sources.

Source	EPA[1] (%)*	DHA[2] (%)*	Reference
Algae			
Ceramiaceae			
Ceramium boydenii Gepp	16.8	-	Li et al. (2002)
Ceramium kondoi Yendo	20.1	-	Li et al. (2002)
Rhodomelaceae			
*Laurencia okamurai*Okam.	23.4	-	Li et al. (2002)
Polysiphonia urceolata (Lightf.) Grev.	37.5	-	Li et al. (2002)
Rhodomela confervoides (Huds.) Silva	24.8	-	Li et al. (2002)
Corallinaceae			
Corallina pilulifera Post. et Rupr.	31.5	-	Li et al. (2002)
Dumontiaceae			
Hyalosiphonia caespitosa Okam.	12.5	-	Li et al. (2002)
Gloiosiphoniaceae			
Gloiosiphonia capillaris (Huds.) Carm	3.6	-	Li et al. (2002)
Gelidiaceae			
Gelidium amansii Lamour	13.3	-	Li et al. (2002)
Soil microorganism			
Trichoderma sp.	0.298	7.47	Gayathri et al. (2010)

* Percent of total fatty acids
1- (20:5 n-3)
2- (22:6 n-3)

Table 3.5. EPA and DHA content in various fish (Newton and Snyder, 1997).

Fish	EPA[1] (%)*	DHA[2] (%)*
Sardine	3	9-13
Pacific anchovy	18	11
Mackerel	8	8
Capelin	9	3
Herring	3-5	2-3
Freshwater fish	5-13	1-5

*Percent of total fatty acids
1-(20:5 n-3)

2- (22:6 n-3)

Table 3.6. Omega 3 fatty acid content in various fish species (Piccolo, 2008).

Fish Species	Omega-3 (EPA+DHA) (g/100g of fish)
Tuna (fresh)	0.28-1.51
Atlantic salmon	1.28-2.15
Mackerel	0.4-1.85
Atlantic herring	2.01
Rainbow trout	1.15
Sardines	1.15-2
Halibut	0.47-1.18
Tuna (canned)	0.31
Cod	0.28
Haddock	0.24
Catfish	0.18
Flounder or sole	0.4
Oyster	0.44
Shrimp	0.32
Scallop	0.20
Cod liver oil capsule	0.19
Omacor (Pronova)	0.85

2005). The blubber oil of marine mammals such as seal, whale and walrus are rich sources of omega-3 fatty acids (Wanasundara, 1996).

3.6.2. Plant sources

The essential alpha-linolenic acid is found in plant sources such as flax seeds, walnuts, butternuts, pumpkin seeds, red and black currant seeds, wheat germ, soy and canola oil and leafy green plants like purslane. *Perilla frutescens* seed oil (PFSO) is rich source of unsaturated fatty acid particularly in omega-3 linolenic acid (Eckert et al., 2010). Increased intake of ALA, similar to the intake of EPA and DHA, may possess favorable effects in health (Simopoulos, 1991).

Inuwa et al. (2012) isolated long chain n-3 unsaturated fatty acids from *Telfairia Occidentalis* using an arginated silica gel column. The dry weight of *Telfairia Occidentalis* leaves had highest percentage of long chain fatty acid. Type n-3 was noted using GC-MS as palmitoleic acid and elaidic acid.

Siriamornpun et al. (2006) studied the extraction of lipid and fatty acid composition of Thai *Perilla frutescens* obtained from different regions (Maehongsorn and Chiang Mai). The lipid content was between 34-36% and triglycerides were the predominant lipid in perilla seed. The main fatty acid was alpha-linolenic acid (50-60% of total fatty acid) and it was also shown that the compositions of lipids and fatty acids in Thai perilla seeds varied considerably with samples from different locations.

Eckert et al. (2010) studied the effect of *Perilla frutescens* seed oil (PFSO) on central nervous system (CNS) and protection of unsaturated fatty acids within the CNS. The results showed that the identified PFSO was a promising nutraceutical which can be used as alternative to fish oil supplement and it has provided mechanisms for neuroprotective actions of unsaturated fatty acids.

3.7. Medical uses of Omega-3 Oil

Omega 3 fatty acids are vital for conception through pregnancy and infancy, essential for normal growth and development and maintaining good health (Connor, 2000). DHA has beneficial effect in the development of retina and brain in human being while EPA has been related to anti-inflammatory properties, reduction of obesity related disorders, shrinking of tumors and reduction of human depression levels (Mitsuyoshi et al., 1991). They are also used in prevention and treatment of coronary artery disease, cancer, diabetes, hypertension, rheumatoid arthritis (Shahidi, 2008; Shahidi and Miraliakbari, 2006). Fish oil and blubbers oil of marine mammals including seal, whale and walrus are the rich source of omega-3 fatty acids and a low dose of seal oil can reduce the risk of atherogenic disease. However, seal oil contains high level of DPA, which is the most important fatty acid that keeps artery walls plaque free (Hu et al., 2002).

3.8. Extraction of Fish Oil

Huss (1988) stated that the amount of fish oil that can be extracted depends on the fish species, age, gender, location, species-origin characteristics (spawning and migration seasons), environmental conditions (temperature) and extraction method. Fish oils are classified into their lipid fractions such as triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), sterylesters, phospholipids, free fatty acids and sterols. Lipids differ between various tissues, organs and species. The kind of fatty acids present as free acid or as neutral lipid depends upon the species and environments (McGill and Moffat, 1992). Currently, fish oil is extracted by cooking and pressing at the commercial scale. However, solvent and enzymatic extractions are performed at the laboratory scale.

3.8.1. Cooking and pressing

Commercially, fish oil is extracted by cooking and pressing. In large scale, the raw material is cooked and the cooked products are transferred to a screw press where most of the liquid is squeezed out to form a press-cake. The press liquid contain sludge (water,

most of oil, salt, protein and fine particles) that is removed in a decanter. The liquid from the decanter is fed to separators and the oil is recovered (SR-MJOL HF, 2011).

3.8.2. Solvent extraction

Solvent extraction is only carried out at the laboratory scale. Based on interactions between the solvents and hydrophobic or hydrophilic regions of the molecule, pure lipids dissolve in variety of solvents. Lipids which have a low polarity functional group such as triacylglycerols or cholesterol are soluble in hydrocarbon solvents and partially soluble in higher polarity solvents, but are insoluble in polar solvents (Christie, 2003). For extraction of lipids from tissues, it is necessary to find the solvent which not only readily dissolves the lipids but also overcomes the interaction between the lipids and tissue matrix. The principle behind the solvent extraction process involves the addition of solvent to the sample mixture in which one or more constituents are removed.

In the extraction process, the solvent added to the mixture may not be miscible, but is capable of selectively dissolving one of its constituents in the mixture. The substances are separated based on the difference in their solubility in appropriate solvent (Alders, 1955). Binary solvent systems are generally used in the extraction of fish oil. In the extraction process, the solvent with higher polarity is called extraction solvent which in the process forms the extract phase. The solvent with less or no polarity is called wash solvent, which forms the residue phase during the process.

There is growing health and environmental concerns with the use of chlorinated solvents such as chloroform that is suspected to be a carcinogenic agent (Reuber, 1979; Radin, 1981). Chloroform-methanol may be the best lipid extractant, but it is definitely not the safest from environmental and health concerns. According to Hara and Radin (1978), extraction of lipids from tissues using hexane:isopropanol had a number of advantage over the commonly used chloroform:methanol method. Hexane has been used extensively as a solvent for oil extraction and has a lower vaporization temperature, higher stability, lower cost, lower corrosiveness and lower toxicity (Akoh et al., 2002).

The most recommended method for total lipid extraction from biological tissues is the Bligh and Dyer method (Smedes and Askland, 1999). The major advantage of Bligh and Dyer method is the reduction of solvent:sample ratio (Bligh and Dyer, 1959). Most of the data published on total lipid content have been derived from Bligh and Dyer method (Iverson et al., 2001). Ozogul et al. (2011) evaluated the efficiency of Bligh and Dyer and Soxhlet methods and noted that Bligh and Dyer extraction method was more efficient in extracting polar and non-polar lipids from fish than Soxhlet method. Norziah et al. (2009) evaluated the yield of fish oil using Bligh and Dyer method in fish leaching waste with different ratios of mixture of chloroform:methanol:water (2:4:1, 4:2:1, 2:2:1, 2:1:2) as extracting solvent. A high yield of fish oil was obtained with chloroform:methanol:water ratio of 2:4:1.

Zuta (2003) studied two extraction methods of oil from mackerel fish using chloroform:methanol (Bligh and Dyer) and hexane:isopropanol. It was reported that the extraction method using chloroform:methanol was discontinued after preliminary analysis as it did not have any advantage over hexane:isopropanol to justify the risk of toxicity associated with exposure of chloroform and methanol. Radin (1981) stated that there is growing health and environmental concerns due use of chlorinated solvents such as chloroform which is suspected to be a carcinogenic agent. In addition, the chlorinated solvents will add to solvent disposal costs. Studies have been made to adapt the Bligh and Dyer method with non-chlorinated solvents (Smedes, 1999).

Saify et al. (2003) studied the fatty acid composition of liver oil obtained from two different sharks (winghead snark and sandbar shark). The isolation, identification and characterization were carried out using gas-liquid chromatography and thin layer chromatography. It was noted that there was a large variation between the two shark oils. Saturated fatty acid e.g. palmitic acid (C16:0), in unsaturated monoenoic fatty acids e.g. oleic and palmitoleic acids were major constituents. Additional polyunsaturated fatty acids e.g. eicosapentaenoic and docosahexaenoic acids, were also found.

Zhong et al. (2007) studied the fatty acid composition of steelhead trout muscle and visceral lipid. It was noted that steelhead trout muscle and viscera had similar fatty acid patterns with monounsaturated fatty acid, polyunsaturated fatty acids (PUFA) and saturated fatty acids. Oleic acid (C18:1) was predominant in both muscle and viscera and the PUFA content was slightly higher in muscle than in viscera. The major n-3 PUFAs were DHA and EPA with 9.70% and 3.28% of total fatty acids respectively in muscle and 7.98% and 3.35%, respectively in viscera.

3.8.3. Enzymatic extraction

Enzymatic extraction is also carried out at the laboratory scale. Extraction using enzymes has increasing importance in the production of food supplements, fine chemicals, medicines as a minimum amount of energy is required for the large-scale production (Katsivela et al., 1995). Fish oil produced from an enzymatic process with proteases provides a good alternative as reactions can be carried out under mild conditions for short periods of time (Linder et al., 2005). Commercial proteases used to extract oil from marine by-products have resulted in improved yields and in addition the hydrolysate provides fish protein (Gbogouri et al., 2006).

Enzymatic hydrolysis of fish sample is mainly performed to extract fish oil and fish protein. The main advantage of lipase is that lipase specificity which differentiates the enzymatic reaction from chemical reaction, molecular properties of enzyme and structures of substrate. Factors affecting binding the enzyme and substrate will control the lipase specificity (Öztürk, 2001). The commercial enzymes used in the enzymatic hydrolysis include Alcalase, Neutrase, Protamex and Lecitase. Alcalase is one of the best enzymes used in hydrolysis process for fish oil and protein hydrolysates (Slizyte et al., 2005). Alcalase is a proteolytic enzyme produced from *Bacillus licheniformis*. The optimal working conditions for Alcalase are temperatures in the range of 55-70°C and pH values of 6.5-8.5.

Batista et al. (2009) studied the extraction of oil from sardines (raw and cooked sample) using commercial enzymes (Alcalase, Neutrase and Protamex). The results showed that

the highest oil was released from raw sardine with the enzymes Alcalase and Protamex. Also the oil obtained was dark with high peroxide value. Liaset et al. (2002) reported on the extraction of fish oil and nitrogen recovery from frames of Atlantic salmon using Protamex protease in which hydrolysing parameters were varied (pH, temperature, enzyme-substrate ratio and frame-water ratio) between low and high levels. It resulted in highest level of enzyme-substrate ratio, temperature, pH and lowest levels of frame-water ratio gave highest nitrogen recovery (76%).

Slizyte et al. (2005) studied the effect of initial heat inactivation of enzyme, addition of water prior to the process and use of different combinations of enzymes on the yield and purity of oil and protein fractions of cod by-product. The results showed that it was not possible to obtain all desired qualities using only one hydrolysis process but the hydrolysis of unheated raw material with *Alcalase* and addition of water was better when compared to other mentioned quality indicators.

Mbatia et al. (2010) recovered oil from Nile perch and salmon head using protease (bromelain and Protex) and found that increases in water content during the hydrolysis resulted in a decrease in oil yield and the maximum oil yield was obtained with Protex at 55°C. Also fatty acid composition of crude oil was evaluated in which nile perch had higher amount saturated fatty acid (36.8 mol %) than salmon head (19 mol %). The total amount of palmitic acid, EPA and DHA were 50, 13 and 48 mol% respectively. Gbogouri et al. (2006) described the fatty acid composition of salmon head oil obtained using proteolytic enzymes. The fish oil contained saturated fatty acids (24.7-27.3%), monounsaturated fatty acids (39.9-40.8%) and PUFA (32.3-35.4%).

3.9. Refining Edible Oils

Most edible oils are refined before final use. The main purpose of the refining process is to remove impurities such as phosphatides, trace metals, pigments and oxidation products. The processes involved in the chemical refining of fats and oils include degumming, neutralization, bleaching and deodorizing (Johnson, 1998).

3.9.1. Degumming

The crude fish oil is degummed to remove phosphatides and ill-defined mucilaginous materials. Degumming is a process in which the oil is treated with water/steam or with an aqueous solution of boric acid or salt such as sodium chloride at 30-50°C to remove the phospholipids and other mucilaginous materials from the crude oil (Zuta, 2003). Deposited hydrated phospholipid is referred to as gums which can be separated from oil by centrifugation. Air should be excluded in the process as it may lead to oxidation. For the effective degumming process, water should be minimized to prevent hydrolysis (Wanasundara, 1996).

3.9.2. Neutralization

Neutralization or alkali refining of fish oil removes free fatty acids as sodium salts which is also refered to as soaps (Zuta, 2003). The process involves reaction of free fatty acids with sodium hydroxides which forms soapstock. Addition of alkali to the oil along with slight heating breaks the emulsion and the soapstock is removed by centrifugation (Carr, 1978). Higher amount of sodium hydroxide could cause saponification of the triglycerides (Chang, 1967). The refined oil is washed with warm water to remove any trace amount of soap (Kwon et al., 1984).

3.9.3. Bleaching

After refining process the oil is bleached which removes coloured material, pigments and any trace amount of soap (Zuta, 2003). Bleaching materials commonly used are natural clay, activated clay and activated carbon. Activated carbon is used at 5-10% in combination with clay. Natural clay is used if the oil is readily bleachable (Wanasundara, 1996). Also bleaching removes secondary oxidation products and peroxides from the oil (Zehnder and McMicheal, 1967).

3.9.4. Deodorization

The deodorization process involves removal of volatile components which are responsible for odor or taste and also reduce free fatty acids content (Zuta, 2003). Deodorizing involves steam distillation at high temperatures (180-270°C) under high vacuum. Cmolik and Porkorny (2000) reported that the drawback of high temperature conditions lead to isomerization of PUFA. Lin et al. (1990) reported that this process also destroys peroxides and removes secondary oxidation products (aldehyde) which lead to autoxidation. Dinamarca et al. (1990) developed a pilot scale method for deodorization by high vacuum distillation with temperature below 150°C to produce fish oil without destroying long chain PUFA.

3.10. Lipid Oxidation

Lipid oxidation is a process that deteriorates the nutritive value and quality of lipids. The fundamental substrates involved in lipid oxidation reactions are unsaturated fatty acids. Different oxidation processes take place including: autoxidation, photooxidation and enzymatic oxidation (Frankel, 1991).

3.10.1. Auto oxidation

Autoxidation is a spontaneous reaction of free radical with oxygen. It is divided into three main stages: initiation, propagation and termination. Hydroperoxide (LOOH) in the presence of metal ions undergoes initiation to give peroxyl radical (LOO•). The peroxyl radical (LOO•) attacks the weak C-H bonds on fat to generate fatty acid radical (L•). The L• reacts with oxygen to form another peroxyl radical (LOO•). The LOO• forms reaction cycle again to form more LOOH and L•. Termination is the reaction between radicals to form non-radical products and the end products of oxidation are aldehydes, ketones, acids and alcohols which affect the overall quality of food (Simic et al., 1988).

Initiation:

$$\mathrm{LOOH} \xrightarrow[\text{catalyst}]{\text{energy}} \mathrm{LOO\bullet} \qquad (3.1)$$

Propagation:

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet \quad (3.2)$$

$$L\bullet + O_2 \rightleftharpoons LOO\bullet \quad (3.3)$$

Termination:

$$L\bullet + L\bullet \longrightarrow L\text{-}L \text{ (non radical monomer products)} \quad (3.4)$$

$$LOO\bullet + LOO\bullet \longrightarrow LOOL + O_2 \quad (3.5)$$

$$LOO\bullet + L\bullet \longrightarrow LOOL \quad (3.6)$$

Higher quantities of n-3 PUFAs containing marine lipids are susceptible to auto oxidation which also leads to successive degradation (Gardner, 1983). EPA and DHA are subjected to oxidation reaction due to the presence of five and six double bonds in their molecular structure. Due to high number of double bonds, the oxidation process forms a complex mixture of hydroperoxide and secondary oxidation product. This reaction readily occurs at room temperature (Frankel, 1998).

Pasoz et al. (2005) stated that auto oxidation degrades PUFAs during the storage and processing of fish oils and fatty fish which leads to the formation of volatiles associated with rancidity. Strlič et al. (2009) studied the autoxidation of lipids in parchment. Experimental evidence on the production of volatile aldehydes (product of lipid autoxidation) was provided through gas chromatographic analysis. Parchment oxidation with different aldehyde emissions was followed in situ using chemiluminometry and the same technique was used for oxidation of different delipidised parchment evaluation. The results showed that the lipid content decreased with the production of peroxides and emission of aldehydes from the material.

Marion and Forsythe (1964) studied the autoxidation of raw, ground turkey tissue with thiobarbituric acid (TBA) method. The samples stored at 4°C showed differences between the rates of autoxidation in one week. The results also showed that there was a delay in autoxidation by addition of 1% egg albumen solid (w/w) and 0.04% butylated hyldroxyanisole to the ground tissue. However, 1% gelatin exhibited no beneficial effect.

3.10.2. Photo oxidation

Photooxidation occurs due to exposure of substrate to light. Lipid photooxidation takes place in fatty fish species during processing and storage. The fish fat is prone to photooxidation both in situ (in the tissue) and when extracted out from the tissue (Undeland, 1998). According to Frankel (1998), photooxidation is a major form of deterioration of food quality and is much faster than autooxidation. However, the light induced oxidation of lipid in food is not only due to absorption by chromophoric groups in lipids but also by consequence of photosensitized oxidation.

Chingmin et al. (1989) studied the extent of oxidation of fish oil with added antioxidants after storage under nitrogen at 1 or -16°C and after exposure to room temperature with direct or indirect sunlight. The thiobarbituric acid value showed that the fish oil was stable for two weeks when stored under dark at 1 or -16°C and was immediately oxidized when exposed to light at room temperature. However, the fish oil exposed to light at room temperature remained stable for at least 8 days.

Sang and Jin (2004) studied the effect of photo-oxidative stress on the stability of oils (crude and refined mackerel liver oil). Oils were stored in artificial light or darkness and with or without antioxidants. The results showed that visible light exposure had a critical role in the acceleration of fish liver oil oxidation.

Akhtar et al. (2010) tested edible films of hydroxypropyl methylcellulose containing different edible colors (blue, green, yellow, red and white) for its ability to avoid photooxidation in salmon oil. The results showed that hydroxypropyl methylcellulose film with edible colors acts as a satisfactory light barrier to avoid photooxidation during extended storage of salmon oil. The white, red and yellow edible colors showed good control over oil photooxidation. Blue and green edible colors showed increased oxidation as time of light exposure increased.

3.10.3. Enzymatic oxidation

Enzymatic oxidation is important in plants and animal system (Gutteridge and Halliwell, 1990). The by-products of enzymatic oxidation affect sensory properties of the oil adversely. Sharp pungent odors along with stale and musty odors confirm rancidity. Factors that contribute to the formation of off-flavors are temperature, light, oxygen, moisture and metals (O'Brien, 2009). Endogenous enzymes liberated from the fish tissue itself can be a source responsible for the initiation of the peroxidation (Kanner and Kinsella, 1983).

Medina et al. (1999) studied the effect of enzymatic oxidative activity of two fatty fish (sardine and herring) during chilled storage. The lipoxygenase enzyme activity was isolated and tested by measuring the hydroperoxide. The lipoxygenase were concentrated in the skin tissue of fish and were active for up to 48 h of chilled storage and the pro-oxidative activity due to haem proteins continued longer than that due to the lipoxygenase.

Saeed and Howell (2002) studied the effect of storage of lipid and protein in Atlantic mackerel for up to 24 months at -20 and -30°C and found an increase in lipid oxidation with storage time and higher storage temperature of -20°C than -30°C. Ke et al. (1977) studied the kinetic variations in lipid oxidation in various parts of mackerel stored at -15, -30 and -40 °C with respect to peroxide value and thiobarbituric acid value. Oxidation of skin was eight times faster than that of white and dark muscles from mackerel at -15°C in two months.

3.11. Use of Antioxidants

To stabilize fish oil against oxidation, exogenous antioxidants are required (Zuta, 2003). Antioxidants are classified as either primary or secondary. The primary antioxidants include hindered phenols such as synthetic antioxidant or as naturally occurring compounds (Gray et al., 1996).

3.11.1. Natural antioxidants

The need for natural antioxidant continues to grow because they are safe since they occur in foods and have been used for centuries (Frankel, 1998). In general, natural antioxidants are plant phenolic compounds derived from different parts of the plants such as fruits, vegetables, seeds, nuts, leaves, bark and roots (Pratt and Hudson, 1990). These plant phenols can also act as free radical terminators, metal chelators, reducing agents and singlet oxygen quenchers (Mathew and Abraham, 2006).

Several studies have been carried out for isolation of natural antioxidant from oilseeds, cereal crop, spices and herbs (Wettasinghe and Shahidi, 1999). The most important natural antioxidants today are: tocopherols, ascorbic and citric acids and their salts (Tsimidou et al., 1995). The most abundant and biologically active tocopherol in food is α – tocopherol.

Zuta (2003) reported that a low level of alpha-tocopherol such as 50 and 100 ppm was found to reduce the oxidation in the fish oil. He also stated that 50 ppm of alpha-tocopherol in combination with storage at low temperature (-40°C) was considered as best level of antioxidant in protecting the fish oil from auto-oxidation.

Jayathilakan et al. (2007) studied the role of natural antioxidants (Maillard reaction products/MRPs, ascorbic acid, cloves and cinnamon) at 0.02% level each in controlling warmed-over-flavour in cooked and refrigerated meat (sheep, pork and beef). The results showed that the susceptibility of these species to lipid oxidation was in order, pork>beef>sheep, and order of natural antioxidants was MRPs>cloves>ascorbic acid> cinnamon.

Adios et al. (2002) noted that during the oil storage, no photo-oxidation was detected; storage at room temperature led to significant auto-oxidation over time from primary to tertiary oxidation products and storage at 50°C had significant increases in secondary and tertiary oxidation products. Free fatty acid (FFA) content remained low at all storage conditions and the one with alpha-tocopherol content remained constant.

3.11.2. Synthetic antioxidants

Synthetic antioxidants such as butylated hydroyanisole (BHA) and butylated hydroxytoluene (BHT) are used as food additives to prevent rancidification (Iqbal and Bhanger, 2005). They are effective and less expensive than natural antioxidants (Suja et al., 2004). However, these compounds may cause many health risks including: cancer and carcinogenesis (Iqbal and Bhanger, 2005). Propyl gallate (PG) and butylated hydroxyanisole (BHA) are synthetic phenolic antioxidants that show high chemical activities in arresting chain initiation or breaking chain propagation of peroxidation. Ascorbyl palmitate, a lipid soluble ester of vitamin C, is an approved synthetic antioxidant that helps delaying rancidity (Beddows et al., 2001).

Jayathilakan et al. (2007) studied the role of synthetic antioxidants (TBHQ, BHA and propyl gallate/PG) at 0.02% level each in controlling warmed-over-flavour in cooked and refrigerated meat (sheep, pork and beef). The results showed that the susceptibility of these species to lipid oxidation in synthetic antioxidants was TBHQ>BHA>PG.

Raza et al. (2009) studied the effect of BHA on the storage stability of freshly prepared butter. After 5 weeks of storage at 45°C, freshly prepared butter containing 200 ppm BHA showed lower values of FFA (0.586%) and peroxide value (7.33 meq/kg). From the results it was noted that the shelf life of butter can be extended for 5 weeks by the addition of an antioxidant like BHA.

3.12. Quality Parameters of Fish Oil

Fish oils are commercially sold and priced according to the level of free fatty acids, impurities, moisture content, unsaponifiable matter, color, *p*-anisidine value, and peroxide value (European Commission, 2006). Some oil properties such as color and fatty acid profile are reported to be similar, regardless of the extraction method used. Thus, comparison of other properties such as oil acidity, total oxidation value (TOTOX) of different methods has been recommended (Rubio-Rodríguez et al., 2008). Table 3.7 shows recommended values of quality parameters for crude fish oil.

Table 3.7. Recommended quality parameters for crude fish oil.

Quality Parameter	Recommended Value	Reference
Moisture	0.5%	Bimbo (1998)
Impurities	(1% maximum)	Bimbo (1998)
Free fatty acids	1-7%	Bimbo (1998)
Peroxide value (PV)	3-20 meq/kg	Bimbo (1998)
p-Anisidine value	4-60	Bimbo (1998)
Total oxidation value	10-60	Bimbo (1998)
Unsaponifiable matter	<2 (wt%)	Scrimgeour (2005)
Color (Gardner scale)	10.5-12.5	Omega protein (2010)

3.12.1. Acid value

According to the Lipid Library (2012), free fatty acids (FFA) are unesterified fatty acids. In animals, dietary lipids are hydrolysed to free acids before they get absorbed and utilized for animal lipid synthesis. A variety of lipolytic enzymes (e.g. lipoprotein lipase, phospholipase A, hormone-sensitive lipase) helps the intact lipid in tissue to hydrolyse to free acids before oxidation, desaturation, elongation or re-esterification.

According to Rubio-Rodríguez et al. (2008), oil acidity is an important quality parameter determining the presence of FFA and other non-lipid acid compounds. FFA is mostly generated by a hydrolysis reaction of triacylglycerides. Oil acidity depends on several factors such as oil composition, the extraction procedure and the raw material freshness.

The acid value quantifies the amount of acid present. It is the mass of potassium hydroxide in mg that is required to neutralize 1g of chemical substance (Wrolstad et al., 2005). As oil go rancid, triacylglyceride (TAG) coverts to fatty acid (FA) and glycerol which increases acid number.

Memon et al. (2010) studied the effect of refrigerated storage on the acid value of Jarko fish oil for up to 120 days. The results showed that the Jarko fish oil remained edible for consumption for 60 days but eventually exceeded the recommended acid value after 60 days of refrigerated storage. Domiszewski et al. (2011) evaluated the acid value of catfish fillet and observed that the raw catfish fillet had an acid value of 6.45±0.06 mg KOH/g lipid.

It is important to determine free fatty acids (FFA) content as it is used for quality criteria of fats and oils. Also, FFA has employed to assess fish deterioration during frozen storage (de Koning and Mol, 1991) and FFA content increases with increase in storage time. According to Miyashita and Takagi (1986), FFA has shown catalytic effect on lipid oxidation development on marine lipids.

Adeniyi and Bawa (2006) extracted mackerel oil and found that the crude oil had an acid value of 2.5 mg/KOH, whereas the refined oil had 2.27 mg/KOH. Aberoumand (2010) studied the quality of different fish oils and reported acid values of 4.12, 1.21, 3.21, 4.28 and 6.72 for *Cyprinodon aphanius dispar*, *Chirocenterous dorab*, *Cybium scomberomorus guttatum*, *Cybium guttatum* and *Hilsa macrura ilisha*, respectively.

3.12.2. p-Anisidine value

The p-anisidine value is used to measure the secondary product of oxidation and determines the aldehyde in the lipid. Aldehyde present in the oil and the p-anisidine reagent react under acidic condition (IUPAC, 1987; O'Brien, 2009). The color obtained not only depends on the aldehyde present, but also their structure. Further degradation of lipids generates off-flavours and off-odours (St. Angelo, 1996).

Hung and Slinger (1981) evaluated the peroxide, anisidine, free fatty acid and thiobarbituric acid value as methods of measuring oxidation in herring oil and found that oxidation occurred when herring oil passed the induction period (792 h).

Pak (2005) studied the stability and quality of fish oil and found the anisidine value to be 19.8. Boonchouy et al. (2009) studied that quality analysis of fish oil using near-infrared spectroscopy. The optimized calibration was developed near 1100-2500 nm. From the result it was observed that the root mean square of p-anisidine value was 6.88 with a correlation coefficient of 0.88.

Klaypradit et al. (2010) studied the oxidation of menhaden fish oil using fourier transform infrared spectroscopy (FTIR) method. Anisidine value was determined each day during 3 weeks of storage. The result showed an anisidine value of 1,746-1,736 with wave number of 2.58. Che Man and Setiowaty (1999) studied the anisidine value of oxidized palm olein by FTIR and an anisidine value was 8.684.

3.12.3. Peroxide value

The peroxide value is the measurement of primary oxidation product hydroperoxide (Adios et al., 2001). The most widely used chemical test for the determination of fats and oil quality is peroxide value test (O'Brien, 2009). The number of peroxides present in the oil is an index of their primary oxidative level and its tendency to go rancid. The lower the peroxide value the better the oil quality and its state of preservation (CDR, 2008).

Aidos et al. (2002) studied the effect of temperature on lipid oxidation of crude herring oil stored at 0, 20, and 50°C using peroxide value (PV) and reported a secondary oxidation stage of oil at all temperatures during storage. The oxidation products exhibited temperature dependency.

Fritsche and Johnston (1988) reported that peroxide value of the diet (containing menhaden oil without antioxidant) had elevated 5-6 folds within 24 h and 12 fold within 48 h when exposed to air at room temperature.
Boran et al. (2006) studied the effect of storage temperature (4 and -18°C) on oil quality of various fish (horse mackerel, shad, garfish and golden mullet) and observed increases in peroxide values at both the temperatures.

Boonchouy et al. (2009) evaluated the quality of fish oil using near-infrared spectroscopy. The optimized calibration was developed near 1100-2200 nm and the result obtained for peroxide value was 1.44 meq/kg. Pak (2005) studied the stability and quality of fish oil which had 0.6 meq/kg peroxide value.

3.13. Concentration and Composition of Omega-3 Fatty Acids

There are various methods to concentrate omega-3 fatty acid including: enzymatic hydrolysis, urea complexation, supercritical fluid extraction and molecular distillation. The challenge is to develop cost-effective procedures to produce omega-3-PUFA concentrates (Wanasundara and Shahidi, 1998).

3.13.1. Enzymatic hydrolysis

Enzymatic extraction is carried out at the laboratory scale. Hydrolysis of edible fats and oils result in the formation of free fatty acids, di- and monoglyceride and glycerol (O'Brien, 2009). Acylglycerols (glycerides) are acyl esters of glycerols known as monoacylglycerols, diacylglycerols and triacylglycerols. Natural oils and fats are mostly triacylglycerols which are known to be the most important class of storage lipids in most animals and plants, but not necessarily in marine organisms (Gurr and James, 1980). According to Lee and Akoh (1998) and Soumanou et al. (1998), enzymatic processes (selective hydrolysis, hydrolysis and selective esterification and transesterification) using lipases are ideal techniques for the production of omega-3 fatty acids (EPA and DHA) that is nutritionally valuable because of their specificity and high activity at low temperatures.

Wanasundara and Shahidi (1998) studied the production of omega-3 fatty acid in small scale from seal blubber oil (SBO) and menhaden oil (MHO) in the form of acylglycerols using various microbial lipases including *Aspergillus niger*, *Candida cylindracea* (now known as *Candida rugosa*), *Chromobacterium viscosum*, *Geotrichum candidum*, *Mucormiehei*, *Pseudomonas* sp., *Rhizopus oryzae*, and *Rhizopusniveus*. The result indicated that all lipases effectively increased the n-3 PUFA content of the acylglycerols of both SBO and MHO. However, the highest concentration of n-3 PUFA was provided by *Candida cylindracea* lipase. After 40 h of hydrolysis, 43.5% of ω-3 in SBO [9.75% eicosapentaenoic acid (EPA), 8.61% docosapentaenoic acid (DPA), and 24.0% docosahexaenoic acid (DHA)] and 44.1% in MHO (18.5% EPA, 3.62% DPA, and 17.3% DHA) were obtained. It was concluded that *Candida cylindracea* lipase was most suitable for preparation of n-3 PUFA in the acylglycerol form from marine oils.

Carvalho et al. (2002) attempted to concentrate the omega-3 fatty acid in small scale using four different lipases (*Candida cylindracea lipase, Rhizopus delemar lipase, Aspergillus oryzae lipase* and *Chromobacterium viscosum*) and found *Candida cylindracea lipase* to be the most effective compared to other lipases.

Okada and Morrissey (2007) studied enzymatic hydrolysis of sardine oil for concentrating omega-3 fatty acid in small scale with *Candida rugosa, Mucor javanicus,* and *Aspergillus niger* and found *Candida rugosa* to be more effective compared to other lipases. Moore and McNeill (1996) studied triglycerides enriched in long chain polyunsaturated fatty acid (PUFA) from fish oil and obtained 51% of total omega-3 fatty acid using *Candida rugosa*. Sun et al. (2002) studied enzymatic hydrolysis for concentrated n-3 PUFA in which *Candida rugosa* and *Pseudomonas cepacia* were found to be most effective. Table 3.8 describes the comparison of different lipase used in enzymatic hydrolysis. *Candida rugosa* gave better yield in limited time.

Zuta (2003) used lipase enzyme to concentrate long chain omega-3 in small scale from natural sources due to the reason that most lipase had low reactivity towards this fatty acid because of their geometric configuration. Concentration of EPA and DHA beyond 300 mg/g was possible only after splitting the lipid followed by non-enzymatic method. Levels above 80% was prepared by the combining lipase catalyzed selective hydrolysis and esterification with various fractionation techniques such as HPLC and molecular distillation.

Mbatia et al. (2010) studied the enzymatic enrichment of omega-3 fatty acid (small scale) with Nile perch viscera oil and reported that the temperature can affect the reaction rate of lipase whichin turn affected the enrichment. Higher enrichment level was linked with more EPA and DHA losses. Chen and Ju (2000) stated that using immobilized enzymes is time consuming and to avoid the PUFA oxidation (with relatively low temperature) additional time is required.

The major factors that affect the economy of producing ω-3 fatty acids concentrates *via* lipase-assisted hydrolysis are enzyme concentration, reaction time, temperature and pH. Also, the temperature of the reaction medium and the reaction time are considered important because they influence the oxidative state of the prepared ω-3 concentrates (Sun et al., 2002).

Table 3.8. Comparison of immobilized lipase used in enzymatic hydrolysis.

Lipase*	Time (h)	Temperature (°C)	Yield (%)			Reference
			EPA	DHA	Total	
Candida rugosa	48	25	18.5	28.8	51.0	Moore and McNeill (1996)
	20	35	37.3	20.6	57.9	Sun et al. (2002)
	8	35	19.7	20.7	40.4	Carvalho (2002)
	40	37	19.5	24	43.5	Wanasundara and Shahid (1998)
Rhizopus delemar	8	35	15.9	15.5	31.4	Carvalho (2002)
Chromobacterium viscosum	8	35	15.7	15.2	30.9	Carvalho (2002)
Aspergillus oryzae	8	35	14.8	13.2	28.0	Carvalho (2002)

*Immobilized form

3.13.1.1. Enzyme load. According to Shuang et al. (2009), adding more lipase than necessary would result in a waste and increase reaction cost. The appropriate amount of enzyme depends on the enzyme activity. However, using smaller amounts of enzyme is simply not possible due to difficulties in handling. Mussatto et al. (2008) studied the effect of enzyme load in enzymatic hydrolysis of BSG cellulose. It was noted that the increase from 5-45 FPU/g of enzyme load increased the glucose yield and cellulose conversion. The enzyme load increase also favored the enzymatic hydrolysis of cellulose from hardwood (Gan et al., 2003), rice husk (Yanez et al., 2006), and dairy manure fibers (Liao et al., 2005).

Wanasundara and Shahidi (1998) studied the effects of enzyme concentration; reaction time and reaction temperature on omega-3 fatty acids from SBO and MHO using three-factor central composite rotatable design. The maximum yield obtained after hydrolysis was 54.3% of total ω-3 fatty acids from SBO at an enzyme (*Candida cylindracea*) concentration of 308 U/g oil, reaction time of 40 h, and reaction temperature of 37°C. Similarly, maximum of 54.5% of total ω-3 fatty acids was obtained from MHO at an enzyme concentration of 340 U/g oil, reaction time of 45 h, and reaction temperature of 38°C.

3.13.1.2. Temperature. As temperature increases, the enzyme activity increases until it reaches its maximum. Senanayake and Shahidi (1999) stated that the temperature increase caused the kinetic energy of the enzyme to increase, which in turn increased the amount of molecules colliding with the enzyme. This increased the rate of reaction but with sufficiently high temperatures the reaction rate began to decrease as the enzyme was denatured and the bonds holding the three-dimensional shape of the enzyme became weak. Akoh et al. (2002) found that as temperature was increased, the rate at which the long-chain fatty acids were released was faster than the rate at which short chain fatty acids were released. Shuang et al. (2009) stated that too high a temperature can affect the enzyme stability and reactivity. Sun et al. (2012) stated that high temperature changes the specific structure and inhibits the enzyme activity.

3.13.1.3. pH. Utsun et al. (1997) reported that variation in the pH for most enzymes within a range of two or three pH units each side of iso-electric points is a reversible process and the extreme pH will cause irreversible denaturation of the enzymes. Also, the pH used depends on the enzyme. The *C. cylindracea* (CC) lipase possessed a wide range of optimal pH activity (5.0-8.0). However, pH 7.0 was found to be most suitable (Hoshino et al., 1990). Eisenberg and Field (1956) studied the enzymatic hydrolysis of glucuronolactone and noticed that at lower pH the enzymatic hydrolysis of glucuronolactone was completely inhibited.

3.13.1.4. Reaction time. Determining the reaction time is important as it is related to energy necessary for shaking and maintaining constant temperature (Ruiz et al., 2009). Also, reaction time can affect the scale-up process. Shuang et al. (2009) reported that longer reaction times resulted in higher infusing of caprylic acid into soybean oil using immobilized enzyme derived from Rhizopus miehei.

3.13.2. Urea complexation

Urea complexation is the simplest and most efficient technique to obtain polyunsaturated fatty acid (PUFA) concentrates in the form of free fatty acids on laboratory scale. It is a well-established technique for elimination of saturatedand monounsaturated fatty acids (Gamez et al., 2003; Tor and Yi, 2001). Figure 3.4 shows the formation of urea crystals in the absence and presence of long chain fatty acids. Detailed procedures for recovery of fatty acids and preparation of omega-3 fatty acids concentration as given by Wanasundara and Shahidi (1999) are shown in Figure 3.5 and 3.6. Also, several researchers used similar procedure (Zuta, 2003; Ratnayake et al., 1988, Liu et al., 2006 and Bharadwaj, 2011).

Ratnayake et al. (1988) demonstrated the use of urea complexation in pilot scale for the concentration of omega-3 fatty acid. Wanasundara and Shahidi (1999) optimized the production of omega-3 fatty acid from seal blubber using urea complexation and obtained a maximum production of omega-3 fatty acid (88.2%) at a urea:fatty acid ratio of 4.5:1, a

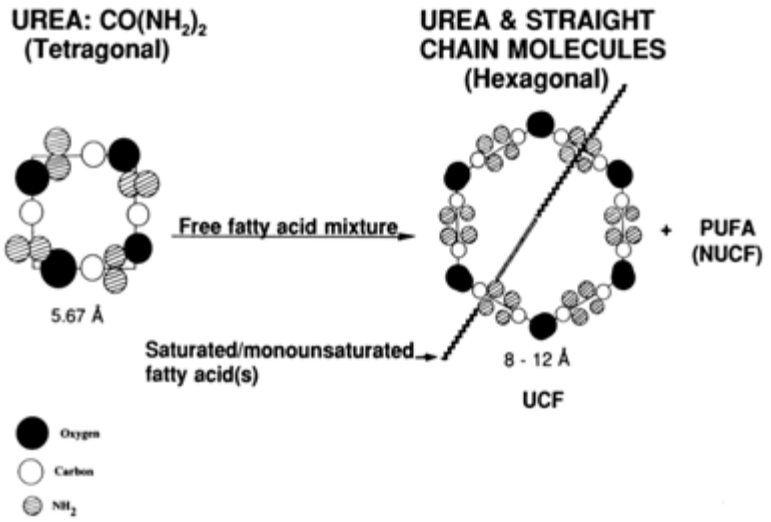


Figure 3.4. Formation of urea crystals in the absence and presence of long chain fatty acids (Wanasundara and Shahidi, 1998).

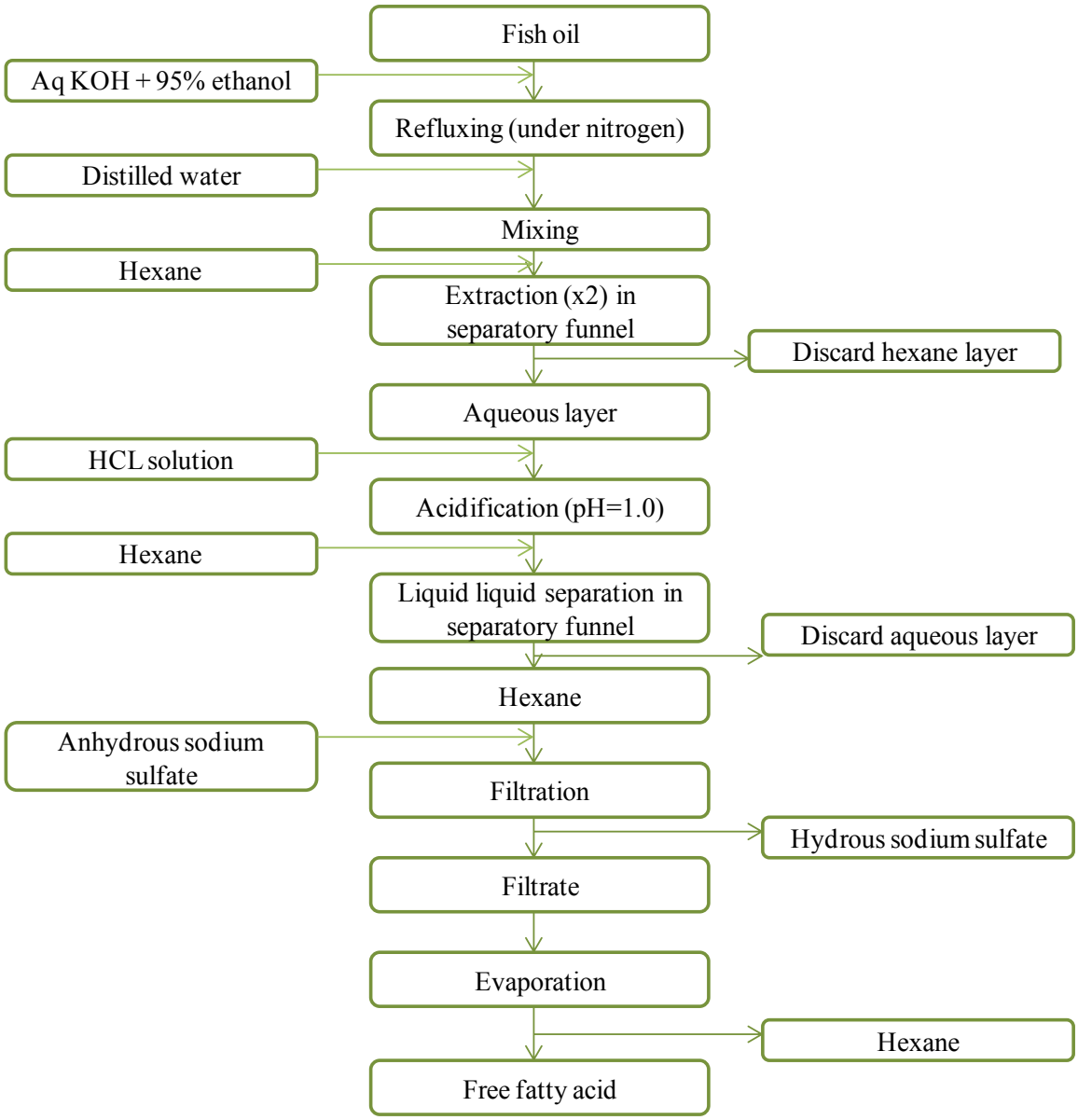


Figure 3.5. Schematic representation of free fatty acids from fish oil (Wanasundara, 1996).

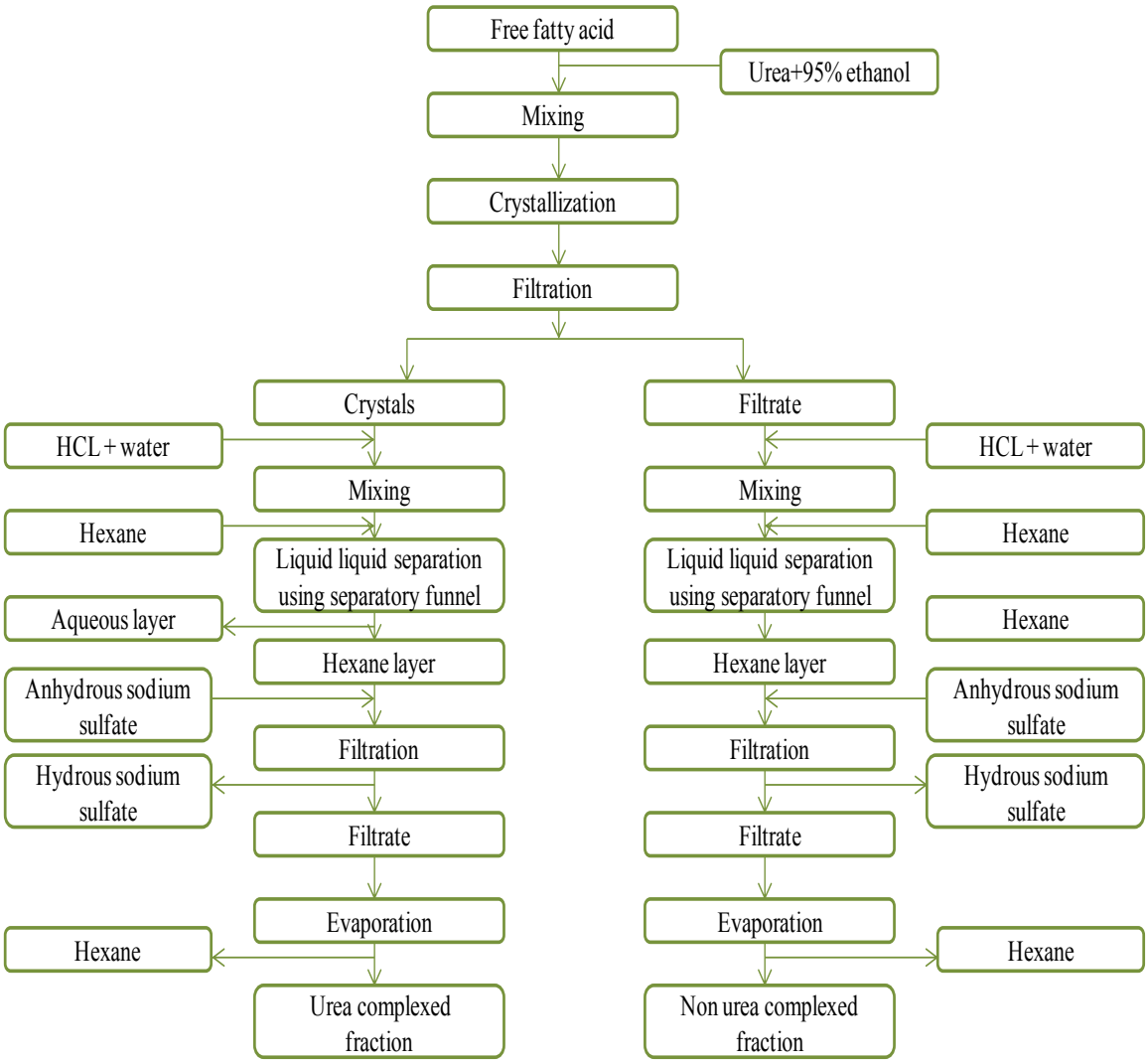


Figure 3.6. Preparation of omega-3 fatty acids concentrates by urea complexation (Wanasundara, 1996).

crystallization time of 24 h, and a crystallization temperature of -10°C under optimum conditions.

According to Strocchi and Bonaga (1975), TAG in the oil splits into their constituent fatty acid by alkaline hydrolysis with alcoholic KOH or NaOH. The obtained free fatty acid again mixed with ethanolic solution of urea for complex formation. Saturated and monosaturated fatty acid complexes with urea were crystallized easily by cooling and were removed by filtration.

Liu et al. (2006) studied the extraction of omega-3 fatty acid from tuna oil using optimized urea complexation procedure. The total DHA and EPA (85.02%) were obtained at a urea-to-fatty acid ratio of 15 (mole/mole), a crystallization temperature of -5 °C and a crystallization time of 20 h. Bharadwaj (2011) reported that on the production of EPA and DHA enriched PUFA from cod liver fish oil using urea complexation and obtained 77.7% PUFA.

In urea complexation method, the important factors are temperatures (-6.9 to 6.9°C), time (14 to 34 h), and ratio of urea to fatty acid (0.9:1 – 6.1:1) (Zuta, 2003).

3.13.2.1. Crystallisation temperature. Temperature has significant effect on the urea complexation (Liu et al., 2006). Temperature should be controlled carefully to achieve a maximum extraction of omega-3 fatty acid in the concentrate with a reasonable recovery. Fei et al. (2010) reported that recovery of polyunsaturated fatty acid varied inversely with increases in urea to fatty acid ratio as well as decreases in crystallisation temperature.

At lower the crystallisation temperature, there is formation of more stable urea complex which in turn reduces the saturated fatty acid. Ratnayake et al. (1988) used the urea complexation method to extract omega-3 PUFA concentrates from fish oils and observed that even at low temperature (-5°C) it was difficult to completely remove the short chain lengths particularly C14:0, C16:0 and C16:1.

3.13.2.2. Crystallisation time. A longer crystallisation time allows for further stabilisation of crystals (Fei et al., 2010). Liu et al. (2006) studied the extraction of EPA and DHA of tuna oil by urea complexation by optimizing the process parameters (crystallisation time, crystallisation temperature, urea to fatty acid ratio). The results indicated that the crystallisation time was insignificant. Similar results were reported by Wanasundar and Shahidi (1999); Abdullah and Salimon (2010); Ratnayake et al. (1988). Zuta (2003) reported that crystallization time was mostly not significant with the range of values used.

3.13.2.3. Urea:fatty acid ratio. Urea:fatty acid ratio affects the urea complexation (Liu et al., 2006). Enrichment of polyunsaturated fatty acid and recovery yield in concentrate vary inversely with increasing urea-to-fatty acid ratio. Lower urea to FA ratio prevents indiscriminate FFA complexation (Hayes et al., 1998). Senanayake and Shahidi (2000) studied the effect of urea:FA ratio (w/w) on the yield of DHA. By keeping the weight of fatty acids constant and varying the weight of urea, the urea complex was allowed to crystallize at 4°C for 24 h. The results showed that the concentration of DHA was maximum at 3:1 urea:fatty acid ratio. Haagsma et al. (1982) reported that the urea complex formation reached its maximum when the urea:FA ratio was 3:1 for cod liver oil fatty acid methyl esters.

3.13.3. Supercritical fluids

Supercritical fluid extraction is a new separation process that is used to avoid the problems associated with conventional separation techniques. Various gases possess selective solvent properties under pressure. Properties that are given importance are viscosity and diffusivity of the supercritical fluid (generally more gas-like than those of typical liquids). CO_2 is used because it has moderate critical temperature and pressure and is inert, inexpensive, non-flammable, environmentally acceptable, readily available and safe. The extraction involved in the separation of polyunsaturated fatty acid (PUFA) depends on the molecular size of the components rather than their degree of un-saturation (Mishra et al., 1993).

In the separation of omega-3 fatty acid by supercritical fluid extraction, the oil used requires preparation steps such as extraction, hydrolysis and esterification by conventional methods as shown in Figure 3.7. Mishra et al. (1993) and Stout and Spinelli (1987) used supercritical fluid extraction to extract omega-3 fatty acid from fish oil and noted that supercritical fluid extraction was effective in refining fish oil and removing cholesterol, vitamin E, polychlorinated biphenyls and other components. Nearly 60-65% of DHA was obtained from fish oil esters that were fractionated by supercritical fluid extraction.

Letisse et al. (2006) used supercritical fluid extraction for the extraction and fractionation of EPA and DHA from sardine fish waste (fish heads produced for canning). They optimized various conditions including temperature, pressure, time and carbon dioxide rate in order to obtain the highest yield of oil and the highest amount of EPA and DHA in the extracted product. The yield of extracted oil was 10.36% and the concentration of EPA and DHA were 10.95 and 13.01%, respectively.

Rubio-Rodriguez et al. (2008) extracted omega-3 rich oil from by-products of the fish industry (off cuts obtained from peeling hake) by supercritical fluid extraction. The extraction process was carried out at different pressures and flow rates. The result obtained indicated that the internal mass transfer controlled the rate of the experiment and the oil obtained contained high omega-3 and high EPA and DHA content.

The main disadvantage of the supercritical fluid extraction process was the high production cost, because of the use of high pressure equipment and the raw material which should be freeze-dried in order to reduce its moisture to values below 20% and keep the omega-3 PUFA unaltered (Rubio-Rodríguez et al., 2008).

3.13.4. Molecular distillation

The molecular distillation method is used for partial separation of FA mixtures of esters. Molecular distillation (MD) is a short path distillation which uses lower temperatures and short heating intervals (Brown and Kolb, 1955). Molecular distillation is industrially used

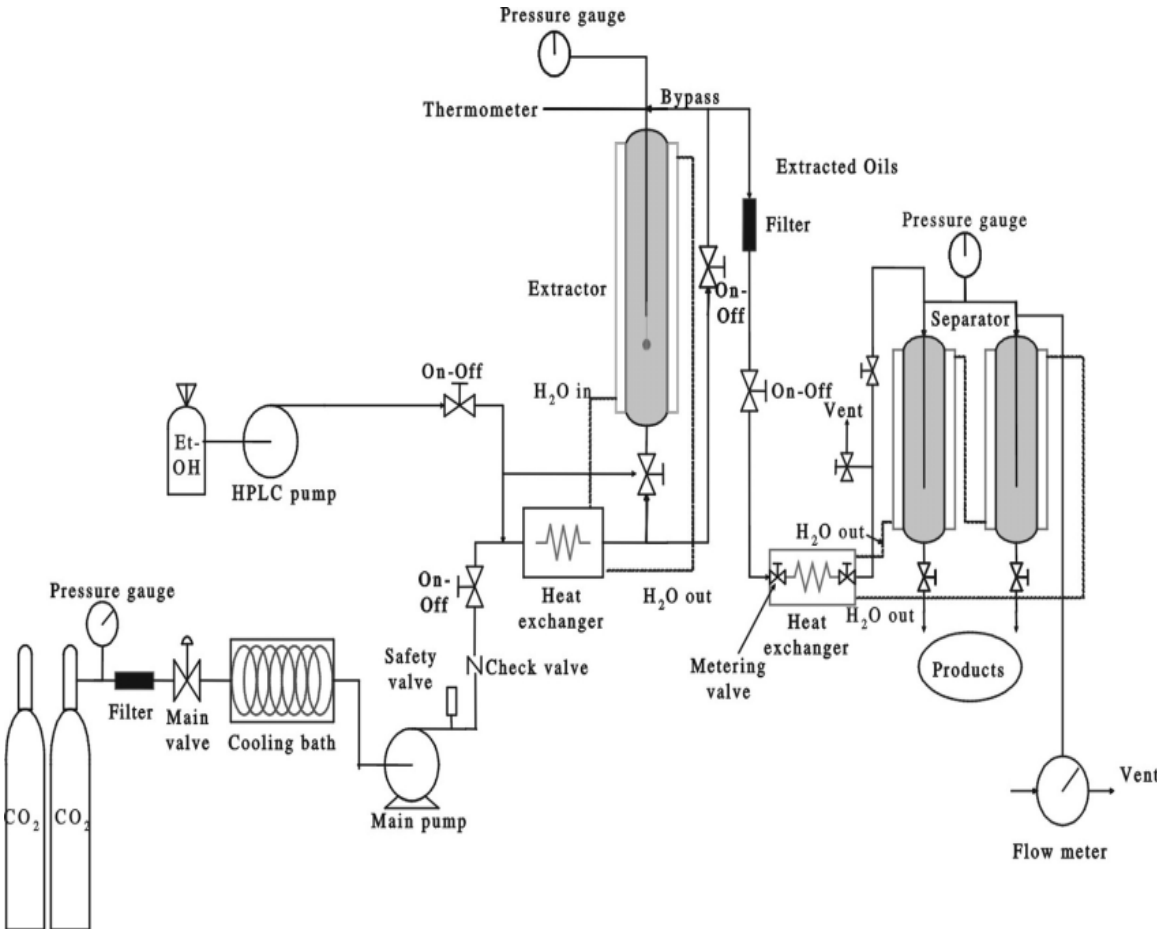


Figure 3.7. Schematic diagram of the supercritical fluid process (Kang et al., 2005).

in the purification of unstable fats and oils and consists of wiped film molecular distillation unit and centrifugal molecular distillation unit (Cermak et al., 2007).

According to Lembke (1997), the free fatty acids and fatty acid ethyl esters have relatively low evaporation temperatures when placed in a strong vacuum. Good separation of C-18 fatty acids can be achieved by maintaining oil under vacuum and temperatures of 140°C and 160°C. The procedure resulted in the concentration of initial EPA and DHA content with increases of 25%. The advantage of molecular distillation is that it is an excellent technology to decontaminate and remove cholesterol from fish oils triglycerides (Lembke, 2011).

Liang and Hwang (2000) studied fractionation of squid visceral oil ethyl esters by short-path distillation. The elimination temperatures of squid visceral oil ethyl esters (SVOEE) ranged from 50 to 140°C, increasing with the carbon number of ethyl esters. It was observed that at 130°C distillate gave 15.5% EPA and 34.7% DHA with 99 mg/100 g of cholesterol and the yield was 21.8%.

Stout et al. (1990) studied molecular distillation of menhaden oil and observed that oil in its ethyl esters form increased the EPA content from 15.9 to 28.4%. However, DHA content increased from 9.0 to 43.9% when oil was in the simple alkyl esters form. Wijesundara et al. (1989) reported that during distillation, exposure of long-chain PUFA to high temperatures may encourage hydrolysis, polymerization, thermal oxidation and isomerisation. Shahidi and Wanasundara (1998) reported that the process for preparation of omega-3 PUFA concentrates should involve low temperature and short time to minimize thermal damage.

3.13.5. Comparison of extraction methods

There are several methods to concentrate omega-3 FA but only few are suitable in large scale production. These extraction methods include enzymatic hydrolysis, urea complexation, supercritical fluids and molecular distillation. A comparative analysis of the extraction methods is presented in Table 3.9.

Table 3.9. Comparison of extraction methods of omega-3 fatty acids

Criteria	Enzymatic Hydrolysis	Urea Complexation	Supercritical Fluid	Molecular Distillation
Capital cost	Moderate	Low	High	Moderate
Total cost	Low	Moderate	Moderate	Low
Recovery yield	43.5-57.9%	69-85%	10.95 -EPA and 13.01%-DHA	65-75%
Final product form	Acylglycerol	free acid or simple ester	Ethyl ester	Ethyl ester
Environmental friendly	Yes	Yes	Yes	Yes
Time	20-24 h	30- 35 h	<5 h	Depends on the condition used
Reusability	Reusable	Not reusable	Not reusable	Not reusable
Temperature	35-40 °C	55-70 °C	<50 °C	140-160 °C
Mode of operation	Easy	Moderate	Moderate	Moderate

The advantage of the enzymatic hydrolysis method is enzymes are environmentally friendly and safe and the process requires low temperature (Ferna´ndez-Lorente et al., 2005), the concentration of PUFA is straightforward (Bottino et al., 1967). The use of immobilized lipases has additional technological and economical advantages as it can be reused (Ferna´ndez-Lorente et al., 2005). Recovery of omega-3 PUFA in glyceride form makes it more nutritionally favorable than methyl or ethyl esters of these fatty acids (FAs) due to improved absorption of glyceride form (Sun et al., 2002). The disadvantage of this method is the initial cost and need for downstream processing (Kahveci and Xu, 2012).

The urea complexation method has advantage of having higher yield and it is cost effective as urea is inexpensive. It is also environmentally friendly as less toxic solvents and uses simple equipment are used for concentration of PUFA of larger sample size (Zuta, 2003). The disadvantages of this method are: the final product (simple ester) is not easily incorporated in human plasma, high temperature and longer time and the need for downstream processing step (Boustani et al., 1987).

In supercritical fluid extraction (SFE) a number of gases can be used. However, carbon dioxide is chosen as it has moderate critical temperature and pressure (31.1°C, 1070 psig) and it is inert, inexpensive, non-inflammable, environmentally friendly and safe (Mishra et al., 1993). The disadvantage of this method is that the final product is in the form of ethyl ester which is not easily absorbed well by humans (Lawson and Hughes, 1988). The use of extremely high pressure and high capital costs limit the widespread use of this method in large processing industries (Pinton, 1998).

The molecular distillation (MD) operates at a temperature in the range of 140-160°C. Due to increased thermal stress, the shelf-life and stability of the unsaturated fatty acids may be impacted (Lembke, 2011). According to Lembke (1997), fish oil passing through the molecular distillation method suffers up to 350% higher thermal stress when compared to a product passing through the supercritical extraction method. However, when comparing molecular distillation and supercritical fluid extraction, the investment for the molecular

distillation is considerably lower than the investment for an industrial supercritical fluid extraction plant. And molecular distillation is the standard process in the fish oil industry.

CHAPTER 4. MATERIALS AND METHODS

4.1. Experimental Materials

4.1.1. Fish

Atlantic Herring (*Clupea harengus*) was obtained from Clearwater, Halifax, Nova Scotia. The samples were homogenized and kept in sealed plastic bags in a freezer at -20°C.

4.1.2. Glassware

The glassware used in the experiments includes test tubes, beakers, conical flasks, pipettes and funnels. They were obtained from Fisher Scientific, Montreal, Quebec, Canada. The glassware were washed with soap, rinsed first with tap water and then rinsed with acetone, dried in the oven. And then a final rinse with methylene chloride was performed before use.

4.1.3. Chemicals and enzymes

Chloroform, hexane, isopropyl alcohol, alpha-tocopherol, starch indicator, sodium thiosulphate, potassium hydroxide, potassium iodide, 1% phenolphthalein indicator, were purchased from Fisher Scientific Limited, Ottawa, Ontario, Canada. Methanol, anhydrous sodium sulphate, butylated hydroxytoluene (BHT), sodium hydroxide, sodium chloride, sulphuric acid, iodine solution, sodium thiosulfate, toluene, isooctane, glacial acetic acid and *p*-anisidine, *Alcalase* (Liquid enzyme) and *Candida rugosa* (previously known as *Candida cylindracea*) Type VII lipase were purchased from Sigma Aldrich, Oakville, Ontario, Canada.

4.1.4. Reagents

Hilditch reagent (100 ml dry methanol + 1.5 ml sulphuric acid), methylene chloride w/0.01% BHT, 3:2 v/v acetic acid-chloroform (3 volumes of reagent-grade glacial acetic acid with 2 volumes of reagent-grade chloroform), *p*-Anisidine reagent (0.25g/100 ml

solution in glacial acetic acid), 3M sodium hydroxide (Molecular weight of NaOH is multiplied by three which is dissolved in 1 L of distilled water) were prepared.

4.1.5. Equipment

The equipment used in this study were a rotary evaporator (RE540- Yamato Scientific America Inc, Orangeburg, NewYork, USA), water-bath shaker (Thermo Model 2870, Thermo Scientific, Ottawa, Ontario, Canada), water bath (Microprocessor Controlled 280 Series, Precision, Ottawa, Ontario, Canada), centrifuge (Sorvall RT1, Thermo Scientific, Ottawa, Ontario, Canada), gas chromatograph (PerkinElmer Auto-system,LAS Canada Inc, Woodbridge, Ontario, Canada), spectrophotometer (Model No. 4001/4, Thermo Scientific, Ottawa, Ontario, Canada), nitrogen evaporator (N-EVAP112, OA-SYS heating system, Organomation associates Inc, Berlin, Massachusetts, USA) and pH meter (Orion 5 Star 1119001, Thermo Scientific, Ottawa, Ontario, Canada).

4.2. Experimental Design

The experimental work was performed on two steps: (a) extraction of fish oil from fish and (b) recovery of omega-3 fatty acid from fish oil and determination of omega-3 fatty acid composition. Table 4.1 and 4.2 represent the optimization conditions for recovery of fish oil and omega-3 fatty acid using enzymatic hydrolysis, respectively.

In step 1, fish oil was extracted from fish sample by enzymatic hydrolysis using the enzyme alcalase. The effects of temperature (55°C and 70°C), pH (7.0, 7.5 and 8.0) and enzyme load (0.5%, 1.0% and 2.0% - by weight of raw material) on the oil yield and quality were investigated. Oil extraction was also performed using the Bligh and Dyer (Chloroform:methanol) solvent extraction method. Three triplicates were carried out. The chemical properties of extracted fish oil (acid value, *p*-anisidine value, and peroxide value), fatty acid composition, and lipid yield were determined. The oils obtained from enzymatic hydrolysis and the Bligh and Dyer (solvent extraction) method were compared with respect to yield, properties and fatty acid composition. Figure 4.1 shows the

Table 4.1. Fish oil extraction by enzymatic hydrolysis.

Temperature (°C)	pH	Enzyme Load (%)
50	7.0	0.5
		1.0
		2.0
	7.5	0.5
		1.0
		2.0
	8.0	0.5
		1.0
		2.0
70	7.0	0.5
		1.0
		2.0
	7.5	0.5
		1.0
		2.0
	8.0	0.5
		1.0
		2.0

No. of replicates = 3
No. of runs = 54

Table 4.2. Recovery of omega-3 fatty acids using enzymatic hydrolysis.

Stirring Level	Value
Stirring level-1	No stirring
Stirring level-2	Intermediate stirring (100 rpm with 4 h interval)
Stirring level-3	Constant-slow stirring (50 rpm)
Stirring level-4	Constant-fast stirring (200 rpm)

No. of replicates = 3
No. of runs = 12

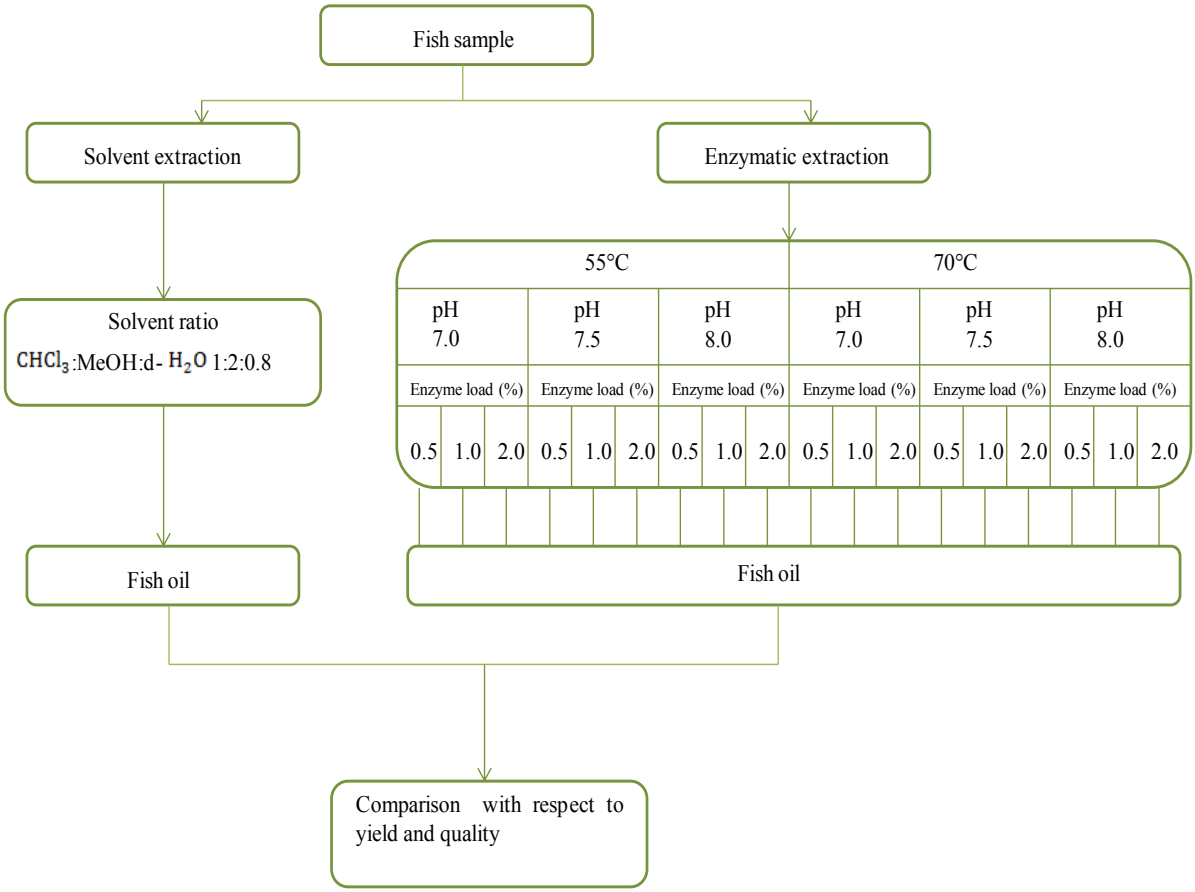


Figure 4.1. Schematic representation of experiment for the recovery of fish oil and comparison of fish oil extraction methods.

schematic representation of the experiments for the recovery of fish oil and comparison of fish oil extraction methods.

In step 2, omega-3 fatty acid was extracted from the fish oil obtained by enzymatic hydrolysis using *Candida rugosa* lipase. After the addition of enzyme, four stirring methods (no stirring, intermediate stirring, constant-slow stirring and constant-fast stirring) were evaluated (Figure 4.2) at optimum environment conditions (20 hr, temperature of 35ºC and enzyme load of 800 Units). The recovery yield and composition of omega-3 fatty acid was determined. Three replicates were carried out.

4.3. Experimental Procedure

4.3.1. Sample preparation

Atlantic herring (*Clupea harengus*) was used in this experiment. 10 kg of fish was thawed and homogenized by directly feeding (no solvent or water added) into a homogenizer (Model No.4532s/s, Hobart Manufacturing Co. Ltd, Ontario, Canada) which gives homogenized sample by leaving bones behind. It was then weighted, packed in plastic bags, sealed and stored at -20°C for later use.

4.3.2. Stabilization of fish oil with antioxidant

Due to the high degree of unsaturation of fatty acids, fish oil was susceptible to auto-oxidation. To stabilize the oil against oxidation, exogenous antioxidants were required. 100 ppm of alpha-tocopherol was prepared in 2-3 ml hexane as recommended by Zuta (2003). The solvent was evaporated under nitrogen and then fish oil was added and mixed thoroughly while still under nitrogen. The sample was then stored at -20°C.

4.3.3. Solvent extraction of fish oil

The solvent extraction was performed according to the procedure described by Bligh and Dyer (1959) as shown in Figure 4.3. A sample of 100 g of homogenized fish, 200 ml

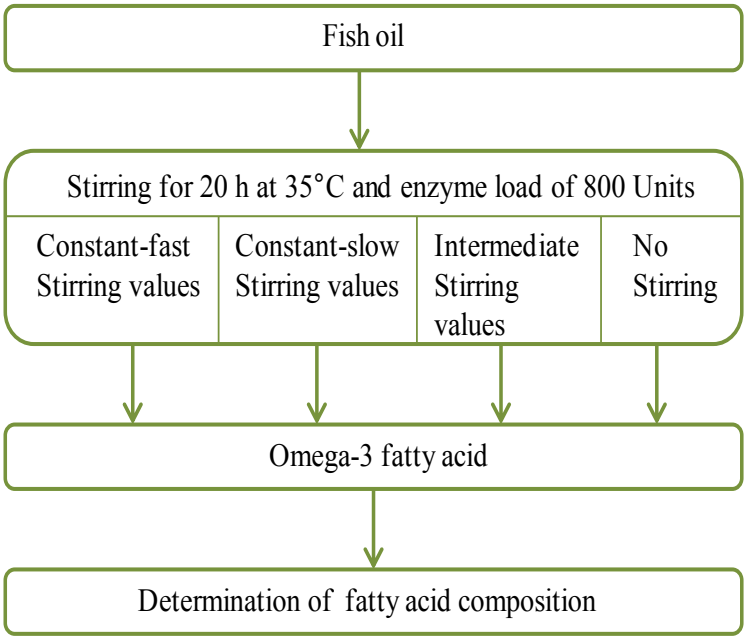


Figure 4.2. Schematic representation of experiment for recovery of omega-3 fatty acid from fish oil.

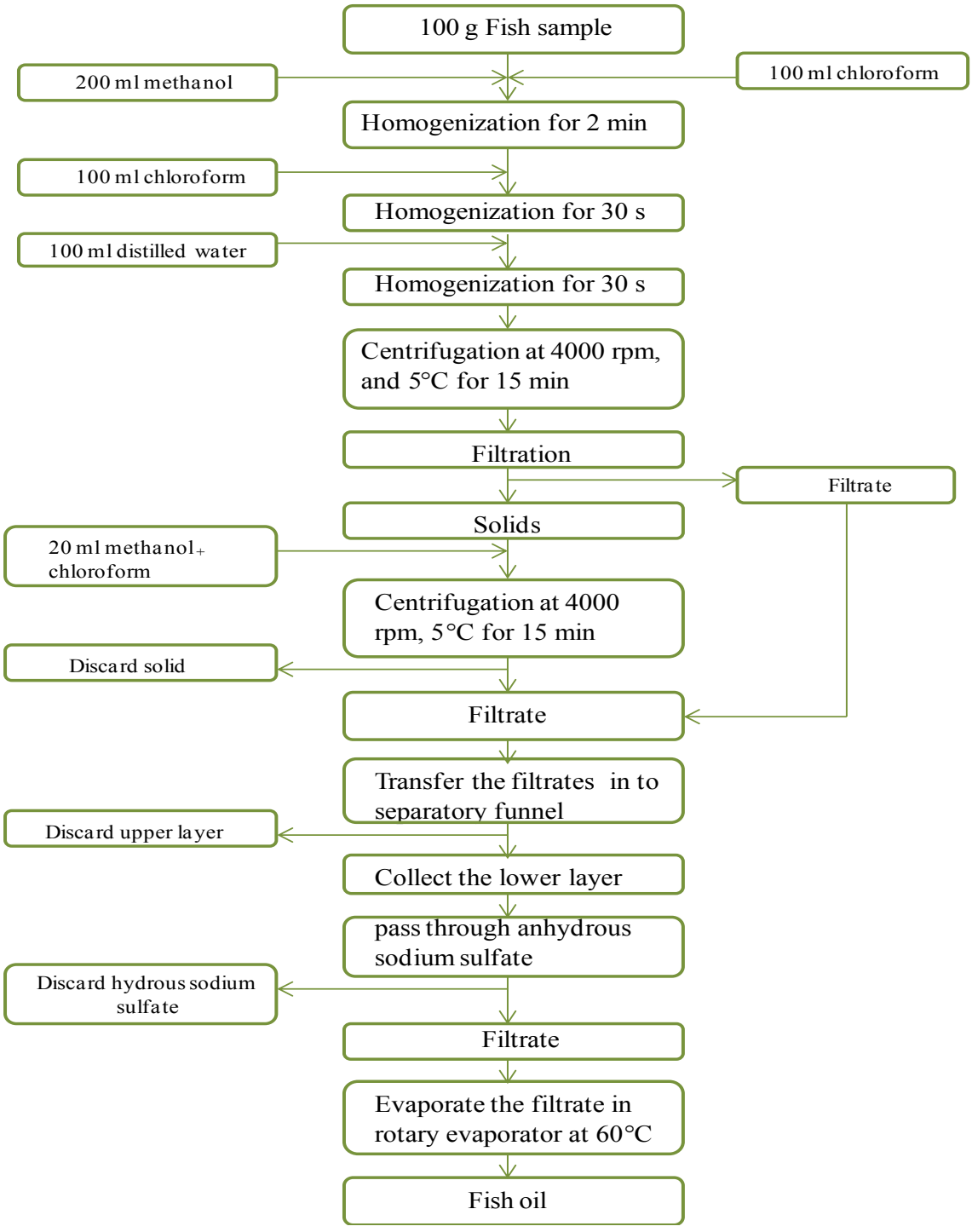


Figure 4.3. Schematic representation of the Bligh and Dyer method for extraction of fish oil.

methanol and 100 ml chloroform were blended for 2 min in a food blender (Model-53257C, Hamilton Beach, Halifax, Canada). Additional 100 ml of chloroform were added and blending continued for 30 s. Finally, 100 ml distilled water were added and blending continued for another 30 s. The homogenate was centrifuged at 4000 rpm (Sorvall RT1 centrifuge, Thermo Scientific, Ontario, Canada) and 5°C for 15 min. The liquid was filtered and the filtrate was kept aside. 20 ml of solvent (Chloroform:methanol) were added to solids and centrifugation was repeated again. The liquid was filtered and solids were discarded. The two filtrates were combined and transferred into separatory funnel. The chloroform layer (lower layer) was collected and filtered by passing it through bed of anhydrous sodium sulfate. The filtrate was evaporated in rotary evaporator at 60°C (RE540, Yamato Scientific America Inc, Orangeburg, NY).

4.3.4. Enzymatic hydrolysis of fish oil

The enzymatic hydrolysis of fish oil described by Liaset et al. (2000) was followed. Figure 4.4 shows enzymatic hydrolysis of fish oil using Alcalase. About 100 g of fish sample was weighed in 250 ml Erlenmeyer flask. The sample was heated at 95°C for 5 min to aid in the inactivation of endogenous enzymes. 100 ml of distilled water was added and the pH was adjusted to the desired level (7.0, 7.5 or 8.0) with 3M NaOH. The flask was transferred to preheated water bath shaker (Model-2870, Precision, Thermo Scientific, Ontario, Canada) at the desired temperature (55°C or 70°C). Enzymatic hydrolysis was started by adding Alcalase (0.5, 1.0, and 2.0 % by weight of raw material). The process was carried out for 2 h at 120 rpm. After hydrolysis, the enzyme was inactivated in the water bath shaker at 90°C for 5 min (Slizyte et al., 2005). It was then cooled at room temperature and centrifuged at 4000 rpm (Sorvall RT1 centrifuge, Thermo Scientific, Ontario, Canada) for 50 min. The top two layers (oil and emulsion) were collected in another centrifuge tube. 2 ml of 0.9% of NaCl solution was added and centrifuged for 5 min at 4000 rpm. The top layer (oil layer) was again transferred to another centrifuge tube with 0.5-1.0 g anhydrous sodium sulfate, mixed gently and allowed to settle. The oil was collected and stored at -20°C. This process was repeated several times to collect the needed amount of fish oil for further use.

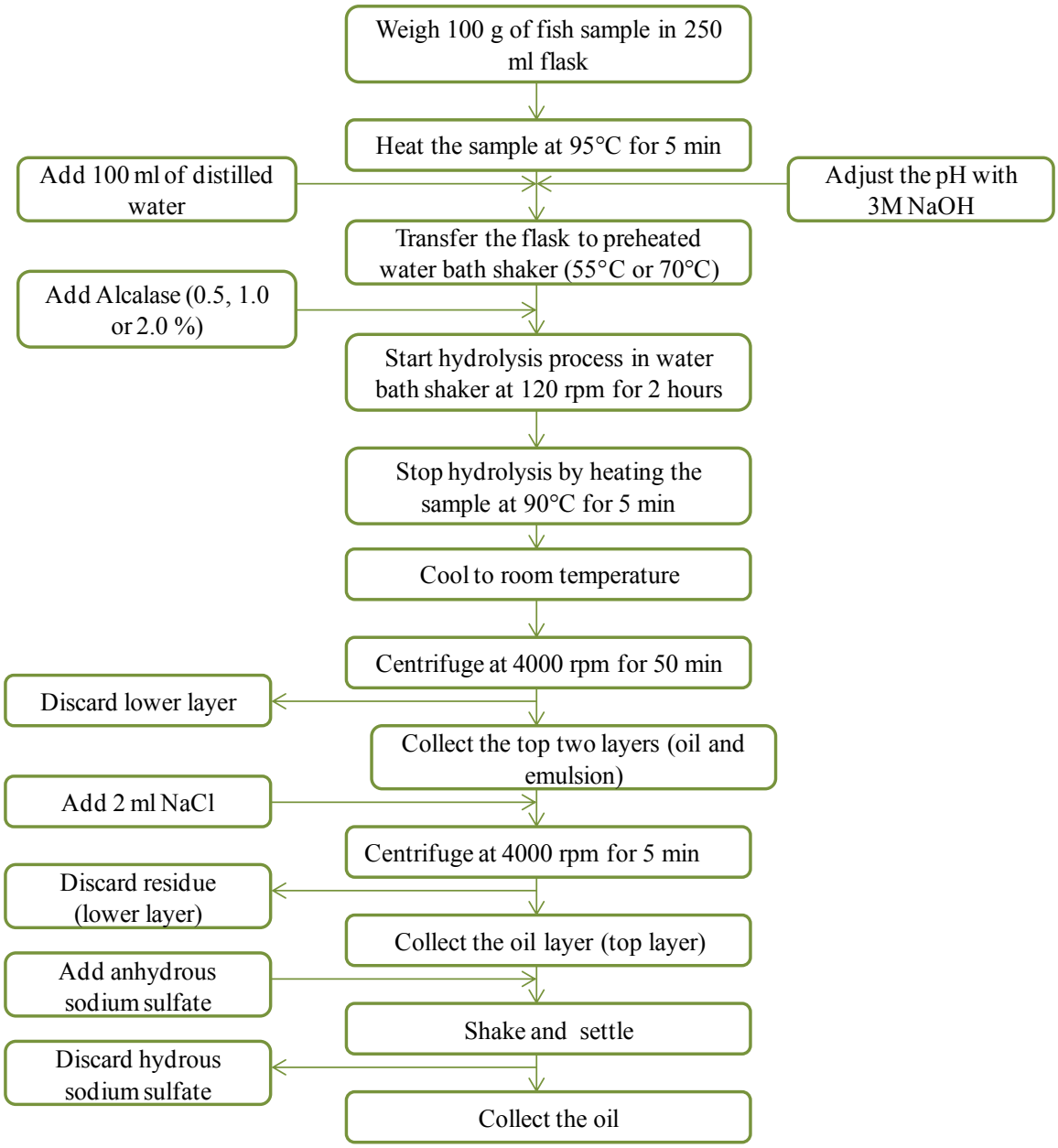


Figure 4.4. Schematic representation of enzymatic hydrolysis for extraction of fish oil.

4.3.5. Enzymatic concentration of omega-3 fatty acids

The procedure for enzymatic hydrolysis of omega-3 fatty acids described by Wanasundara and Shahidi (1998) was followed. Figure 4.5 shows enzymatic process of omega-3 fatty acid extraction using *Candida rugosa* lipase. 4 g oil (herring oil obtained from chemical extraction), 6 ml of 1M phosphate buffer to maintain pH of 7.5 and 800 Units (200 U/g) of lipase were added into a 50 ml conical flask. The flask was flushed with nitrogen and then sealed with a rubber cap and parafilm as recommended by Gamez et al. (2003). The flask was transferred to preheated water bath shaker (Model-2870, Precision, Thermo Scientific, Ontario, Canada) which was maintained at 35°C. The hydrolysis was started with the desired stirring level (Constant- fast, constant-slow, intermediate or no stirring) for 20 hours. The hydrolysis process was stopped by adding 2 ml methanol to the mixture as recommended by Carvalho et al. (2002). An amount of 0.5N KOH was added to neutralize the fatty acids released during hydrolysis. The mixture was transferred into a separatory funnel (Kimble Kimax, Fisher Scientific, Ontario, Canada) and thoroughly mixed with 100 ml hexane and 50 ml distilled water as recommended by Gamez et al. (2003). The lower aqueous layer was separated and discarded. The upper layer (hexane), which containing acylglycerols, was washed two times with 50 ml distilled water and then passed through a bed of anhydrous sodium sulfate. The acylglycerols were recovered after hexane removal at 45°C in a rotary evaporator (RE540, Yamato Scientific America Inc, Orangeburg, NY) as recommended by Carvalho et al. (2002) and Wanasundara and Shahidi (1998).

4.3.6. Preparation of fatty acid methyl esters (FAME)

100 mg lipids were placed in heavy duty centrifuge tube. 1.5 ml of methylene chloride, 0.01% BHT and 3.0 ml Hilditch reagent were added and the tube was flushed with nitrogen, capped and vortexed for 30 s. The centrifuge tubes were placed in the heating block (VWR Analog heat block, S.N 061024002, USA) for 1 hour. The tubes were then cooled at room temperature. 1.5 ml of hexane and 1 ml of distilled water were added and the tube was capped, and vortexed for 30 s. The top layer was removed to new tube (#2) without disturbing the interface. The hexane extraction was repeated twice with original

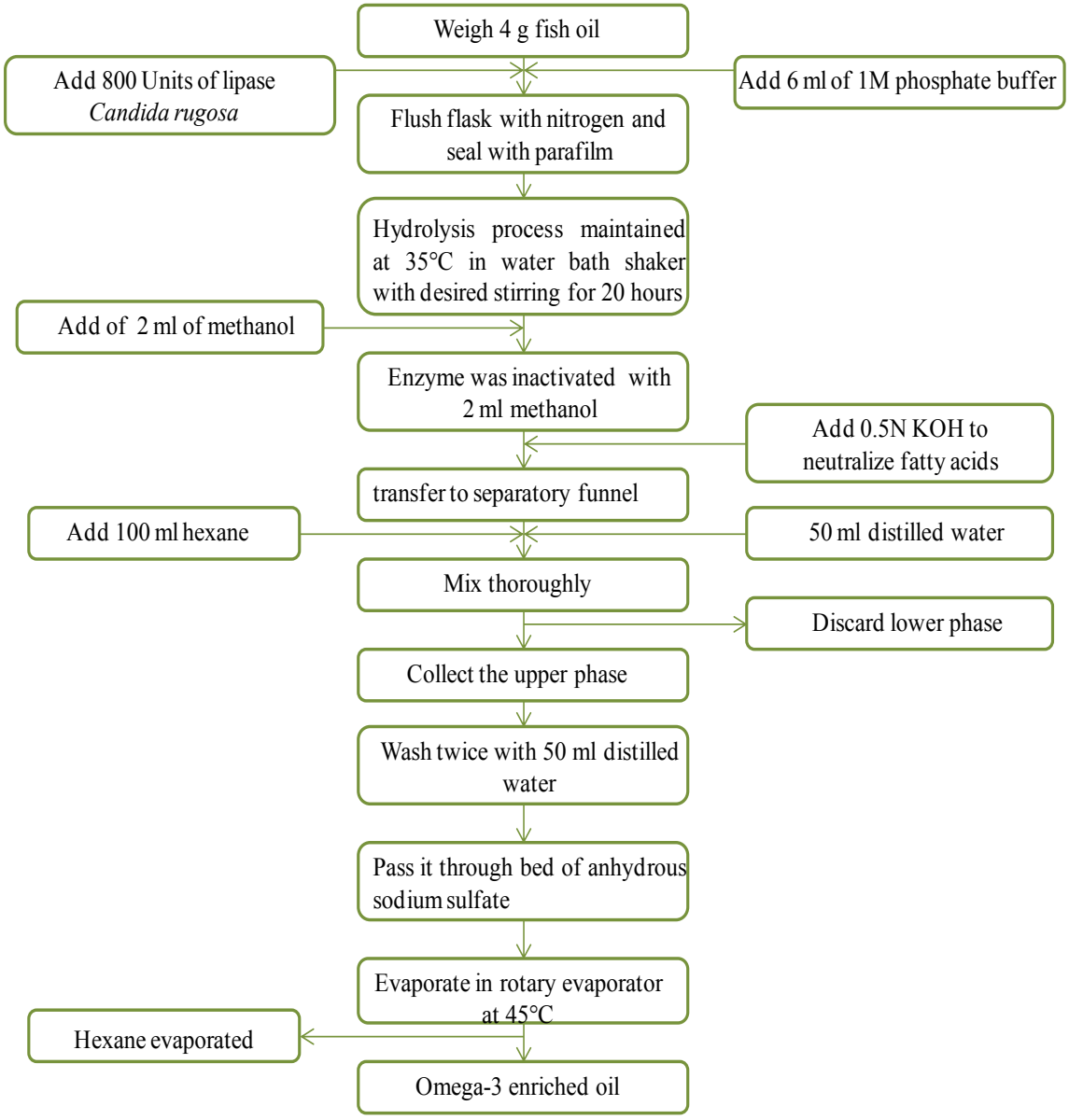


Figure 4.5. Schematic representation of enzymatic hydrolysis for extraction of omega-3 fatty acids from fish oil.

tube (#1). The top layer was removed to tube #2 every time. 2 ml of distilled water was added to the tube #2. The tube was capped, vortexed for 30 s and then centrifuged for 2 min. The top layer from tube #2 was removed and transferred to tube #3, to which few scoops of anhydrous sodium sulfate was added. Tube #3 was mixed gently and allowed to settle. The solvent from tube #3 was transferred to pre-weighed tube #4 and was evaporated under nitrogen. The outside of the tube was wiped with kimwipe, weighed and subtracted from the pre-weighed tube weight to get the total FAME. Hexane was added to a final concentration of 50 mg FAME/ml hexane. 5 and 0.5 dilutions was made for split and splitless injection into the GC and transferred to GC vials as recommended by Budge et al. (2006).

4.4. Experimental Analysis

4.4.1. Determination of acid value

The acid value of the oil sample was determined according to the procedure described in the AOCS official methods (AOCS, 1989). 125 ml of 1:1 toluene-isopropyl alcohol and 2 ml of phenolphthalein indicator were added into 250 ml glass Erlenmeyer flask and neutralized with 0.1N potassium hydroxide to a faint but permanent pink color. In another Erlenmeyer flask, 0.5-2.5 g of oil sample was weighed and 125 ml of neutralized solvent mixture were added to the sample and mixed thoroughly. Titration was then carried out with 0.1N potassium hydroxide to permanent pink color. The acid value (mg KOH/g of sample) was given by the following formula:

$$\text{Acid value} = \frac{(A-B) \times N \times 56.1}{W} \qquad (4.1)$$

Where:

A= ml of standard alkali used in the titrating the sample

B= ml of standard alkali used in the titrating the blank

N= normality of standard alkali

W= grams of sample

56.1= molecular weight of KOH in grams

4.4.2. Determination of p-anisidine value

The *p*-Anisidine value of oil sample was determined using the procedure described in the AOCS official method (AOCS, 1989). 0.7 g of oil was placed into a 25 ml volumetric flask. It was dissolved and diluted to 25 ml with iso-octane. The absorbance was measured at 350 nm with spectrophotometer (Genesys 20, Thermo Scientific, Ottawa, Ontario, Canada). In a test tube, 5 ml of iso-octane and 1 ml of *p*-anisidine reagent were added and used as blank. To another test tube, 5 ml of oil was transferred, and exactly 1 ml of *p*-anisidine reagent was added to the test tube. After 10 minutes, the absorbance was measured at 350 nm. The *p*-anisidine value was given by the following formula:

$$p\text{-anisidine value} = \frac{25 \times (1.2\ A_S - A_B)}{W} \qquad (4.2)$$

Where:

AS= absorbance of the oil solution after reaction with the *p*-anisidine reagent

AB= absorbance of the oil solution

W= grams of sample

25= size of volumetric flask used

1.2= correction factor

4.4.3. Determination of peroxide value

The peroxide value of oil sample was determined using the procedure described in the AOCS official method (AOCS, 1989). 5 g of oil sample were added into a 250 ml Erlenmeyer flask. Then, 30 ml of 3:2 acetic acid-chloroform were added and the flask was swirled to dissolve the sample. 0.5 ml potassium iodide solution was added and the solution was allowed to stand with occasional shaking for exactly 1 min. Then, 30 ml of distilled water were added. This mixture was titrated against 0.1N sodium thio-sulfate until the yellow color disappeared. About 0.5 ml of starch indicator solution was added and the titration continued with constant agitation till the blue color disappeared. A blank was prepared without adding the oil sample. The peroxide value (milliequivalents peroxide/1000g sample) was given by the following formula:

$$\text{Peroxide value} = \frac{(S-B) \times N \times 1000}{W} \quad (4.3)$$

Where:

B= ml of standard potassium thiosulfate used for titration of blank

S= ml of standard potassium thiosulfate used for titration of sample

N= normality of sodium thiosulfate solution

1000= per 1000g of sample

W= gram of sample

4.4.4. FAME analysis

The split injection procedure described by Budge et al. (2006) was followed. Using syringe, 1 µl of a sample was delivered with a concentration of 50 mg/mL to an injector of gas chromatography (GC-FID, Perkin Elmer Autosystem, Ontario, Canada) held at a constant temperature of 250°C. To generate a split ratio of approximately 1:100, the helium split flow was set at a rate of 100 mL/min. The carrier gas flow rate (He) was 1 mL/min. To the detector, the flow rates of air and hydrogen were 450 and 45 mL/min respectively. The detector was held at 250°C and the temperature program for oven began at 153°C. The temperature was held at 153°C for 2 min and then ramped at a rate of 2.3°C/min to 174°C. The temperature of 174°C was maintained for 0.2 min and then ramped at 2.5°C to 210°C. This final temperature was held for 2 min. The runtime of the program was approximately 32 min. The gas chromatography protocol for FAME analysis is shown in Table 4.3.

The splitless injection was also carried out as described by Budge et al. (2006). Quantity of the sample for injection was the same except a sample concentration of 0.5 mg/mL was used. The temperatures of the injector and detector, flow rates of gases to the column and detector also remained the same. The split flow rate was set at 30 mL/min. The temperature program was modified, where the initial temperature was set at 50°C and held for 1 min. Then the temperature was rapidly ramped at 45°C/min to 153°C. The program described above was then followed.

Table 4.3. Protocol for fatty acid analysis by gas chromatography.

Instrumental Parts	**Particulars**
Detector	Flame ionization detector
Column	polar capillary column (30 m × 0.25 mm × 0.25 μm) flexible fused silica column coated with 50% cyanopropyl polysiloxane.
Carrier gas	helium
Carrier gas flow rate	1ml/min
Temperature (°C)	250°C
Oven	153°C held for 2 min and then ramped at a rate of 2.3°C/min to 174°C. That temperature was maintained for 0.2 min and then ramped at 2.5°C to 210°C. This final temperature was held for 2 min
Detector	250°C
Injector	250°C

4.4.5. Statistical analysis

The experiments were carried out in triplicates. The results were presented as average and standard deviation. Analysis of variance and Tukey grouping were performed on the data using MINITAB (Okada and Morrissey, 2007).

CHAPTER 5. RESULTS

5.1. Fish Composition

Fish sample was given to Nova West Laboratory to determine the composition of herring fish (whole). The analysis (Table 5.1) showed that the fish had a moisture content of 70.36% and the fat, protein, ash and carbohydrate were 8.82%, 18.46%, 2.12% and 0.24%, respectively.

5.2. Enzymatic Extraction of oil

5.2.1. Oil recovery

Crude fish oil was extracted from whole herring fish (100g) using enzymatic hydrolysis. During the hydrolysis optimization process, three factors were studied: temperature (55°C and 70°C), pH (7.0, 7.5 and 8.0) and enzyme load (0.5%, 1.0% and 2.0%). The amount of recovered oil and recovery yield are presented in Table 5.2.

Analysis of variance (ANOVA) and Tukey's grouping were performed on the recovery yield data using MINITAB (General linear model, Minitab Inc. State College, Pennsylvania, USA). The ANOVA results (Table 5.3) showed that the effects of temperature (T), pH and enzyme load (EL) were highly significant at the 0.001 level. There were also significant two way interactions between T and pH (at the 0.006 level), T and EL (at the 0.05 level) and pH and EL (at the 0.001 level). The three way interaction between the temperature, pH and enzyme load was also highly significant at the 0.001 level.

The Tukey's grouping results (Table 5.4) showed that the two temperatures were significantly different from one another at the 0.05 level with the temperature of 55°C achieving the highest average oil recovery yield of 14.99%. The three levels of pH were significantly different from one another at the 0.05 level with the pH 7.5 achieving the

Table 5.1. Composition of whole herring (Nova West Laboratory).

Parameter	Value
Fat (%)	8.82
Protein (%)	18.46
Moisture (%)	70.36
Ash (%)	2.12
Carbohydrate (%)	0.24

Table 5.2. Extracted fish oil using enzymatic hydrolysis.

Temperature (°C)	pH	Enzyme Load (%)	Oil Recovered (g)	Recovery Yield* (%)
55	7	0.5	0.96	10.9±0.62
		1.0	1.11	12.6±0.39
		2.0	1.44	16.3±0.51
	7.5	0.5	1.19	13.5±0.64
		1.0	1.60	18.1±0.51
		2.0	1.78	20.2±0.42
	8.0	0.5	1.00	11.4±0.23
		1.0	1.33	15.1±0.47
		2.0	1.46	16.6±0.45
70	7	0.5	0.40	4.5±0.36
		1.0	0.58	6.7±0.39
		2.0	0.67	7.6±0.17
	7.5	0.5	0.64	7.3±0.75
		1.0	0.81	9.2±0.51
		2.0	1.38	15.6±0.88
	8.0	0.5	0.51	5.8±0.53
		1.0	0.82	9.3±0.28
		2.0	0.92	10.5±0.59

Total oil content of herring – 8.82g/100g (Nova West lab)
Results are mean±SD of three replicates
* = (oil recovered/total oil content) x 100

Table 5.3. ANOVA for oil recovery yield.

Source	DF	SS	MS	F	P
Total	53	1063.68			
Model					
T	1	563.83	563.83	2096.04	0.001
pH	2	161.29	80.65	299.80	0.001
EL	2	278.63	139.31	517.90	0.001
T*pH	2	3.22	1.61	5.99	0.006
T*EL	2	1.70	0.85	3.16	0.050
pH*EL	4	25.29	6.32	23.50	0.001
T* pH *EL	4	20.03	5.01	18.62	0.001
Error	36	9.68	0.27		

T: Temperature
EL: Enzyme load
DF: Degree of freedom
SS: Sum of squares
MS: Mean of squares
R^2 : 99.09%

Table 5.4. Tukey's grouping for oil recovery yield.

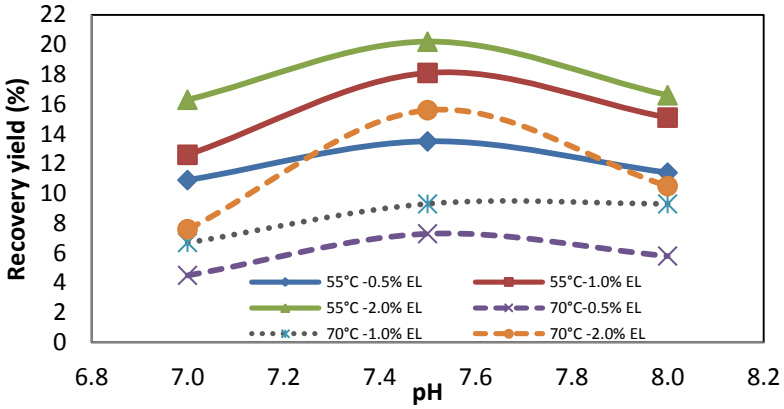
Parameter	Number of Observations	Mean Recovery Yield (%)	Grouping
Temperature (°C)			
55	27	14.99	A
70	27	8.53	B
pH			
7.0	18	9.81	A
7.5	18	14.01	B
8.0	18	11.46	C
Enzyme load (%)			
0.5	18	8.94	A
1.0	18	11.85	B
2.0	18	14.50	C

highest average oil recovery yield of 14.01%. The three levels of enzyme load were significantly different from one another at the 0.05 level with the enzyme load of 2.0% achieving the highest average oil recovery yield of 14.5%.

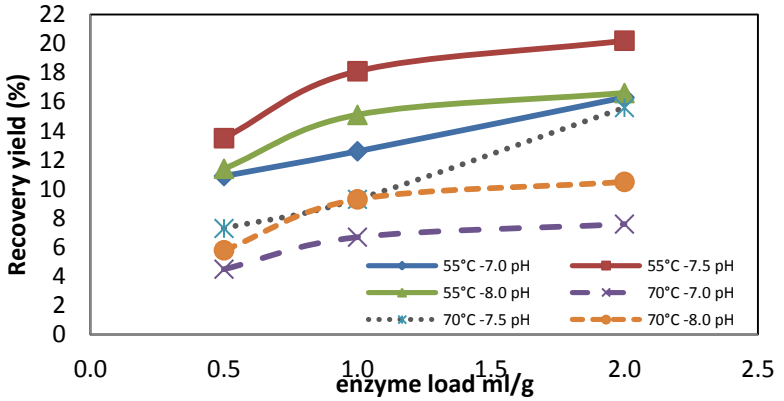
Figure 5.1 shows the effects of pH, enzyme load (EL) and temperature on the oil recovery yield. The pH 7.5 appeared to produce the highest oil recovery yield at all temperatures and enzyme loads. When the pH was increased from 7.0 to 7.5, the oil recovery yield increased from 10.9 to 13.5% (24%), from 12.6 to 18.1% (44%), from 16.3 to 20.2% (24%), from 4.5 to 7.3% (62%), from 6.7 to 9.3% (39%) and 7.6 to 15.6% (105%) and then decreased from 13.5 to 11.4% (16%), from 18.1 to 15.1% (17%), from 20.2 to 16.6% (18%), from 7.3 to 5.8% (21%) and from 15.6 to 10.5% (33%) when the pH was further increased to 8.0 for the combinations 55°C-0.5%EL, 55°C-1.0%EL, 55°C-2.0%EL, 70°C-0.5%EL and 70°C-2.0%EL, respectively. However, for the combination 70°C-1%EL, the oil recovery yield slightly increased from 9.2 to 9.3% (1.1%) when the pH was increased from 7.5 to 8.0.

Increasing the enzyme load increased the oil recovery yield at all temperatures and pH levels. When enzyme load was increased from 0.5% to 2.0%, the oil recovery yield increased from 10.9 to 16.3% (50%), from 13.5 to 20.2% (50%), from 11.4 to 16.6% (46%), from 4.5 to 7.6% (69%), from 7.3 to 15.6% (114%) and from 5.8 to 10.5% (81%) for the combinations 55°C-7.0pH, 55°C-7.5pH, 55°C-8.0pH, 70°C-7.0pH, 70°C-7.5pH and 70°C-8.0pH, respectively.

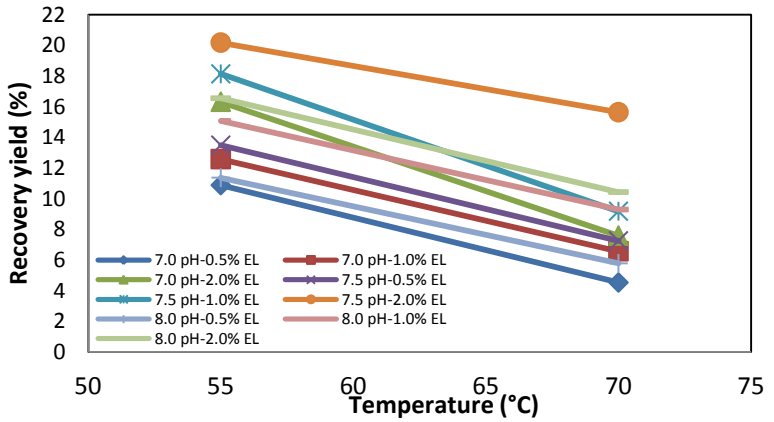
Higher oil recovery yields were obtained with the lower temperature for all enzyme loads and pH levels. When temperature was increased from 55°C to 70°C, the recovery yield decreased from 10.9 to 4.5% (59%), from 12.6 to 6.7% (47%), from 16.3 to 7.6% (53%), from 13.5 to 7.3% (46%), from 18.1 to 9.3% (49%), from 20.2 to 15.6% (23%), from 11.4 to 5.8% (49%), from 15.1 to 9.3% (38%) and from 16.6 to 10.5% (37%) for the combinations 7.0pH-0.5%EL, 7.0pH-1.0%EL, 7.0pH-2.0%EL, 7.5pH-0.5%EL, 7.5pH-1.0%EL, 7.5pH-2.0%EL, 8.0pH-0.5%EL, 8.0pH-1.0%EL and 8.0pH-2.0%EL, respectively.



(a) effect of pH



(b) effect of enzyme load



(c) effect of temperature

Figure 5.1. Effects of pH, enzyme load and temperature on oil recovery yield obtained by enzymatic hydrolysis.

The highest recovery yield of 20.2% was obtained at a temperature of 55°C, a pH of 7.5 and an enzyme load of 2.0%.

5.2.2. Quality of oil

Chemical analyses were performed on the extracted crude fish oil to determine its quality. These were: peroxide value, acid value and *p*-anisidine value. The results are presented in Table 5.5.

5.2.2.1. Peroxide value. The results of ANOVA and Tukey grouping performed on the peroxide value data are presented in Tables 5.6 and 5.7, respectively. The effects of temperature, pH and enzyme load were highly significant at the 0.001 level. Also, the two way and three way interactions among these parameters were highly significant at the 0.001 level. The Tukey's grouping showed that all the levels of each parameter were significantly different from one another at the 0.05 level.

Figure 5.2 shows the effects of pH, enzyme load and temperature on peroxide value of crude fish oil obtained by enzymatic hydrolysis. The effect of pH on the peroxide value was influenced by the enzyme load and temperature. When the pH was increased from 7.0 to 7.5, the peroxide value decrease from 32.20 to 31.44 Meq/g (2%), from 37.16 to 36.66 Meq/g (1%) and from 40.70 to 38.95 Meq/g (4%) and then increased from 31.44 to 38.96 Meq/g (24%), from 36.66 to 40.82 Meq/g (11%) and from 38.95 to 41.79 Meq/g (7%) when pH was further increase to pH 8.0 for the combinations 55°C-2.0%EL, 70°C-0.5%EL and 70°C-2.0%EL, respectively. However, when the pH was increased from 7.0 to 8.0, the peroxide value increased from 28.14 to 35.37 Meq/g (26%) and from 26.78 to 37.58 Meq/g (40%) for the combinations 55°C-1.0%EL and 55°C-0.5%EL and decreased from 39.13 to 37.32 Meq/g (5%) for the combination 70°C-1.0%EL.

The results also showed that the effect of enzyme load on the peroxide value was influenced by pH and temperature. When enzyme load increased from 0.5 to 2.0%, the peroxide value increased slightly from 26.78 to 32.20 Meq/g (20%), from 37.16 to 40.70

Table 5.5. Chemical analysis of fish oil.

Temperature (°C)	pH	Enzyme Load (%)	Peroxide Value (Meq/g)	Acid Value (mgKOH/g)	*p*-Anisidine Value
55	7	0.5	26.78±0.48	7.76±0.38	56.93±0.63
		1.0	28.14±0.44	9.27±0.23	59.96±0.60
		2.0	32.20±0.43	10.01±0.16	64.01±0.46
	7.5	0.5	36.12±0.44	8.39±0.36	58.85±0.42
		1.0	28.14±0.68	10.11±0.06	59.36±0.35
		2.0	31.44±0.33	11.13±0.46	58.27±0.19
	8.0	0.5	37.58±0.44	8.27±0.56	64.30±0.42
		1.0	35.37±0.34	8.43±0.66	60.02±0.40
		2.0	38.96±0.07	8.64±0.53	62.33±0.21
70	7	0.5	37.16±0.21	9.22±0.59	61.09±0.75
		1.0	39.13±0.42	12.53±0.18	64.39±0.10
		2.0	40.70±0.21	15.28±0.43	66.14±0.32
	7.5	0.5	36.66±0.54	12.92±0.26	62.29±0.40
		1.0	38.56±0.23	14.73±0.84	65.20±0.26
		2.0	38.95±0.64	17.17±0.47	67.20±0.43
	8.0	0.5	40.82±0.48	16.26±0.21	68.73±0.86
		1.0	37.32±0.54	18.05±0.33	65.25±0.37
		2.0	41.79±0.32	19.81±0.28	69.83±0.21

Results are mean ± SD of three replicates

Table 5.6. ANOVA for peroxide value.

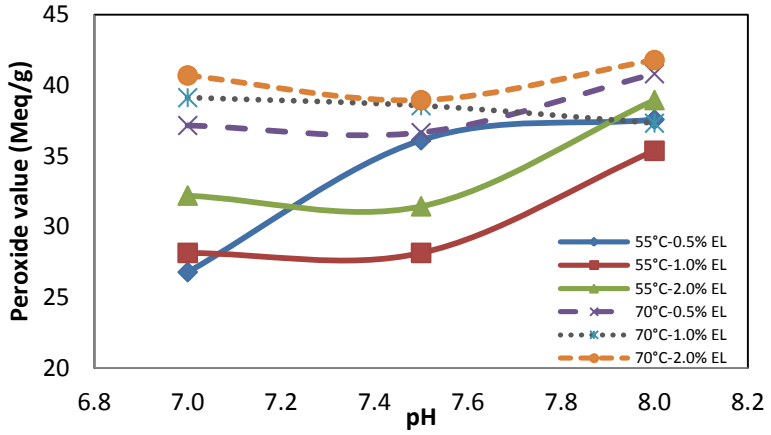
Source	DF	SS	MS	F	P
Total	53	1094.55			
Model					
T	1	529.47	529.47	2798.39	0.001
pH	2	214.14	107.07	565.88	0.001
EL	2	75.36	37.68	199.15	0.001
T*pH	2	119.32	59.66	315.32	0.001
T*EL	2	21.18	10.59	55.97	0.001
pH*EL	4	65.75	16.44	86.88	0.001
T* pH *EL	4	62.51	15.63	82.60	0.001
Error	36	6.81	0.19		

T: Temperature
EL: Enzyme load
DF: Degree of freedom
SS: Sum of squares
MS: Mean of squares
R^2 : 99.38%

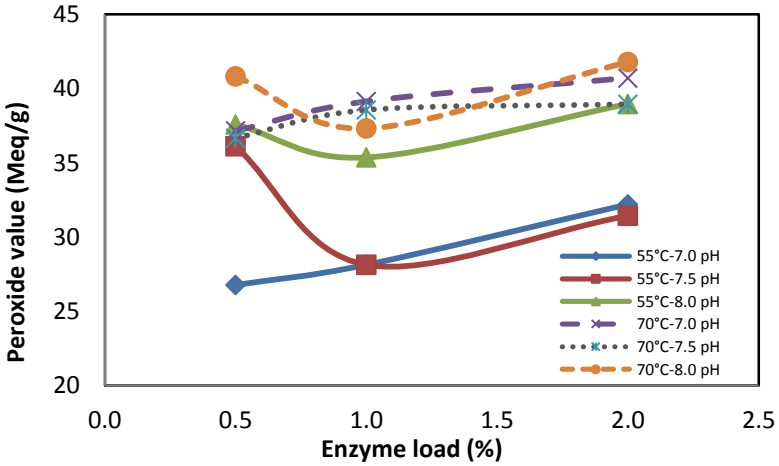
Table 5.7. Tukey's grouping for peroxide value.

Parameter	Number of Observations	Mean Peroxide Value (Meq/g)	Grouping
Temperature (°C)			
55	27	32.75	A
70	27	39.01	B
pH			
7.0	18	34.02	A
7.5	18	34.98	B
8.0	18	38.64	C
Enzyme load (%)			
0.5	18	34.45	A
1.0	18	35.86	B
2.0	18	37.34	C

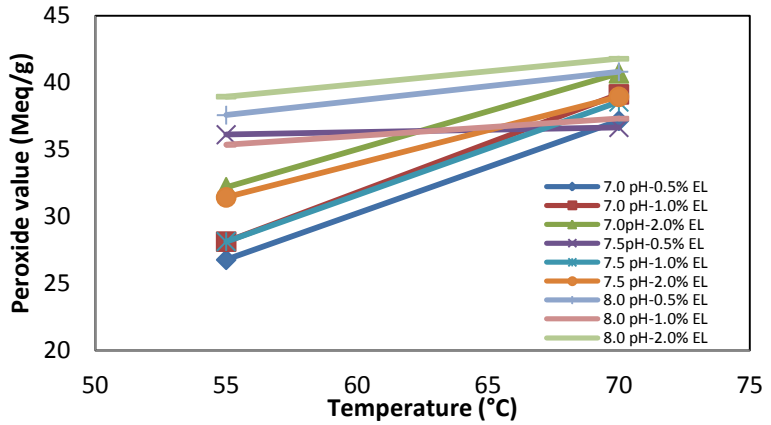
Groups with the same letter are not significantly different from each other at the 0.05 level.



(a) effect of pH



(b) effect of enzyme load



(c) effect of temperature

Figure 5.2. Effects of pH, enzyme load and temperature on peroxide value of fish oil obtained by enzymatic hydrolysis.

Meq/g (10%) and from 36.66 to 38.95 Meq/g (6%) for the combinations 55°C-7.0pH, 70°C-7.0pH and 70°C-7.5pH, respectively. However, when the enzyme load was increased from 0.5 to 1.0%, the peroxide value decreased from 36.12 to 28.14 Meq/g (22%), from 37.58 to 35.37 Meq/g (6%) and from 40.82 to 37.32 Meq/g (9%) and then increased from 28.14 to 31.44 Meq/g (12%), from 35.37 to 38.96 Meq/g (10%) and from 37.32 to 41.79 Meq/g (12%) when the enzyme load was further increase to 2.0% for the combinations 55°C-7.5pH, 55°C-8.0pH and 70°C-8.0pH, respectively.

Higher peroxide values were recorded with the higher temperature at all enzyme loads and pH levels. When temperature was increased from 55°C to 70°C, the peroxide value increased from 26.78 to 37.16 Meq/g (39%), from 28.14 to 39.13 Meq/g (39%), from 32.20 to 40.70 Meq/g (26%), from 36.12 to 36.66 Meq/g (2%), from 28.14 to 38.56 Meq/g (37%), from 31.44 to 38.95 Meq/g (24%), from 37.58 to 40.82 Meq/g (9%), from 35.37 to 37.32 Meq/g (6%) and 38.96 to 41.79 Meq/g (7%) for the combinations 7.0pH-0.5%EL, 7.0pH-1.0% EL, 7.0pH-2.0% EL, 7.5pH-0.5% EL, 7.5pH-1.0%EL, 7.5pH-2.0% EL, 8.0pH-0.5% EL, 8.0pH-1.0% EL and 8.0pH-2.0% EL, respectively.

The lower peroxide value the better the quality. The lowest peroxide value of 26.78 Meq/g was obtained with a temperature of 55°C, an enzyme load of 0.5% and pH of 7.0 and the highest value of 41.79 Meq/g were obtained with a temperature of 70°C, an enzyme load of 2.0% and a pH of 8.0. All peroxide values were higher than the recommended range of 3-20 meq/kg indicating that the fish oil was oxidized.

5.2.2.2. Acid value. Table 5.8 shows the ANOVA results of acid value. The effects of pH, temperature and enzyme load on the acid value were highly significant at the 0.001 level. All the two way interactions among these factors were highly significant at the 0.001 level. However, the three way interactions among these parameter was significant at the 0.02 level. The results of Tukey's grouping are presented in Table 5.9. All the levels of each of these parameters were significantly different from one another at the 0.05 level.

Table 5.8. ANOVA for acid value.

Source	DF	SS	MS	F	P
Total	53	768.62			
Model					
T	1	485.04	485.04	2522.02	0.001
pH	2	61.57	30.78	160.07	0.001
EL	2	92.47	46.24	240.41	0.001
T*pH	2	94.04	47.02	244.50	0.001
T*EL	2	18.28	9.14	47.53	0.001
pH*EL	4	7.88	1.97	10.24	0.001
T* pH *EL	4	2.41	0.60	3.13	0.020
Error	36	6.92	0.19		

T: Temperature
EL: Enzyme load
DF: Degree of freedom
SS: Sum of squares
MS: Mean of squares
R^2 : 99.10%

Table 5.9. Tukey's grouping for acid value.

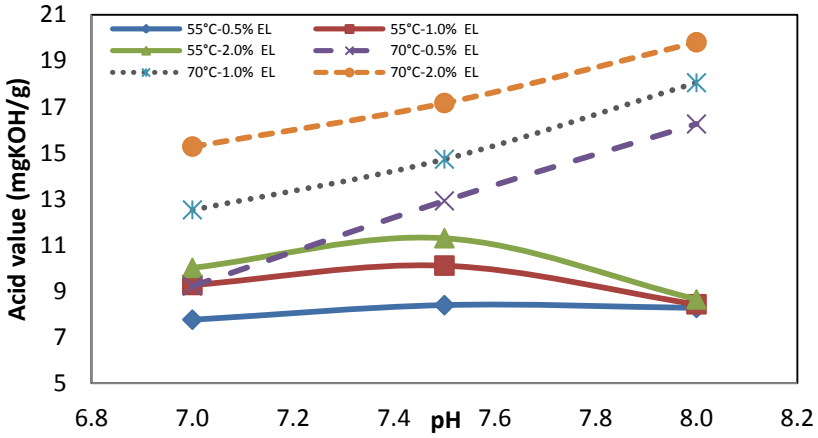
Parameter	Number of Observations	Mean Acid Value (mg KOH/g)	Grouping
Temperature (°C)			
55	27	9.11	A
70	27	15.11	B
pH			
7.0	18	10.68	A
7.5	18	12.41	B
8.0	18	13.25	C
Enzyme load (%)			
0.5	18	10.47	A
1.0	18	12.19	B
2.0	18	13.68	C

Groups with the same letter are not significantly different from each other at the 0.05 level.

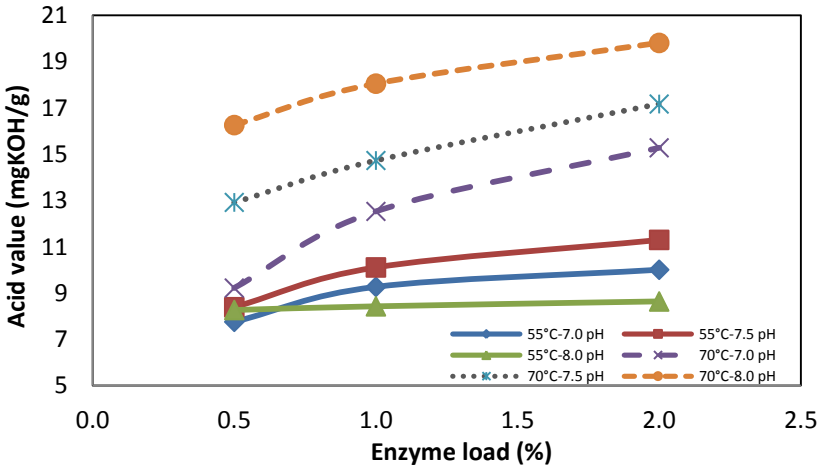
Figure 5.3 shows the effects of pH, enzyme load and temperature on the acid value of crude oil obtained by enzymatic hydrolysis. The effect of pH on acid value appeared to be dependent on the temperature and enzyme load. When the pH was increased from 7.0 to 7.5, the acid value increased from 7.76 to 8.39 mg KOH/g (8%), from 9.27 to 10.11 mg KOH/g (9%) and from 10.01 to 11.13 mg KOH/g (11%) and then decreased from 8.39 to 8.27 mg KOH/g (1%), from 10.11 to 8.43 mg KOH/g (17%) and from 11.13 to 8.64 mg KOH/g (22%) when pH was further increased to 8.0 for the combinations 55°C-0.5%EL, 55°C-1.0%EL, 55°C-2.0%EL. However, when the pH was increased from 7.0 to 7.5, the acid value increased from 9.22 to 12.92 mg KOH/g (40%), from 12.53 to 14.73 mg KOH/g (18%) and from 15.28 to 17.17 mg KOH/g (12%) and then increased from 12.92 to 16.26 mg KOH/g (26%), from 14.73 to 18.05 mg KOH/g (23%) and from 16.26 to 19.81 mg KOH/g (22%) when pH was further increased to 8.0 for the combinations 70°C-0.5%EL, 70°C-1.0%EL and 70°C-2.0%EL, respectively.

Increasing the enzyme load increased the acid value at all temperatures and pH levels. When enzyme load was increased from 0.5 to 2.0%, the acid value increased from 7.76 to 10.01 mg KOH/g (29%), from 8.39 to 11.13 mg KOH/g (33%), from 8.27 to 8.64 mg KOH/g (5%), 9.22 to 15.28 mg KOH/g (66%), 12.92 to 17.17 mg KOH/g (33%) and 16.26 to 19.81 mg KOH/g (22%) for the combinations 55°C-7.0pH, 55°C-7.5pH, 55°C-8.0pH, 70°C-7.0pH, 70°C-7.5pH and 70°C-8.0pH, respectively.

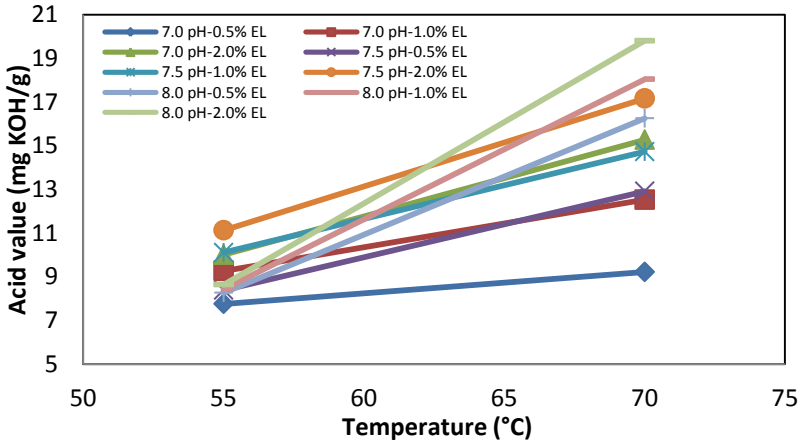
The highest acid value was obtained at the higher temperature for all enzyme loads and pH levels. When temperature was increased from 55°C to 70°C, the acid values increased from 7.76 to 9.22 mg KOH/g (19%), from 9.27 to 12.53 mg KOH/g (35%), from 10.01 to 15.28 mg KOH/g (53%), from 8.39 to 12.92 mg KOH/g (54%), from 10.11 to 14.73 mg KOH/g (46%), from 11.13 to 17.17 mg KOH/g (54%), from 8.27 to 16.26 mg KOH/g (97%), from 8.43 to 18.05 mg KOH/g (114%) and from 8.64 to 19.81 mg KOH/g (129%) for the combinations 7.0pH-0.5%EL, 7.0pH-1.0%EL, 7.0pH-2.0%EL, 7.5pH-0.5%EL, 7.5pH-1.0%EL, 7.5pH-2.0%EL, 8.0pH-0.5%EL, 8.0pH-1.0%EL and 8.0pH-2.0%EL, respectively.



(a) effect of pH



(b) effect of enzyme load



(c) effect of temperature

Figure 5.3. Effects of pH, enzyme load and temperature on acid value of fish oil obtained by enzymatic hydrolysis.

The lower the acid value the better the quality of oil. The recommended range of acid value is <5 mg KOH/g. All the acid values were above the recommended level. The highest value of 19.81 mg KOH/g was obtained with a temperature of 70°C, an enzyme load of 2.0% and a pH of 8.0 whereas the lowest acid value of 7.76 mg KOH/g was observed with a temperature of 55°C, an enzyme load of 0.5% and a pH of 7.0.

5.2.2.3. p-Anisidine value. Tables 5.10 and 5.11 showthe results of ANOVA and Tukey's grouping of *p*-anisidine value, respectively. The effects of temperature, pH and enzyme load were highly significant at the 0.001 level. Also the two way and three way interactions among these factors were highly significant at the 0.001 level. The Tukey's grouping showed that the levels of each factor were significantly different from one another at the 0.05 level.

Figure 5.4 shows the effects of pH, enzyme load and temperature on *p*-anisidine value of crude fish oil obtained using enzymatic hydrolysis. The effect of pH on the *p*-anisidine value seemed to be dependent on the enzyme load and temperature. When the pH was increased from 7.0 to 8.0, the *p*-anisidine increased slightly from 56.93 to 64.30 (13%), from 61.09 to 68.73 (13%), from 64.39 to 65.25 (1%) and from 66.14 to 69.83 (6%) for the combinations 55°C-0.5%EL, 70°C-0.5%EL, 70°C-1.0%EL and 70°C-2.0%EL, respectively. However, when pH was increased from 7.0 to 7.5, the *p*-anisidine value decreased from 59.96 to 59.36 (1%) and then increased from 58.27 to 62.33 (7%) with further increase in pH to 8.0 for the combination 55°C-1.0%EL. On the other hand, when pH was increased from 7.0 to 7.5, the *p*-anisidine value increased from 59.36 to 60.02 (1%) and then decreased from 64.01 to 58.27 (9%) with further increase in pH to 8.0 for the combination 55°C-2.0%EL.

The effect of enzyme load on the *p*-anisidine value also appeared to be influenced by temperature and pH level. When enzyme load was increased from 0.5 to 2.0%, the *p*-anisidine value increased from 56.93 to 64.01 (12%), from 61.09 to 66.14 (8%) and from 62.29 to 67.20 (8%) for the combinations 55°C-7.0pH, 70°C-7.0pH and 70°C-7.5pH, respectively. However, when the enzyme load was increased from 0.5 to 1.0%, the

Table 5.10. ANOVA for *p*-anisidine value.

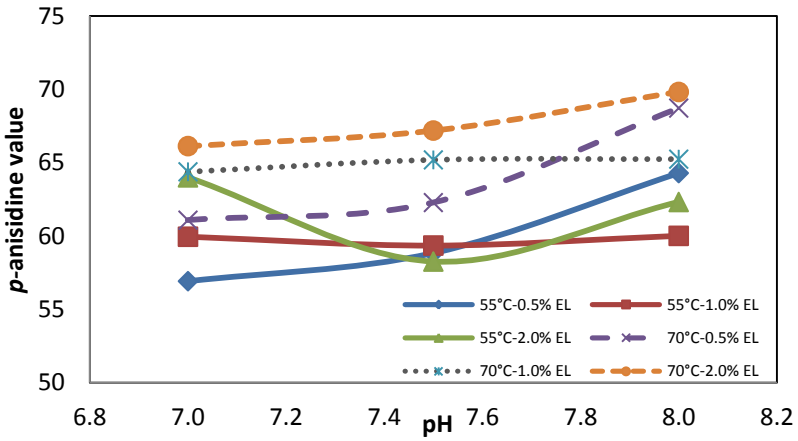
Source	DF	SS	MS	F	P
Total	53	709.13			
Model					
T	1	354.15	354.15	1690.78	0.001
pH	2	115.83	57.91	276.51	0.001
EL	2	71.90	35.95	171.64	0.001
T*pH	2	16.46	8.23	39.29	0.001
T*EL	2	10.63	5.31	25.37	0.001
pH*EL	4	108.36	27.09	129.33	0.001
T* pH *EL	4	24.26	6.06	28.96	0.001
Error	36	7.54	0.21		

T: Temperature
EL: Enzyme load
DF: Degree of freedom
SS: Sum of squares
MS: Mean of squares
R^2 : 98.94%

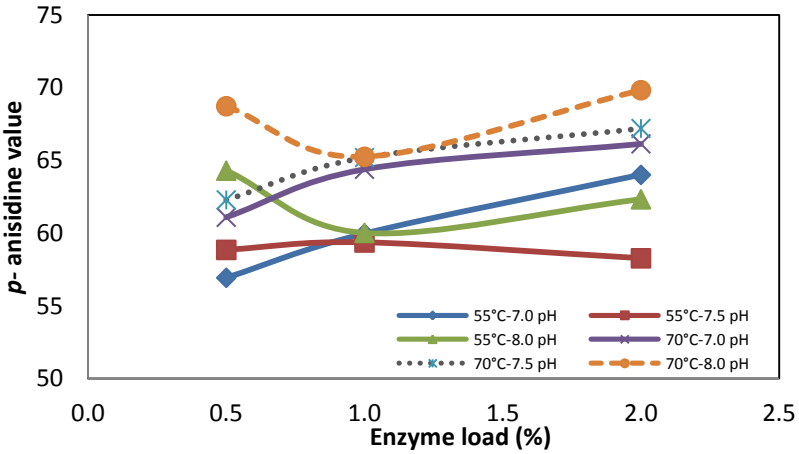
Table 5.11. Tukey's grouping for *p*-anisidine.

Parameter	Number of Observations	Mean *p*-anisidine Value	Grouping
Temperature(°C)			
55	27	60.45	A
70	27	65.57	B
pH			
7.0	18	61.87	A
7.5	18	62.09	A
8.0	18	65.08	B
Enzyme load (%)			
0.5	18	62.03	A
1.0	18	62.37	A
2.0	18	64.63	B

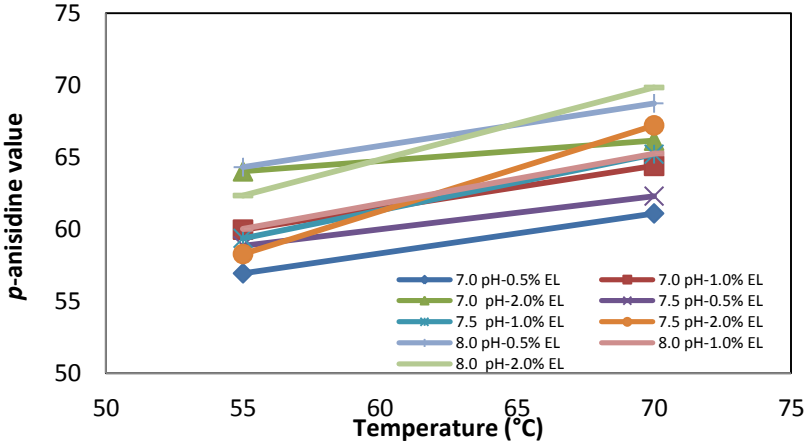
Groups with the same letter are not significantly different from each other at the 0.05 level.



(a) effect of pH



(b) effect of enzyme load



(c) effect of temperature

Figure 5.4. Effects of pH, enzyme load and temperature on *p*-anisidine value of fish oil obtained by enzymatic hydrolysis.

p-anisidine value decreased from 64.30 to 60.02 (7%) and 68.73 to 65.25 (5%) and then increased from 60.02 to 62.33 (4%) and from 65.25 to 69.83 (7%) when enzyme load increased further to 2.0% for the combinations 55°C-8.0pH and 70°C-8.0pH, respectively. For the combination 55°C-7.5pH, when enzyme load increased from 0.5 to 1.0% the *p*-anisidine value first increased slightly from 58.85 to 59.36 (1%) and then slightly decreased from 59.36 to 58.27 (2%) with further increase in enzyme load to 2.0%.

The highest *p*-anisidine value was obtained with the higher temperature at all enzyme loads and pH levels. When temperature was increased from 55°C to 70°C, the *p*-anisidine value increased from 56.93 to 61.09 (7%), from 59.96 to 64.39 (7%), from 64.01 to 66.14 (3%), from 58.85 to 62.29 (6%), from 59.36 to 65.20 (10%), from 58.27 to 67.20 (15%), from 64.30 to 68.73 (7%), from 60.02 to 65.25 (9%) and from 62.33 to 69.83 (12%) for the combinations 7.0pH-0.5%EL, 7.0pH-1.0%EL, 7.0pH-2.0%EL, 7.5pH-0.5%EL, 7.5pH-1.0%EL, 7.5pH-2.0%EL, 8.0pH-0.5%EL, 8.0pH-1.0%EL and 8.0pH-2.0%EL, respectively.

The lowest *p*-anisidine value of 56.93 was obtained at a temperature of 55°C, a pH of 7.0 and an enzyme load of 0.5% whereas the highest value of 69.83 was obtained with a temperature of 70°C, pH of 8.0 and an enzyme load of 2.0%. The recommended range for *p*-anisidine value for crude fish oil is 4-60. The combinations of 55°C-7.0 pH-2% EL, 55°C-8.0 pH-0.5% EL, 55°C-8.0 pH-2% EL and all samples at 70°C had *p*-anisidine values outside the recommended range.

5.2.3. Fatty acid composition of oil

There were about 78 fatty acids identified in crude herring oil using the gas chromatography analysis but only 12 fatty acids had values >0.5%. Fatty acid composition (saturated, monounsaturated and polyunsaturated fatty acids) of crude oil obtained by enzymatic hydrolysis are shown in Table 5.12 and 5.13, respectively. Oleic acid (18:1 n-9) had the highest concentration followed by palmitic acid (16:0).

Table 5.12. Fatty acid composition of fish oil obtained using enzymatic hydrolysis at 55°C.

pH	EL (%)	Saturated Fatty Acid			Monounsaturated Fatty Acid			Polyunsaturated Fatty Acid					
		Myristic acid (14:0)	Palmitic acid (16:0)	Stearic acid (18:0)	Palmitoleic acid (16:1n-7)	Oleic acid (18:1n-9)	Vaccenic acid (18:1n-7)	Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)	Arachidonic (20:4n-6)	EPA (20:5n-3)	DPA (22:5n-3)	DHA (22:6n-3)
7.0	0.5	6.11 (±0.2)	14.85 (±0.5)	1.69 (±0.1)	7.41 (±0.2)	22.28 (±1.0)	3.53 (±0.1)	1.41 (±0.1)	0.53 (±0.1)	0.38 (±0.0)	4.90 (±0.1)	0.52 (±0.0)	5.56 (±0.2)
	1.0	6.30 (±0.9)	14.92 (±2.1)	0.86 (±1.1)	7.51 (±1.1)	18.77 (±1.1)	3.62 (±0.5)	1.49 (±0.2)	0.54 (±0.1)	0.44 (±0.0)	6.24 (±0.8)	0.64 (±0.1)	7.45 (±0.9)
	2.0	5.39 (±0.0)	12.85 (±0.2)	1.58 (±0.1)	6.87 (±0.1)	19.98 (±0.1)	3.28 (±0.0)	1.36 (±0.0)	0.50 (±0.0)	0.42 (±0.0)	5.84 (±0.0)	0.59 (±0.0)	7.20 (±0.0)
7.5	0.5	6.60 (±0.0)	15.12 (±0.0)	1.68 (±0.0)	7.76 (±0.0)	22.71 (±0.5)	3.64 (±0.0)	1.54 (±0.0)	0.60 (±0.0)	0.42 (±0.0)	5.97 (±0.0)	0.71 (±0.0)	6.84 (±0.1)
	1.0	6.22 (±0.6)	14.11 (±1.4)	0.74 (±0.9)	7.46 (±0.7)	21.61 (±2.1)	3.51 (±0.3)	1.46 (±0.1)	0.57 (±0.0)	0.39 (±0.0)	5.75 (±0.5)	0.64 (±0.1)	6.60 (±0.6)
	2.0	5.81 (±0.1)	13.10 (±0.2)	0.73 (±0.9)	6.88 (±0.1)	19.84 (±0.3)	3.23 (±0.1)	1.38 (±0.0)	0.36 (±0.1)	0.38 (±0.0)	5.50 (±0.1)	0.58 (±0.0)	6.41 (±0.1)
8.0	0.5	6.29 (±0.1)	15.12 (±0.2)	0.86 (±1.1)	7.89 (±0.1)	23.72 (±0.3)	3.81 (±0.0)	1.55 (±0.0)	0.56 (±0.1)	0.44 (±0.0)	6.15 (±0.0)	0.57 (±0.1)	7.11 (±0.1)
	1.0	6.20 (±0.0)	15.13 (±0.0)	1.69 (±0.0)	7.84 (±0.0)	23.64 (±0.0)	3.82 (±0.0)	1.51 (±0.0)	0.60 (±0.0)	0.43 (±0.0)	6.08 (±0.0)	0.63 (±0.0)	7.11 (±0.0)
	2.0	6.30 (±0.1)	15.42 (±0.1)	1.67 (±0.0)	7.88 (±0.1)	23.82 (±0.1)	3.81 (±0.0)	1.51 (±0.0)	0.59 (±0.0)	0.42 (±0.0)	6.11 (±0.0)	0.63 (±0.0)	7.14 (±0.1)

Value in () are ± SD
T- Temperature
EL- Enzyme load
EPA- Eicosapentaenoic acid
DPA- Docosapentaenoic acid
DHA- Docosahexaenoic acid

Table 5.13. Fatty acid composition of fish oil obtained using enzymatic hydrolysis at 70°C.

pH	EL (%)	Saturated Fatty Acid			Monounsaturated Fatty Acid			Polyunsaturated Fatty Acid					
		Myristic acid (14:0)	Palmitic acid (16:0)	Stearic acid (18:0)	Palmitoleic acid (16:1n-7)	Oleic acid (18:1n-9)	Vaccenic acid (18:1n-7)	Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)	Arachidonic (20:4n-6)	EPA (20:5n-3)	DPA (22:5n-3)	DHA (22:6n-3)
7.0	0.5	6.28 (±1.0)	14.98 (±2.4)	1.73 (±0.3)	8.02 (±1.3)	20.69 (±0.0)	4.04 (±0.6)	1.53 (±0.2)	0.62 (±0.1)	0.47 (±0.0)	6.07 (±0.9)	0.59 (±0.1)	7.66 (±1.2)
	1.0	5.68 (±0.1)	13.42 (±0.2)	1.53 (±0.0)	7.13 (±0.1)	21.65 (±0.3)	3.47 (±0.1)	1.38 (±0.0)	0.58 (±0.0)	0.39 (±0.0)	5.46 (±0.1)	0.56 (±0.0)	6.53 (±0.1)
	2.0	6.91 (±0.0)	16.21 (±0.0)	1.85 (±0.0)	8.06 (±0.0)	21.69 (±0.1)	4.45 (±0.0)	1.76 (±0.0)	0.73 (±0.0)	0.51 (±0.0)	7.02 (±0.0)	0.72 (±0.0)	8.39 (±0.0)
7.5	0.5	7.39 (±0.7)	15.63 (±1.5)	1.74 (±0.2)	8.00 (±0.7)	20.30 (±1.5)	3.73 (±0.4)	1.58 (±0.1)	0.75 (±0.1)	0.46 (±0.0)	6.41 (±0.6)	0.71 (±0.1)	7.45 (±0.7)
	1.0	7.16 (±0.4)	14.87 (±1.0)	1.62 (±0.1)	7.67 (±0.4)	22.13 (±1.4)	3.50 (±0.2)	1.54 (±0.1)	0.75 (±0.0)	0.45 (±0.0)	6.48 (±0.5)	0.71 (±0.1)	7.55 (±0.5)
	2.0	7.62 (±0.2)	17.03 (±0.6)	1.91 (±0.1)	6.41 (±0.8)	19.96 (±0.5)	4.05 (±0.1)	1.63 (±0.1)	0.77 (±0.0)	0.47 (±0.0)	6.57 (±0.2)	0.72 (±0.0)	5.15 (±1.2)
8.0	0.5	6.63 (±0.5)	14.93 (±1.2)	1.34 (±0.3)	7.34 (±0.7)	18.89 (±4.6)	3.68 (±0.3)	1.53 (±0.1)	0.74 (±0.1)	0.46 (±0.0)	6.22 (±0.4)	0.69 (±0.1)	6.03 (±0.5)
	1.0	6.69 (±0.1)	14.85 (±0.2)	1.70 (±0.0)	8.06 (±0.2)	19.30 (±2.1)	3.90 (±0.1)	1.43 (±0.0)	0.59 (±0.0)	0.40 (±0.0)	5.10 (±0.1)	0.57 (±0.0)	6.00 (±0.1)
	2.0	7.65 (±0.2)	16.81 (±0.4)	1.91 (±0.0)	8.86 (±0.1)	14.18 (±3.5)	4.23 (±0.2)	1.67 (±0.0)	0.80 (±0.0)	0.51 (±0.0)	6.49 (±0.1)	0.74 (±0.0)	7.69 (±0.1)

Value in () are ± SD
T- Temperature
EL- Enzyme load
EPA- Eicosapentaenoic acid
DPA- Docosapentaenoic acid
DHA- Docosahexaenoic acid

Tables 5.14 and 5.15 show the ANOVA and Tukey's grouping of saturated fatty acids (myristic, palmitic and stearic acids), respectively. The ANOVA results indicated that the effect of temperature on the three saturated fatty acids was significant at 0.05 level. However, the pH and enzyme load did not seen to have any significant effect on the three saturated fatty acids. The Tukey's grouping results showed that the differences among the temperature levels were significant for the three saturated fatty acids at the 0.05 level. However, the differences among the levels of pH and enzyme load were not significant for the three saturated fatty acids at the 0.05 level.

Tables 5.16 and 5.17 show the ANOVA and Tukey's grouping of monounsaturated fatty acids (palmitoleic, oleic and vaccenic acids), respectively. The temperature had a significant effect on the three monounsaturated fatty acids at the 0.05 level. However, the pH and enzyme load did not have any significant effect on the three monounsaturated fatty acids at the 0.05 level. The Tukey's grouping showed that the differences among the levels of temperature were significant for the three monounsaturated fatty acids at the 0.05 level. However, the differences among the levels of pH and enzyme load were not significant for the three monounsaturated fatty acids at the 0.05 level.

Tables 5.18 and 5.19 show the ANOVA and Tukey's grouping of polyunsaturated fatty acids (linoleic, alpha-linolenic and arachidonic acids), respectively. The effect of temperature on the three polyunsaturated fatty acids was significant at the 0.01 level. However, the effects of pH and enzyme loads on the three polyunsaturated fatty acids were not significant at the 0.05 level. The Tukey's grouping showed that differences among the levels of temperature were significant for the three polyunsaturated fatty acids at the 0.05 level. However, the differences among the levels of pH and enzyme load were insignificant for the three polyunsaturated fatty acids at the 0.05 level.

Tables 5.20 and 5.21 show the ANOVA and Tukey's grouping of polyunsaturated fatty acids (eicosapentaenoic, docosapentaenoic and docosahexaenoic acids), respectively. The effect of temperature on the three polyunsaturated fatty acids was significant at the 0.05

Table 5.14. ANOVA for saturated fatty acids (myristic, palmitic and stearic)

Source	DF	SS	MS	F	P
		Myristic Acid			
Total	35	17.22			
Model					
T	1	5.07	5.07	25.79	0.001
pH	2	3.05	1.52	7.78	0.104
EL	2	0.36	0.18	0.92	0.415
T*pH	2	1.00	0.50	2.56	0.015
T*EL	2	2.91	1.45	7.41	0.004
pH*EL	4	0.69	0.17	0.88	0.496
T* pH *EL	4	0.58	0.14	0.74	0.577
Error	18	3.54	0.19		
		Palmitic Acid			
Total	35	60.54			
Model					
T	1	7.20	7.21	6.91	0.017
pH	2	4.18	2.09	2.01	0.164
EL	2	3.15	1.58	1.51	0.247
T*pH	2	3.27	1.64	1.57	0.235
T*EL	2	18.07	9.04	8.66	0.220
pH*EL	4	2.78	0.69	0.67	0.623
T* pH *EL	4	3.05	0.76	0.73	0.582
Error	18	18.78	1.04		
		Stearic Acid			
Total	35	10.27			
Model					
T	1	1.60	1.60	6.14	0.023
pH	2	0.13	0.06	0.26	0.770
EL	2	0.38	0.19	0.74	0.492
T*pH	2	0.36	0.18	0.69	0.512
T*EL	2	0.24	0.12	0.48	0.628
pH*EL	4	2.07	0.51	1.98	0.141
T* pH *EL	4	0.75	0.18	0.72	0.588
Error	18	4.70	0.26		

T: Temperature
EL: Enzyme load
DF: Degree of freedom
SS: Sum of squares
MS: Mean of squares
R^2 :79.46%, 68.97% and 54.20%.

Table 5.15. Tukey's grouping for saturated fatty acids (myristic, palmitic and stearic)

Parameter		Number of Observations	Mean (wt%)	Grouping
		Myristic Acid		
Temperature (°C)	55	18	6.14	A
	70	18	6.89	B
pH	7.0	12	6.11	A
	7.5	12	6.79	A
	8.0	12	6.62	A
Enzyme load (%)	0.5	12	6.55	A
	1.0	12	6.37	A
	2.0	12	6.61	A
		Palmitic Acid		
Temperature (°C)	55	18	15.41	A
	70	18	14.51	B
pH	7.0	12	14.54	A
	7.5	12	14.97	A
	8.0	12	15.37	A
Enzyme load (%)	0.5	12	15.10	A
	1.0	12	14.55	A
	2.0	12	15.23	A
		Stearic Acid		
Temperature (°C)	55	18	1.27	A
	70	18	1.70	B
pH	7.0	12	1.53	A
	7.5	12	1.40	A
	8.0	12	1.53	A
Enzyme load (%)	0.5	12	1.50	A
	1.0	12	1.35	A
	2.0	12	1.60	A

Groups with the same letter are not significantly different from each other at the 0.05 level.

Table 5.16. ANOVA for monounsaturated fatty acids (palmitoleic, oleic and vaccenic)

Source	DF	SS	MS	F	P
		Palmitoleic Acid			
Total	35	20.15			
Model					
T	1	1.01	1.01	3.22	0.040
pH	2	2.25	1.12	3.57	0.099
EL	2	0.09	0.04	0.15	0.864
T*pH	2	1.07	0.53	1.71	0.208
T*EL	2	1.42	0.71	2.27	0.132
pH*EL	4	5.16	1.29	4.10	0.160
T* pH *EL	4	3.45	0.86	2.74	0.061
Error	18	5.66	0.31		
		Oleic Acid			
Total	35	235.55			
Model					
T	1	34.57	34.57	12.16	0.003
pH	2	1.52	0.76	0.27	0.768
EL	2	15.97	7.98	2.81	0.087
T*pH	2	88.02	44.01	15.48	0.300
T*EL	2	12.30	6.15	2.16	0.144
pH*EL	4	11.28	2.81	0.99	0.437
T* pH *EL	4	20.71	5.18	1.82	0.169
Error	18	51.17	2.84		
		Vaccenic Acid			
Total	35	4.61			
Model					
T	1	0.86	0.86	13.00	0.002
pH	2	0.42	0.21	3.16	0.067
EL	2	0.26	0.12	1.93	0.173
T*pH	2	0.22	0.11	1.67	0.216
T*EL	2	1.14	0.57	8.57	0.200
pH*EL	4	0.19	0.04	0.72	0.591
T* pH *EL	4	0.30	0.07	1.14	0.371
Error	18	1.20	0.06		

T: Temperature
EL: Enzyme load
DF: Degree of freedom
SS: Sum of squares
MS: Mean of squares
R^2 :71.88%, 78.27% and73.95%.

Table 5.17. Tukey's grouping for monounsaturated fatty acids (palmitoleic, oleic and vaccenic)

Parameter		Number of Observations	Mean (wt%)	Grouping
		Palmitoleic Acid		
Temperature (°C)	55	18	7.49	A
	70	18	7.83	B
pH	7.0	12	7.67	A
	7.5	12	7.36	A
	8.0	12	7.97	A
Enzyme load (%)	0.5	12	7.73	A
	1.0	12	7.61	A
	2.0	12	7.65	A
		Oleic Acid		
Temperature (°C)	55	18	21.82	A
	70	18	19.86	B
pH	7.0	12	20.84	A
	7.5	12	21.08	A
	8.0	12	20.58	A
Enzyme load (%)	0.5	12	21.42	A
	1.0	12	21.18	A
	2.0	12	19.91	A
		Vaccenic Acid		
Temperature (°C)	55	18	3.58	A
	70	18	3.89	B
pH	7.0	12	3.73	A
	7.5	12	3.61	A
	8.0	12	3.87	A
Enzyme load (%)	0.5	12	3.74	A
	1.0	12	3.63	A
	2.0	12	3.84	A

Groups with the same letter are not significantly different from each other at the 0.05 level.

Table 5.18. ANOVA for polyunsaturated fatty acids (linoleic, alpha-linolenic and arachidonic)

Source	DF	SS	MS	F	P
		Linoleic Acid			
Total	35	0.57			
Model					
T	1	0.07	0.07	7.28	0.015
pH	2	0.01	0.01	0.60	0.561
EL	2	0.04	0.02	1.92	0.175
T*pH	2	0.02	0.01	1.20	0.325
T*EL	2	0.15	0.07	7.23	0.050
pH*EL	4	0.02	0.01	0.64	0.642
T* pH *EL	4	0.03	0.01	0.82	0.527
Error	18	0.19	0.01		
		Alpha-linolenic Acid			
Total	35	0.50			
Model					
T	1	0.24	0.24	77.49	0.001
pH	2	0.02	0.01	3.59	0.149
EL	2	0.01	0.02	0.79	0.470
T*pH	2	0.03	0.01	5.05	0.018
T*EL	2	0.07	0.03	11.19	0.010
pH*EL	4	0.05	0.01	4.04	0.116
T* pH *EL	4	0.01	0.03	1.24	0.330
Error	18	0.05	0.03		
		Arachidonic Acid			
Total	35	0.07			
Model					
T	1	0.01	0.01	17.71	0.001
pH	2	0.01	0.01	0.56	0.582
EL	2	0.06	0.03	3.13	0.068
T*pH	2	0.01	0.01	0.85	0.444
T*EL	2	0.01	0.01	7.91	0.030
pH*EL	4	0.04	0.01	1.09	0.389
T* pH *EL	4	0.06	0.01	1.61	0.215
Error	18	0.01	0.01		

T: Temperature
EL: Enzyme load
DF: Degree of freedom
SS: Sum of squares
MS: Mean of squares
R^2 :66.05%, 88.60% and 74.79%

Table 5.19. Tukey's grouping for polyunsaturated fatty acids (linoleic, alpha-linolenic and arachidonic)

Parameter		Number of Observations	Mean (wt %)	Grouping
		Linoleic Acid		
Temperature (°C)	55	18	1.47	A
	70	18	1.56	B
pH	7.0	12	1.49	A
	7.5	12	1.52	A
	8.0	12	1.53	A
Enzyme load (%)	0.5	12	1.52	A
	1.0	12	1.47	A
	2.0	12	1.55	A
		alpha-linolenic Acid		
Temperature (°C)	55	18	0.53	A
	70	18	0.70	B
pH	7.0	12	0.58	A
	7.5	12	0.63	A
	8.0	12	0.64	A
Enzyme load (%)	0.5	12	0.63	A
	1.0	12	0.60	A
	2.0	12	0.62	A
		Arachidonic Acid		
Temperature (°C)	55	18	0.41	A
	70	18	0.46	B
pH	7.0	12	0.43	A
	7.5	12	0.43	A
	8.0	12	0.44	A
Enzyme load (%)	0.5	12	0.44	A
	1.0	12	0.42	A
	2.0	12	0.45	A

Groups with the same letter are not significantly different from each other at the 0.05 level.

Table 5.20. ANOVA for polyunsaturated fatty acids (eicosapentaenoic, docosapentaenoic and docosahexaenoic)

Source	DF	SS	MS	F	P
		Eicosapentaenoic Acid			
Total	35	12.72			
Model					
T	1	1.17	1.17	6.80	0.018
pH	2	0.21	0.11	0.62	0.549
EL	2	1.05	0.53	3.07	0.071
T*pH	2	1.42	0.71	4.12	0.054
T*EL	2	2.40	1.20	6.98	0.060
pH*EL	4	1.98	0.49	2.88	0.153
T* pH *EL	4	1.35	0.34	1.97	0.143
Error	18	3.10	0.17		
		Docosapentaenoic Acid			
Total	35	0.21			
Model					
T	1	0.02	0.02	9.53	0.006
pH	2	0.03	0.01	6.03	0.110
EL	2	0.01	0.01	1.88	0.181
T*pH	2	0.02	0.01	0.36	0.700
T*EL	2	0.03	0.01	6.20	0.009
pH*EL	4	0.03	0.07	2.78	0.058
T* pH *EL	4	0.01	0.04	1.58	0.223
Error	18	0.05	0.02		
		Docosahexaenoic Acid			
Total	35	22.69			
Model					
T	1	1.12	1.12	3.53	0.016
pH	2	0.64	0.32	1.02	0.381
EL	2	0.43	0.21	0.69	0.515
T*pH	2	1.27	0.63	2.01	0.164
T*EL	2	2.70	1.35	4.25	0.310
pH*EL	4	6.18	1.54	4.87	0.800
T* pH *EL	4	4.59	1.14	3.61	0.250
Error	18	5.72	0.31		

T: Temperature
EL: Enzyme load
DF: Degree of freedom
SS: Sum of squares
MS: Mean of squares
R^2 :75.59%, 75.65% and74.78%.

Table 5.21. Tukey's grouping for polyunsaturated fatty acids (eicosapentaenoic, docosapentaenoic and docosahexaenoic)

Parameter		Number of Observations	Mean (wt%)	Grouping
		Eicosapentaenoic Acid		
Temperature (°C)	55	18	5.83	A
	70	18	6.19	B
pH	7.0	12	5.92	A
	7.5	12	6.10	A
	8.0	12	6.02	A
Enzyme load (%)	0.5	12	5.95	A
	1.0	12	5.84	A
	2.0	12	6.25	A
		Docosapentaenoic Acid		
Temperature (°C)	55	18	0.61	A
	70	18	0.66	B
pH	7.0	12	0.60	A
	7.5	12	0.67	A
	8.0	12	0.63	A
Enzyme load (%)	0.5	12	0.63	A
	1.0	12	0.62	A
	2.0	12	0.66	A
		Docosahexaenoic Acid		
Temperature (°C)	55	18	6.82	A
	70	18	7.17	B
pH	7.0	12	7.12	A
	7.5	12	6.81	A
	8.0	12	7.05	A
Enzyme load (%)	0.5	12	6.98	A
	1.0	12	6.87	A
	2.0	12	7.14	A

Groups with the same letter are not significantly different from each other at the 0.05 level.

level. However, the effects of pH and enzyme load on the three polyunsaturated fatty acids were not significant at the 0.05 level. The Tukey's grouping showed that the differences among the levels of temperature were significant for the three polyunsaturated fatty acids at the 0.05 level. However, the differences among the levels of pH and enzyme load were not significant for the three polyunsaturated fatty acids.

5.3. Chemical Extraction of Oil

Crude fish oil was extracted according to Bligh and Dyer method. The recovery yield and values of quality parameters are shown in Table 5.22 and the compositions of fatty acids are shown in Table 5.23. The recovery oil yield of crude fish oil was 97.5 %. About 90.5 % of the solvent was recovered, the remaining was evaporated in the process. The peroxide value, acid value and *p*-anisidine value were 12.04 Meq/g, 5.11 mg KOH/g and 9.33, respectively. The peroxide and *p*-anisidine value were within the recommended range but acid value was slightly higher (due to exposure of sample to air) than the recommended range. Among the 12 fatty acids shown in Table 5.23, oleic acid (18:1n-9) and palmitic acid (16:0) showed the highest fatty acid contents of 19.76 and 14.64, respectively.

5.4. Comparing Enzymatic and Chemical Extraction Methods of Fish Oil

Table 5.24 shows the yield and quality parameters of oils obtained by enzymatic and chemical extraction methods. The combination of enzymatic hydrolysis 55°C-7.5 pH-2.0% enzyme load that produced the highest oil recovery yield was choosen for the comparison with the chemical extraction method. The results showed that the chemical extraction method gave much higher yield and better quality. Figure 5.5 and 5.6 shows a comparison of both methods based on yield and peroxide value, acid value and *p*-anisidine value, respectively.

Table 5.25 shows the fatty acids composition of oils obtained by enzymatic and chemical extraction methods. Only 12 FAs were tabulated under three groups: saturated,

Table 5.22. Recovery and chemical analysis of fish oil extracted using the Bligh and Dyer method.

Parameter	Values
Recovered oil (g)	8.60 ± 0.30
Recovery oil yield (%)	97.16 ± 3.40
Solvent recovery (%)	90.50 ± 1.30
Peroxide value (Meq/g)	12.04 ± 0.01
Acid value (mg KOH/g)	5.11 ± 0.06
p-Anisidine value	9.33 ± 0.33

Oil content of herring - 8.82g/100g
Results are mean ± SD of three replicates

Table 5.23. Fatty acid composition of fish oil obtained using the Bligh and Dyer method.

Fatty Acid	Amount (%)*
Saturated fatty acid	
Myristic acid (14:0)	5.46 ± 0.01
Palmitic acid (16:0)	14.64 ± 0.33
Stearic acid (18:0)	1.76 ± 0.001
Monounsaturated fatty acid	
Palmitoleic acid (16:1 n-7)	6.69 ± 0.09
Oleic acid (18:1n-9)	19.76 ± 0.19
Vaccenic acid (18:1n-7)	3.41 ± 0.0
Polyunsaturated fatty acid	
Linoleic acid (18:2n-6)	1.17 ± 0.0
Alpha-Linolenic acid (18:3n-3)	0.47 ± 0.01
Arachidonic (20:4n-6)	0.38 ± 0.0
Eicosapentaenoic acid (20:5n-3)	4.46 ± 0.0
Docosapentaenoic acid (22:5n-3)	0.44 ± 0.0
Docosahexaenoic acid (22:6n-3)	5.92 ± 0.01

*by weight

Results are mean ± SD of three replicates

Table 5.24. Comparison of chemical and enzymatic method with respect to yield, peroxide value, acid value and *p*-anisidine value

Parameter	Recommended Value*	Chemical Method	Enzymatic Method**
Recovery oil yield (%)	-	97.16±3.4	20.2 ± 0.42
Peroxide value (Meq/g)	3-20	12.04 ± 0.01	31.44±0.33
Acid value (mg KOH/g)	<5.0	5.11 ± 0.06	11.13±0.46
p-Anisidine value	4-60	9.33 ± 0.33	58.27±0.19

Results are mean ± SD of three replicates

*Source: Jensen et al. (1990)

**At the optimum condition of 7.5 pH, 55°C and 2.0% enzyme load.

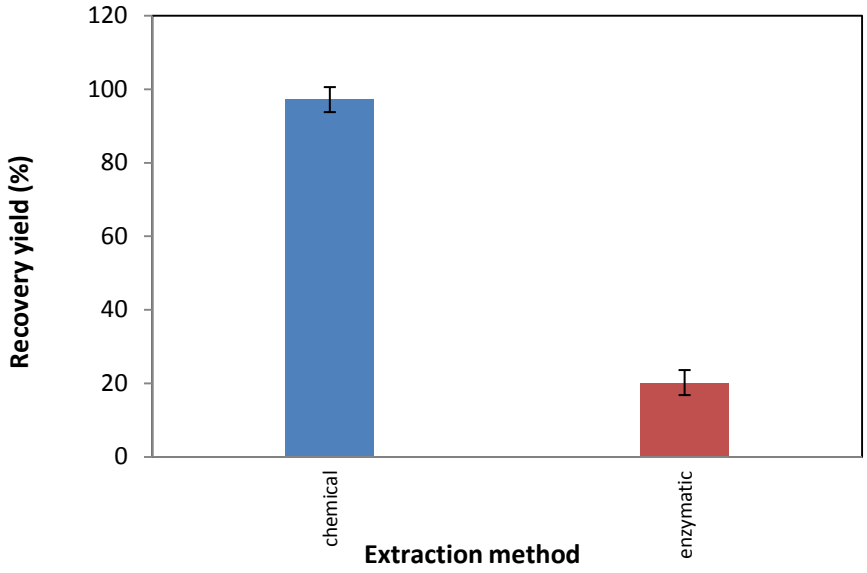
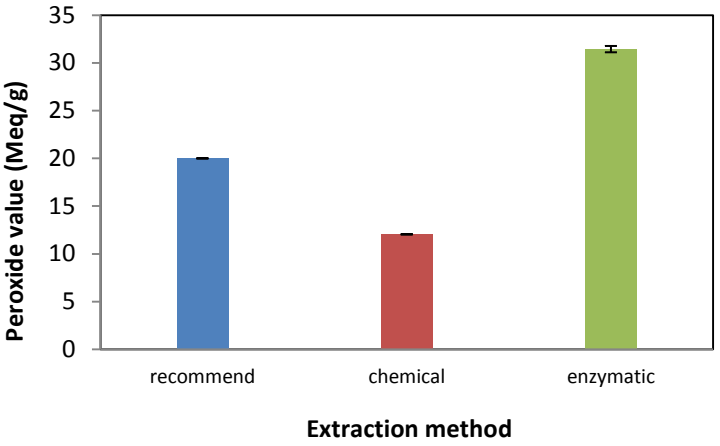
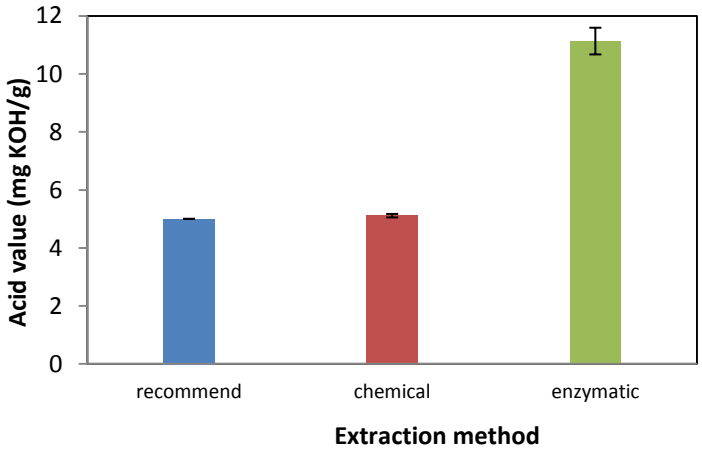


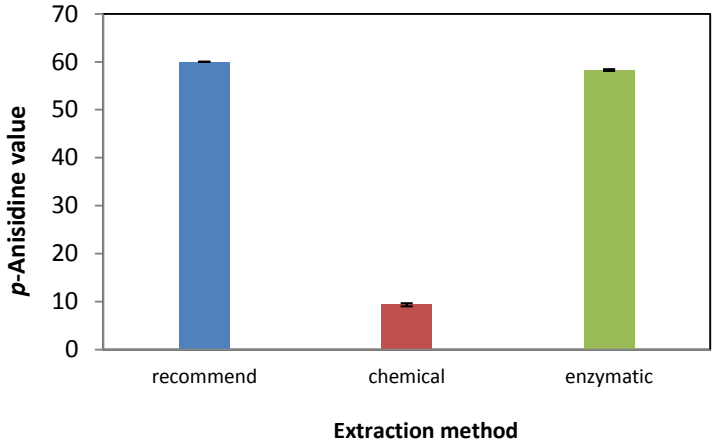
Figure 5.5. Comparison of oil recovery yield of chemical and enzymatic extraction methods.



(a) peroxide value of oil



(b) acid value of oil



(c) *p*-anisidine value of oil

Figure 5.6. Comparison of the oil quality parameters for the enzymatic and chemical extraction methods.

Table 5.25. Comparison of fatty acid composition by chemical and enzymatic methods

Fatty Acid	Weight (%)	
	Chemical Method	Enzymatic Method
Saturated fatty acids		
Myristic acid (14:0)	5.46 ± 0.01	5.81 ± 0.10
Palmitic acid (16:0)	14.64 ± 0.33	13.10 ± 0.23
Stearic acid (18:0)	1.76 ± 0.001	0.73 ± 0.98
Subtotal	21.86	19.64
Monounsaturated fatty acids		
Palmitoleic acid (16:1 n-7)	6.69 ± 0.09	6.88 ± 0.12
Oleic acid (18:1n-9)	19.76 ± 0.19	19.84 ± 0.36
Vaccenic acid (18:1n-7)	3.41 ± 0.0	3.23 ± 0.03
Subtotal	29.86	29.95
Polyunsaturated fatty acids		
Linoleic acid (18:2n-6)	1.17 ± 0.0	1.38 ± 0.02
Alpha-Linolenic acid (18:3n-3)	0.47 ± 0.01	0.36 ± 0.07
Arachidonic (20:4n-6)	0.38 ± 0.0	0.38 ± 0.00
Eicosapentaenoic acid (20:5n-3)	4.46 ± 0.0	5.50 ± 0.09
Docosapentaenoic acid (22:5n-3)	0.44 ± 0.0	0.58 ± 0.00
Docosahexaenoic acid (22:6n-3)	5.92 ± 0.01	6.41 ± 0.13
Subtotal	12.84	14.61
Other FAs	35.44	35.80
Total	100	100

monounsaturated and polyunsaturated fatty acids. The saturated FAs of chemical method were higher than those of the enzymatic method. However, the monounsaturated and polyunsaturated FAs of the enzymatic method were higher than those of the chemical method. By comparing the two methods on the basis of entire fatty acid composition, the enzymatic method produced slightly higher fatty acids compared to the chemical method.

5.5. Concentration of Omega-3 Fatty Acids

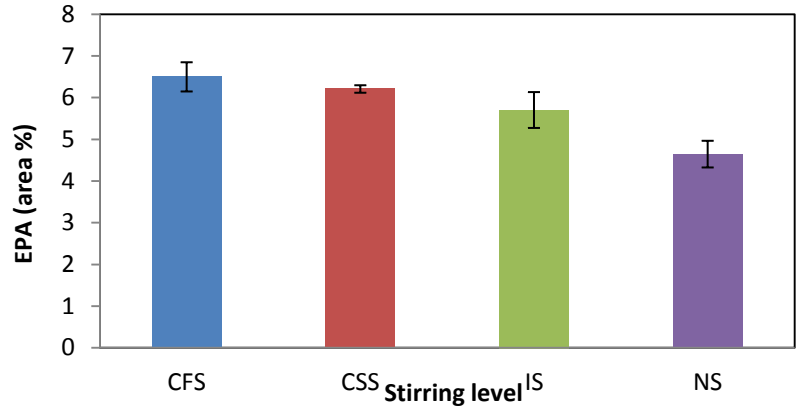
Table 5.26 and Figure 5.7 show the fatty acid composition of omega-3 fatty acid (EPA, DPA and DHA) at four stirring levels (constant fast stirring at 200 rpm, constant slow stirring at 50 rpm, intermediate stirring at 100 rpm every 4 hrs and no stirring). Among the four stirring levels 'no stirring' showed the least value of EPA, DPA and DHA and 'constant fast stirring' showed higher values of EPA, DPA and DHA of 6.49, 0.91 and 11.17%, repectively. Constant slow stirring and intermediate stirring showed lower values of EPA, DPA and DHA than those of constant fast stirring. No stirring showed the least omega-3 FA content as enzymes are not oil soluble. Enzymes possess small functional regions known as the active sites. The substrate molecule is held within the active site and the enzyme breaks the bonds holding the substrate molecules into smaller molecules known as products. This structure is known as the enzyme-substrate complex. Mixing allows good contact between the enzyme and substrate and speeds up the enzymatic reaction rate.

Tables 5.27 and 5.28 show the ANOVA and Tukey's grouping results performed on the data. The effect of mixing was significant at the 0.001 and 0.029 levels for EPA and DHA, respectively. However, there appeared to be no effect of stirring on the DPA. This may be due to the fact that DPA has one less double bond and less bent structure. The results obtained from Tukey's grouping indicated that the three levels of mixing were significantly different from no mixing but were not significantly different from one another at the 0.05 level for all three omega-3 fatty acids.

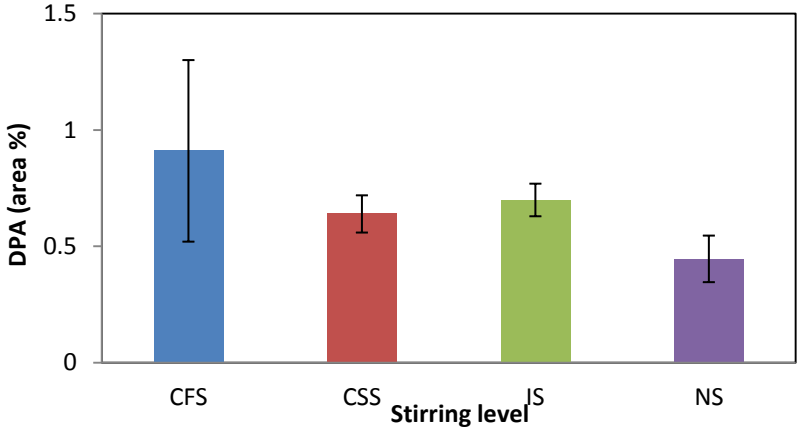
Table 5.26. Fatty acid composition of omega-3 fatty acids.

Sample	EPA (wt%)	DPA (wt%)	DHA (wt %)
CFS	6.49±0.35	0.91±0.39	11.17±0.14
CSS	6.20±0.09	0.63±0.08	10.69±0.74
IS	5.70±0.43	0.69±0.07	10.43±0.94
NS	4.64±0.32	0.44±0.10	8.91±0.88

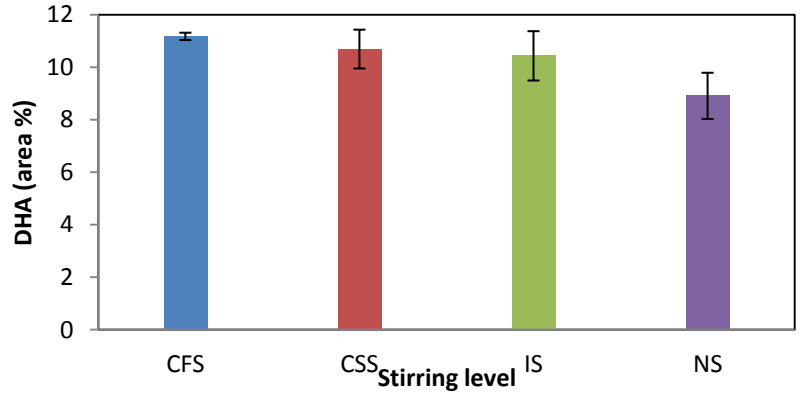
Results are mean ± SD of three replicates
CFS= Constant fast stirring
CSS= Constant slow stirring
NS= No stirring
IS= Intermediate stirring
EPA- Eicosapentaenoic acid
DHA- Docosahexaenoic acid
DPA- Docosapentaenoic acid



(a) EPA



(b) DPA



(c) DHA

Figure 5.7. Effect of stirring level (CFS= constant fast stirring, CSS= constant slow stirring, IS= intermediate stirring, NS= no stirring) on omega-3 fatty acids content

Table 5.27. ANOVA for EPA and DHA.

Source	DF	SS	MS	F	P
		EPA			
Total	11	6.837			
Stirring level	3	5.970	1.990	18.37	0.001
Error	8	0.866	0.108		
		DHA			
Total	11	13.127			
Stirring level	3	8.614	2.871	5.09	0.029
Error	8	4.513	0.564		
		DPA			
Total	11	0.6849			
Stirring level	3	0.3296	0.1099	2.47	0.136
Error	8	0.3553	0.0444		

DF: Degree of freedom,
SS: Sum of squares,
MS: Mean of squares,
R^2: 87.33, 65.62, 48.12 %

Table 5.28. Tukey's grouping for stirring levels.

Factor	Number of Observations	Mean (wt %)	Grouping
Stirring level			
	EPA		
CFS	3	6.49	A
CSS	3	6.20	A
IS	3	5.70	A
NS	3	4.64	B
	DHA		
CFS	3	11.17	A
CSS	3	10.69	A
IS	3	10.43	A
NS	3	8.91	B
	DPA		
CFS	3	0.9107	A
CSS	3	0.6393	A
IS	3	0.6997	A
NS	3	0.4460	A

Groups with the same letter are not significantly different from each other at the 0.05 level.

CFS= Constant fast stirring
CSS= Constant slow stirring
NS= No stirring
IS= Intermediate stirring

Table 5.29 shows the composition of omega-6, omega-7 and omega-9 fatty acids. The value of omega-6 FAs (arachidonic and linoleic acid) was higher with constant fast stirring and lower with no stirring. Similar results were observed with omega-7 FAs (palmitoleic acid and vaccenic acid). However, the value of omega-9 FAs (oleic acid) was higher with constant slow stirring and lower with no stirring. Many lipases are specific toward particular fatty acid substrates and the lipases that are specific to fatty acids hydrolyse the glyceride bonds formed by cis-9 unsaturated fatty acids (Oleic and linoleic acid). Also the chain specificity of *Candida rugosa* lipase is in the order of oleic > palmitic > DHA > EPA > myristic.

Table 5.30 shows the composition of omega-3 fatty acids before and after enrichment of fish oil. Composition of FAs obtained by constant fast stirring was compared with the composition of fish oil obtained by chemical extraction method. The contents of omega-3 FAs increase when mixing was applied. The EPA increased from 4.46% to 6.46% (45%), the DPA increased from 0.44% to 0.82% (86%) and DHA from 5.92% to 11.17% (89%). Also, there was an enrichment of omega-6 and omega-7 FAs whereas omega-9 FAs decrease. Decrease in omega-9 FAs is due to the fact that oleic acids possess higher molecular weight thereby strongly inhibited the lipase activity.

Table 5.29. Fatty acid composition of omega-6, omega-7 and omega-9 fatty acids.

Sample	Omega-6 FA		Omega-7 FA		Omega-9 FA
	Arachidonic acid	Linoleic acid	Palmitoleic acid	Vaccenic acid	Oleic acid
CFS	0.63 ± 0.19	1.19 ± 0.07	7.17 ± 0.66	3.74 ± 0.59	17.52 ± 0.30
CSS	0.60 ± 0.09	1.15 ± 0.03	7.07 ± 0.42	3.49 ± 0.08	18.87 ± 0.20
IS	0.54 ± 0.20	1.09 ± 0.11	6.92 ± 0.10	3.37 ± 0.41	16.73 ± 0.12
NS	0.40 ± 0.14	1.05 ± 0.35	6.70 ± 0.21	3.06 ± 0.05	14.90 ± 0.08

Results are mean ± SD of three replicates
CFS= Constant fast stirring
CSS= Constant slow stirring
NS= No stirring
IS= Intermediate stirring

Table 5.30. Comparison of fatty acids composition of fish oil and omega-3 enriched oil.

Fatty Acids	Fish Oil Wt (%)	Omega-3 Enriched Oil* Wt (%)
Saturated fatty acids		
Myristic acid (14:0)	5.46 ± 0.01	5.42 ± 0.25
Palmitic acid (16:0)	14.64 ± 0.33	15.88 ± 0.06
Stearic acid (18:0)	1.76 ± 0.001	1.75 ± 0.02
Monounsaturated fatty acids		
Palmitoleic acid (16:1 n-7)	6.69 ± 0.09	7.17 ± 0.66
Oleic acid (18:1n-9)	19.76 ± 0.19	17.21 ± 0.30
Vaccenic acid (18:1n-7)	3.41 ± 0.0	3.74 ± 0.59
Polyunsaturated fatty acids		
Linoleic acid (18:2n-6)	1.17 ± 0.0	1.19 ± 0.07
Alpha-Linolenic acid (18:3n-3)	0.47 ± 0.01	0.43 ± 0.32
Arachidonic (20:4n-6)	0.38 ± 0.0	0.63 ± 0.19
Eicosapentaenoic acid (20:5n-3)	4.46 ± 0.0	6.49 ± 0.35
Docosapentaenoic acid (22:5n-3)	0.44 ± 0.0	0.82 ± 0.39
Docosahexaenoic acid (22:6n-3)	5.92 ± 0.01	11.17 ± 0.14

*Omega-3 enriched oil obtained by constant fast stirring

CHAPTER 6. DISCUSSION

6.1. Oil Extraction

Gbogouri et al. (2006) stated that during enzymatic hydrolysis the enzyme breaks down the protein networks which lead to the release of lipid in the fish slurry. Slizyte et al. (2005) reported that when the fish slurry is desludged by centrifugation, four phases are observed: oil on top layer, emulsion, fish protein hydrolysate and sludge at the bottom of centrifuge tube. In this study, the four phases were observed and the upper phase (first phase) was pipetted and the emulsion phase was scooped to extract the remaining trapped oil.

The recovered lipid yield using alcalase enzyme under different conditions of temperature, pH and enzyme load was 20.2%. This may be due to low centrifugal force (low centrifugation speed) applied during the centrifugation step. Sun et al. (2002) stated that in centrifugation step, some lipids are lost in the aqueous layer. Therefore, in order to achieve a higher separation of oil, a centrifugal force >9000 g is recommended for removing the oil from the slurry. In this study, a centrifugal speed of 4000 rpm (2683 g) was used. The results showed that the efficiency of enzymatic hydrolysis in extracting lipid is low. This could be due to the higher amount of emulsion giving a lower amount of separated oil and vice versa. However, Slizyte et al. (2005) stated that the amount of lipids in the oil and emulsion fractions were dependent on each other. The lowest emulsion and greatest amount of separated oil can be obtained by using alcalase without added water. However, in this study water and NaOH was added in order to have desired pH.

In this study frozen homogenate was used to extract fish oil which might be reason for deterioration of oil quality. The characteristics and properties of crude fish oil are strongly influenced by the process and raw material (EFSA, 2010). According to Aubourg and Medina (1999) the free fatty acids (FFA) have been produced during the frozen storage of fish as a result of enzyme catalysis. This effect was increased with

time and temperature of the reaction mixture and with the FFA content (Miyashita and Takagi, 1986) and leading to a high proportion of polyunsaturated FFA (Aubourg et al., 1996). Due to the catalytic effect of the carboxyl group there is a formation of free radicals which decompose hydroperoxides by deteriorating the quality of oil. To avoid the extensive release of FFA, freshly prepared sample should be used.

6.1.1. Effect of temperature

In this study, two different temperatures (55°C and 70°C) were investigated in order to determine the effect of temperature on the oil recovery yield. The higher oil recovery yield (20.2%) was obtained at 55°C compared to 70°C (15.6%). There was a significant effect of temperature on oil recovery yield.

Linder et al. (2005) extracted lipids from salmon heads with neutrase at a temperature of 55°C and obtained a yield of 17.2%. Gbogouri et al. (2006) extracted lipids from salmon heads with neutrase at a temperature 55°C and reported a recovery yield of 14.4%. In general, the rate of an enzyme-catalyzed reaction increases as the temperature rises. Small variation in reaction temperature (1-2°C) may change the results by 10-20%. However, many enzymes have unfavorable response to high temperatures. An increase in the temperature may increase the reaction rate up to a maximum level after which the reaction rate is decreased suddenly with a further increase in the temperature (WBC, 2012). Amy (2008) investigated the effect of temperature on alcalase and found that alcalase to be high temperature protease. The activity of alcalase increased with increased temperature up to optimum temperature of 50-60°C. In this study, temperatures of up to 70°C were investigated as the enzyme alcalase has a temperature range of 50°C-70°C.

6.1.2. Effect of pH

In this study, three different pH levels (7.0, 7.5 and 8.0) were studied in order to determine the optimum pH for enzymatic extraction of fish oil. The recovery yield of oil increased from 16.3 to 20.2% when the pH was increased from 7.0 to 7.5 and then

decreased from 20.2 to 16.6% when the pH was further increased to 8.0. This is due to the reasons that, at the optimum pH enzymes are most active. Dominguez et al. (1994) reported that increasing the pH beyond its optimum level decreases the enzyme activity and yield.

In general, the enzymes and their activity are affected by the change in pH. Increasing or decreasing the pH value results in a complete loss of enzyme activity. Stability of enzymes also depends on the pH. However, the optimum pH value will vary greatly from one enzyme to another (WBC, 2012). Dominguez et al. (1994) stated that effect of pH on the enzyme stability and activity depends on the type of enzyme. In this study, the pH range of alcalase tested was 6.5 to 8.5. The optimum pH for extracting oil from herring was 7.5

In this study, the recovery yield obtained at the optimum pH of 7.5 was 13.5-20.2% and 7.3-15.6% for lower and higher temperature, respectively. Gbogouri et al. (2006) studied the hydrolysis of salmon head with alcalase at a pH of 8.0 and reported a recovery yield of 19.6%. Kechaou et al. (2009) studied the enzymatic hydrolysis of cuttlefish and sardine viscera using alcalase and protamex at a pH of 8.0 and reported 12% total lipids. Nilsang et al. (2005) reported that the enzymatic hydrolysis of fish soluble concentrate (by-product of canned fish industry) using flavourzyme at a pH of 6.0 resulted in 12.9% of oil recovery yield. Batista et al. (2009) extracted lipid from sardine by-products using enzyme alcalase at a pH of 8.0 and reported a recovery yield of 40%. See et al. (2011) reported that enzymatic hydrolysis of salmon skin at a pH of 8.39 yielded 1.65% of fat. These studies indicated that the effect of pH on enzymatic extraction of lipids may depend on the type of fish/fish waste and the extraction technique used. The higher yield reported by Batista et al. (2009) is probably due to sardine containing high amount of lipid whereas the low yield reported by See et al. (2011) was probably due to high pH (higher than the optimum pH), sample size and the total oil in fish sample.

6.1.3. Effect of enzyme load

In this study, three different enzyme loads (0.5, 1.0 and 2.0%) were investigated. The 0.5% (w/w) alcalase load was chosen according to the procedure used by Batista et al. (2009) and 1.0 and 2.0% enzyme loads were selected to determine the effect of increasing the enzyme load on the oil yield. It was observed that increasing the enzyme load showed a steady increase (from 10.9 to 16.3%) in the oil recovery yield. Mbatia et al. (2010) reported maximum oil yield (11.2%) with 0.5% (w/w) bromelain with Nile perch. Batista et al. (2009) extracted lipid from sardine by-products and obtained a higher recovery yield (40%) with 0.5% (w/w) alcalase. Chua et al. (2012) stated that increase of enzyme concentration would proportionally increase the reaction rate but increasing the enzyme load beyond the critical value would not increase the reaction rate. These studies indicated that the effect of enzyme and its concentration vary for different fish samples.

Kulkarni and Pandit (2005) and Ghodsvali et al. (2008) reported that increasing enzyme concentration increased the extent of hydrolysis and oil yield. Dominguez et al. (1994) stated that increasing enzyme concentration made the emulsion more unstable. According to WBC (2012), the rate of reaction is dependent on substrate concentration. Therefore, enzyme assay should be designed so that the observed activity is proportional to the amount of substrate present.

6.2. Oil Quality Parameters

6.2.1. Peroxide value

The peroxide value is the number that expresses in milliequivalents of active oxygen the quantity of peroxide contained in 1000 g of the substance (European Pharmacopoeia, 2005). The number of peroxides present in the oil is an index of their primary oxidative level and its tendency to go rancid. The lower the peroxide value the better the oil quality and its state of preservation (CDR, 2008). Eyo (1993) stated that pH is an

indicator of the extent of microbial spoilage in fish. In this study, the effects of pH, enzyme load and temperature on peroxide value of crude fish oils were investigated.

All peroxide values first slightly decreased when the pH was increased from 7.0 to pH 7.5 and then increased when the pH was increased to pH 8.0. Mancuso et al. (1999) reported that low pH decreased lipid oxidation rates. Srinivasan et al. (1996) stated the lipid oxidation can be lowered by lowering the pH. Sang et al. (2012) reported that lower range of pH decreased the peroxide value and higher range of pH increased the peroxide value. This is explained by the collision theory which states that chemical reactions takes place during particles of reactants colliding which requires certain minimum energy called activation energy.

Most of the samples showed that the peroxide value increased slightly when enzyme load was increased from 0.5 to 2.0% while some showed an initial decrease in the peroxide value when the enzyme load was increased from 0.5 to 1.0% and then an increase with further increases in the enzyme load to 2.0%. DeRouchey et al. (2004) reported that the reaction rate initially increased as enzyme concentration increased and then leveled off. Kareska (2010) reported that doubling the enzyme concentration increased the peroxide value. Ghodsvali et al. (2008) stated that the change in peroxide value depends upon the enzyme type and its concentration. They also stated that for certain enzymes, it was observed that at lower enzyme concentration the peroxide value increased and at higher enzyme concentration the value decreased. To obtained lower peroxide value minimum amount of enzyme load should be used.

There was a steady increase of peroxide value at temperature of 70°C. Saeed and Howell (2002) reported that higher temperatures accelerated oxidation which initially increased the peroxide value to its maximum but it rapidly declined indicating the instability of product. Rabiei et al. (2011) and Capitani et al. (2011) reported that the peroxide value increased with increases in temperature. This is due to the initial propagation stage of lipid autooxidation phenomenon with chain reaction leading to

increases in peroxide formation. Inorder to obtain lower peroxide value, experiments should be carried out at lower temperature.

The recommended range of peroxide value is 3-20 Meq peroxide per kg. The peroxide values obtained in this study (26.78-41.79 Meq/g) were higher than the recommend range which could be due to oil oxidation. Batista et al. (2009) reported that the oil from raw sardine by-products had a high peroxide value in the range of 45.3-68.3 meq peroxide/kg oil and the oil appeared very dark in color indicating that the oil was oxidized. Swapna et al. (2011) obtained fish oil from fish viscera waste from catla and rohu (Indian major carps) using enzymatic hydrolysis and reported a peroxide value of 40.48 meq peroxide/kg oil which is also higher than the recommended range (3-20 Meq peroxide/ kg).

Peroxide value is a measure of oxidation or rancidity and can reduce the quality of food. Connell (1995) reported that peroxide values above 10-20 Meq/kg develop rancid tastes and smells. Mukherjee and Mitra (2009) reported that using the oxidized oil leads to potential dangers to the physiological and biochemical functions of the body. Ahmed and Mahendrakar (1996) stated that the change in PV is indicator of lipid oxidation (oxidation increases the peroxide value). However, lower value can still be achieved due to the breakdown of oxidized products. Wannahari and Nordin (2012) reported 21% reduction in the peroxide value in oils using bagasse as absorbent. Palanisamy et al. (2011) reported that bleaching can be used to remove certain minor constituents (colour pigments, free fatty acid, peroxides, odour and non-fatty materials) from crude fats and oils.

6.2.2. Acid value

Determining the acid value is important because it is still a reliable parameter for oil quality (FAO, 1986). The pH value is a reliable indicator of the degree of freshness or spoilage in fish (Eyo, 1993). The effects of pH, enzyme load and temperature on acid value were studied.

The measured acid value showed a steady increase when the pH was increased from 7.0 to 8.0 with the higher temperature (70°C). However, with the lower temperature (55°C), there was an increase in acid value when the pH was increased from 7.0 to 7.5 which then decreased when the pH was increased from 7.5 to 8.0. Ivanovic et al. (2012) observed higher acid values with higher pHs and lower acid values with lower pHs. Buckley and Fan (2005) reported that the acid value decreased with increasing pH. Sang et al. (2012) reported that effect of pH on the acid value increased when the pH was above 8.0. It was also stated that with the change in pH, the weak bonds holding the enzyme together break leading to deactivation of active site.

All samples showed increased acid values when the temperature was increased from 55 to 70°C. Xiangjin and Yali (2011) stated that at higher temperatures, the acid value increases without delay. Khan et al. (2001) and Zhang et al. (2005) reported that the acid value increased as the temperature was increased. Lee (2010) reported that at high temperature, the enzyme may be denatured and loses its activity whereas at low temperature it may be inactive. Nonetheless, varing the temperature may lead to increase or decrease of rate of reaction depending on the optimum temperature. Inorder to obtain lower acid value, lower tempearature should be used.

Increasing the enzyme load from 0.5 to 2.0% resulted in a steady increase in acid value. DeRouchey et al. (2004) reported that the acid value increased with increased addition of lipase. Sang et al. (2012) reported that the enzyme load increased along with the increase of acid value. Liman et al. (2010) reported that the lipase concentration increased with increased acid value. They also noticed that the lipase activity increased with increases in the substrate concentration for oil til an optimum substrate concentration. Lower acid value can be obtained by using minimum amount of enzyme load (0.5%).

The recommended range of acid value is <5 mg KOH/g. The obtained acid values were higher than the recommended range. The acid value quantifies the amount of acid present. It is the mass of potassium hydroxide in mg that is required to neutralize 1g of

chemical substance (Wrolstad et al., 2005). Swapna et al. (2011) reported that the acids value of oil obtained from fish viscera waste from catla and rohu (major Indian oily freshwater fish of the family Cyprinidae) using nutrease and alcalase were 22 and 20.4 mg KOH/g, respectively. Gbogouri et al. (2006) obtained acid values of 0.7 and 1.2 mg KOH/g of oil from salmon head and fillets, respectively. Swapna et al. (2011) obtained high acid value of fish oil using enzymatic hydrolysis and stated that there was no significant difference in acid value with or without the addition of anitioxidant.

As oil goes rancid, triacylglyceride (TAG) are converted to fatty acid (FA) and glycerol which increases acid number (Memon et al., 2010). Rancidity in fats and oils causes undesirable chemical changes in flavour, colour, odour and nutritional value of oil. Palanisamy et al. (2011) suggested that the nitrogen trap technique was as effective as the continuous nitrogen flow technique and is recommended due to its cost effectiveness. Turner et al. (2006) reported that fish oil is easily oxidized and some fish oils contain higher than recommended levels of oxidised products. In recent investigation it was observed that dietary oxidised fats developed atherosclerosis and thrombosis (Turner et al., 2006).

6.2.3. p-anisdine value

The non-volatile secondary oxidation end-products are measured by *p*-anisidine value. The p-anisidine value is usually associated with the term oxidative rancidity which is an indicator of the level of off-flavour and off-odours (St. Angelo, 1996). Megahed (2011) stated that *p*-anisidine is an indicator of secondary oxidation of unsaturated compounds. *p*-Anisidine is a reagent that reacts with aldehydes to give products that can be measured at an absorbance of 350 nm (Crapiste et al., 1999). The effects of pH, enzyme load and temperature on *p*-anisidine value of crude fish oil were investigated.

The results showed a slight increase in the *p*-anisidine value with increases in the pH. Bangash et al. (2004), Kumar and Kabra (2008) and Dayana and Hayati (2012) stated that an increase in pH increases the secondary oxidation products thereby increasing *p*-anisidine value. Arnoldi (2004) reported that at low pH values, metal ions are more

soluble than at higher pH values and this explains why lipid oxidation is slow at high pH values. Shelbaya et al. (2011) reported that when pH was increased the *p*-anisidine values increased. Baiano et al. (2005) reported that the hydroperoxides break down into aldehyde and ketone which are responsible for off-flavors (secondary oxidation).

There was a slight increase in the *p*-anisidine value with increases in the enzyme load. DeRouchey et al. (2004) reported that as lipase concentration was increased, the *p*-anisidine value remained unchanged. However, Abdulkarim et al. (2006) reported minimal increases of *p*-anisidine values with increases in enzyme concentration. In this study, the colour of the oils became darker with increases in the enzyme load. Similar results were observed by Zuniga et al. (2003) in which the oil appeared to be dark brown in colour with increased enzyme concentration. It was observed that at lower enzyme concentrations (up to 1.0%), a marked effect on the oil extractability was observed. The quality parameters improved slightly when the enzyme concentration was raised above 1.0%. However, the enzyme concentration to be used should be a compromise between the upgrading of the oil quality and the cost of the enzyme (Zhang et al., 2007).

In this study, there was a slight increase in the *p*-anisidine value with increased temperature. Labrinea et al. (2001) stated that the baseline absorbance increased rapidly as the temperature increased. Crapiste et al. (1999) and Kosoko et al. (2009) observed higher *p*-anisidine value as the temperature was increased. Crapiste et al. (1999) reported that the *p*-anisidine value, as an indicator of the aldehyde content, remained constant at the earlier stages of oxidation but then increased sharply following the decomposition of primary oxidation products.

The recommended range for *p*-anisidine value for crude fish oil is 4-60. The obtained *p*-anisidine values were in the range of 56.93-69.83. Anisidine value is an empirical test which determines the advanced oxidative rancidity of oils and fats (Rossell, 1994).

Crapiste et al. (1999) observed no significant oxidation, as measured by p-anisidine value, in samples stored under nitrogen atmosphere or with low oxygen concentration. Harald and Irene (2003) reported a method for reducing the p-anisidine value of oils which involved treating the oil with urea. Bleaching the oil is another way to reduce the p-anisidine value of oils (Palanisamy et al., 2011). EFSA (2010) reported that lipid oxidation in fish oil can be prevented by cold storage in darkness, without exposure to oxygen. Esterbauer (1993) observed that heavily oxidized oils given orally are not extremely toxic to humans. However, low molecular aldehydes are readily absorbed and can create pathological effects (damage to liver, thymus and kidney).

6.3. Fatty Acid Composition

6.3.1. Saturated fatty acids

Saturated fatty acids (SFA) are fatty acids which do not possess double bond (EFSA, 2010). The most important saturated fatty acids are myristic, palmitic and stearic acid which can be synthesized by body in low amount. In this study, the total saturated fatty acid percentage was 24-25%. The saturated fatty acids with values above 1% were tabulated and they are: myristic acid (14:0) which ranged between 6.11 and 7.65%, palmitic acid (16:0) which ranged between 14.85 and 16.81% and stearic acid (18:0) which ranged between 1.69 and 1.91%. The effects of temperature, pH and enzyme load on saturated fatty acids were investigated.

In this study, there was slight increase of saturated fatty acid from 6.11 to 6.28% with increased temperature (from 55 to 70°C). Kleinschmidt and Mcmahon (1970) reported that the saturated fatty acids content decreased at a temperature >55°C. Lanna et al. (2005) reported that palmitic and stearic acid were not affected by the temperature. Kates and Baxter (1962) reported decreased saturated fatty acids such as palmitate and stearate at lower temperatures. This was due to increased polyunsaturated fatty acids like linoleic and linolenic acids.

The results showed an increase in the saturated fatty acids content when the pH was increased from 7.0 to 7.5 which then decreased when the pH was increased from 7.5 to 8.0. Similar results were observed by Bodnaruk and Golden (1996) where the saturated fatty acids content increased at lower pH values and then decreased as pH was increased. Nikkila et al. (1995) also reported an increase in saturated fatty acid at low pHs which then decreased at higher pH values. Beal et al. (2001) reported that a decrease in the pH lowered the palmitic acid.

The results showed that the saturated fatty acids content increased as the enzyme load increased from 0.5 to 1.0% and then decreased with further increases in enzyme load (from 1.0 to 2.0%) at the low temperature. However, there was a decrease in the saturated fatty acids content with initial increases in the enzyme load (from 0.5 to 1.0%) followed by increases with a further increase of enzyme load from 1.0 to 2.0% at the higher temperature. Kempka (2011) stated that a higher enzyme concentration does not lead to increased fatty acid content. Pawar and Tidwell (1968) reported that initial increases in the enzyme concentration increased the saturated fatty acid content. Hari Krishna et al. (2001) reported that increases in the enzyme beyond optimum level reduced the amount of fatty acids.

In this study, the highest saturated fatty acid obtained was palmitic acid. Swapna et al. (2011) evaluated the composition of fatty acids in fish oil from fish viscera waste of catla and rohu and found palmitic acid (16:0) to be the major fatty acid among all the saturated fatty acids. Sathivel et al. (2002) reported on the fatty acid (FA) composition of catfish viscera in which palmitic acid had the highest amount. Wu and Bechtel (2008) obtained saturated fatty acids from salmon by-product in which palmitic acid had the highest concentration (13.9-14.8). Mozaffarian et al. (2010) reported that it is recommended to reduced saturated fat (SFA) consumption in order to reduce coronary heart disease (CHD).

6.3.2. Monounsaturated fatty acids

Monounsaturated fatty acids (MUFA) are fatty acids which possess only one double bond (EFSA, 2010). In this study, the total monounsaturated fatty acids percentage was 39-40%. The monounsaturated fatty acids with values above 1% were tabulated which included palmitoleic acid (16:1n-7) in the range of 7.41-8.86%, oleic acid (18:1n-9) in the range of 22.28-14.18% and vaccenic acid (18:1n-7) in the range of 3.53-4.23%. The effects of temperature, pH and enzyme load on the monounsaturated fatty acids were investigated.

In this study, half of the samples showed increased MUFA as the temperature increased from 55 to 70°C whereas the other half of the samples showed decreased MUFA content as temperature increased from 55 to 70°C, especially oleic acid. Farkas and Herodek (1964) reported that an increase in temperature decreased the amount of unsaturated fatty acids. Olson and Ingram (1975) also reported that monounsaturated fatty acids decreased with increases in temperature. Fulco (1974) stated that in variety samples, environmental conditions especially temperatures have altered the fatty acid composition. Lanna et al. (2005) reported that the oleic acid content decreased at low temperature.

The results obtained from this study showed that the monounsaturated fatty acid content increased with increases in the pH (from 7.0 to 8.0) with lower temperature whereas with the higher temperature the monounsaturated fatty acids decreased with increases in the pH (from 7.0 to 8.0). Rosa et al. (1974) stated that temperature and pH are interdependent and it is difficult to explain the effect of variation of pH. They reported that lower pH gave rise to unsaturated fatty acid content. Bodnaruk and Golden (1996) reported that as the pH increased, the unsaturated fatty acids content decreased. Nikkila et al. (1995) reported that vaccenic acid was much higher at higer pH.

It is difficult to explain the effect of enzyme load/concentration on MUFA. Most of the monounsaturated fatty acids decreased with increased enzyme load at the lower temperature. However, at the higher temperature, few monounsaturated fatty acids

increased with increased enzyme load whereas few other monounsaturated fatty acids decreased when the enzyme load increased from 0.5 to 1.0% and then increased when the enzyme load increased from 1.0 to 2.0%. Chopra et al. (2011) reported that increases in enzyme concentration decreased the monounsaturated fatty acids and the highest yield for MUFA was obtained with enzyme concentration of 1.0%. Brenes et al. (2008) and Bharadwaj (2011) reported that monounsaturated fatty acids especially oleic acid decreased with increased in enzyme concentration.

Swapna et al. (2010) reported that mixed visceral waste oil of catla, rohu, mrigal (Indian oily freshwater fish of the family Cyprinidae) and tilapia was a good source of unsaturated fatty acids. Swapna et al. (2011) reported that among the unsaturated fatty acids in fish viscera waste from catla and rohu, oleic acid was the dominant. Sathivel et al. (2002) observed higher oleic acid among monounsaturated fatty acid in catfish viscera. Pontes-Arruda (2009) reported that oleic acid was the major content in monounsaturated fatty acids which has an importance in cell and heart function, anti-inflammatory effects in the body, treat arthritis, asthma, allergies and skin conditions. EFSA (2010) reported that consuming diets comprising of *trans*-monounsaturated fatty acids results in reduced blood HDL cholesterol concentrations and increases the total cholesterol to HDL cholesterol ratio. However, MUFA can be synthesized by human body and hence there is no need for MUFA as such in the diet.

6.3.3. Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) possess two or more double bonds (EFSA, 2010). n-3 PUFA are polyunsaturated fatty acids with one of the double bonds located at the third carbon atoms from the methyl end. The important n-3 PUFA are: alpha-linolenic acid, eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid (Kinsella et al., 1990). In this study, it was observed that the total polyunsaturated fatty acids percentage was 32-35%. The polyunsaturated fatty acids with values above 1% were tabulated which included linoleic acid (18:2 n-6), alpha-linolenic acid (18:3 n-3), arachidonic (20:4n-6), eicosapentaenoic acid (20:5 n-3), docosapentaenoic acid (22:5 n-3) and docosahexaenoic acid (22:6 n-3) in the ranges of 1.41-1.67, 0.53-0.80, 0.38-0.51,

4.90-6.49, 0.52-0.74 and 5.56-7.69%, respectively. Also, it was observed that increases in PUFA decreased saturated and monounsaturated fatty acids. The effects of temperature, pH and enzyme load on the polyunsaturated fatty acids were investigated.

The results showed that when the temperature was increased from 55 to 70°C, the polyunsaturated fatty acids content increased. Similar results were observed by Olson and Ingram (1975) who reported increases in the polyunsaturated fatty acids content with increased temperature. Canvin (1965) and Lanna et al. (2005) reported that at low temperature, linoleic and linolenic acid contents increased whereas at higher temperature, linoleic and linolenic acid contents decreased. The increased PUFA content was due to the reduced amount of saturated fatty acids that were produced.

In this study, the polyunsaturated fatty acids content increased with increases in the pH (from 7.0 to 8.0) at the lower temperature whereas the polyunsaturated fatty acids content increased with initial increases in the pH (from 7.0 to 7.5) and then decreased as the pH was increased from 7.5 to 8.0 at the higher temperature. AbuGhazaleh and Jacobson (2007) and Sackman et al. (2003) reported that increases in the pH increased the polyunsaturated fatty acids content. Sang et al. (2012) reported that the pH had greater effect on EPA and the highest amounts of PUFA were observed at medium pH.

The results showed that most of the polyunsaturated fatty acids decreased as enzyme load increased from 0.5 to 2.0% at the lower temperature whereas some polyunsaturated fatty acids decreased when the enzyme load was increased from 0.5 to 1.0% and then increased when the enzyme load was further increased from 1.0 to 2.0% at the higher temperature. Chopra et al. (2011) stated that an increase in value of enzyme concentration will decrease PUFA. Lucas (2000) stated that an increase in enzyme concentration will increase the rate of reaction at which lipids are hydrolysed to fatty acids and glycerol. Hamam et al. (2005) reported that increasing the enzyme concentration from 2 to 4% increased the EPA content gradually, reaching its maximum at 4%.

Swapna et al. (2011) reported that among the polyunsaturated fatty acids from fish viscera waste from catla and rohu, EPA and DHA were 1.5% and 0.8%, respectively. Aidos et al. (2002) reported that high value of polyunsaturated fatty acid was obtained from herring by-product in which EPA and DHA ranged from 57 to 73 g/kg. Wu and Bechtel (2008) stated that abundant PUFAs of crude oil of salmon by-product include the long-chain omega-3 EPA with 9.3-11.3%, docosapentaenoic acid with 2.6% and DHA (the most abundant omega-3 PUFA in the salmon by-product) with 12.3-13.1%. EFSA (2010) reported that arachidonic acid can be synthesised by the body from linoleic acid and is, therefore, not an essential fatty acid. Also human body can synthesise eicosapentaenoic acid **(**EPA) and docosahexaenoic acid (DHA) from alpha-linolenic acid. However, it was suggested that the use of oily fish or dietary n-3 long-chain polyunsaturated fatty acids supplements decreases the risk of coronary heart disease (CHD) and sudden cardiac death (Kinsella et al., 1990).

6.4. Enzymatic Extraction vs Chemical Extraction

In this study, enzymatic and chemical extractions were compared. The chemical extraction method gave much higher oil recovery yield (97.16%) than the enzymatic extraction method (20.2%). The Bligh and Dyer method was carried out under mild conditions (neither heat nor high pressures was applied) which makes it a better method as it did not cause any alterations of the extracted oil (Aryee and Simpson, 2009). The Bligh and Dyer is a commonly used method for comparison with other methods as it gives maximum attainable oil yield. In this study, the Bligh and Dyer method was used in order to compare the yield and quality of crude fish oil from the same material. Oliveira (2009) obtained commercial Pollock oil and observed the POV, FFA to be 3.5 meq/Kg and 7.8%, respectively.

According to EFSA (2010), quality analysis is carried out in two groups: firstly to check the fundamental parameters which includes the moisture, impurities, appearance (namely colour), FFA, soap and iodine value (IV). And secondly, to determine refining procedures which usually focuses on the evaluation of peroxide value and *p*-anisidine value. The peroxide and *p*-anisidine value are the most reliable chemical methods for

rancidity measurements of fish oils. Miller (2010) observed that there was decrease in peroxide value as secondary oxidation product appeared. The refined oil had an acid value of <1.0 %, peroxide value of <2.5 meq O2/kg and *p*-anisidine value of <20 (FDA GRAS, 2002).

In this study, the quality parameters (peroxide, acid and *p*-anisidine value) showed that the chemical extraction was better than enzymatic extraction. The obtained peroxide, acid and *p*-anisidine value were 12.04 Meq/g, 5.11 mg KOH/g and 9.33, for chemical extraction and 31.44 Meq/g, 11.13 mg KOH/g and 58.27 for enzymatic extraction, respectively. The values were higher than the recommended values (3-20 Meq/g, <5.0 mg KOH/g, 4-60 for peroxide, acid and *p*-anisidine values, respectively) showing that the oil was oxidized and apperared to be dark in color. The dark color may be due to the presence of products resulting from hemoglobin degradation as reported by Batista (2009). The differences in the quality could be due to agitation/stirring and initial heating of sample (above 90°C) in enzymatic extraction. In the solvent extraction procedure, the samples were not subjected to high temperature. Riaz and Shaheen (1994) reported that agitation is a stimulatory factor in the formation of lipid peroxides. Tian et al. (2010) reported that stirring speed can enhance the oxidation rate. Slizyte et al. (2005) reported that initial heating of raw material had a significant effect on yield and quality of oil. Mishra and Singhai (1992) reported that subjecting lipids to high temperature was found to induce lipid peroxidation.

In present study, the conditions used in enzymatic method which gave higher yield did not give better quality. The enzymatic oil obtained from all the experimental conditions was oxidized. According to Badr and Sitohy (1992), the cell wall degradation increases with increases in the amount of enzyme, leading to the extraction of higher amounts of oil. It may also be due to the higher quantity of oxidized fish oil producing more oxidation products. For commercial purposes, the fish oil with nutraceutical properties should be of a good quality and have low peroxide value, free fatty acids and *p*-anisidine value. Refining the crude oil would help in removing the impurities and reduce the oxidation product, however it would add to the cost of production.

In this study, it was observed that the amount of PUFA obtained by enzymatic method was higher than the chemical extraction method. This is probably due to the use of lipase-catalyzed enzymatic hydrolysis reactions, which are more productive, compared to other concentration methods: esterification and interesterification (Xuebing, 2000). However, in this process, the utilization of lipases was based on the fatty acids-specific selectivity. According to Wanasundara and Shahidi (1998) the lipases exhibit toward SFAs and MUFAs, leaving n-3 PUFAs intact on the glyceride moiety. Also in the present study, the enzymatic method gave very low total oil yield (in g) but produced a very high omega-3 percentage. The problem of low yield could be overcome by using a conventional method for extracting fish oil to obtain higher oil yield and followed by enzymatic hydrolysis for concentration of omega-3 fatty acids. The FAs data were generated by using a blank sample - another way to express FAs data by GC is by using an internal standard as a reference. An internal standard is a compound added to the sample in a known amount and helps to increase the accuracy and precision of analytical methods that have large inherent variability.

Gunnlaugsdottir and Ackman (1993) extracted lipid from menhaden using Bligh and Dyer method (Bligh and Dyer, 1959) and a method proposed by Hara and Radin (1978) using hexane/isopropanol and found Bligh and Dyer to be better method in extraction of lipid. Gbogouri et al. (2004) stated that the difference of the extraction rates between enzymatic hydrolysis and the Bligh and Dyer method did not exceed 2% for the triacylglycerides (TAG) content. Chantachum et al. (2000) reported that among the methods used for extracting lipids, Bligh and Dyer allows the best recovery of lipids.

According to Christie (1992), solvents or solvent mixture used for extraction should be sufficiently polar to remove lipids from cell constituents, but not too polar so that the solvents do not readily dissolve all the TAGs and other non-polar lipids. Norziah et al. (2009) stated that penetration of polar solvents into the cells is necessary to extract the lipid from the cell membrane and muscle fibres that includes the phospholipids materials. In this study, chloroform:methanol solvent system was used. Bligh and Dyer (1959) stated that the major advantage of Bligh and Dyer method is the reduction of

solvent:sample ratio. However, Zuta (2003) reported that there is risk of toxicity related with exposure of chloroform and methanol and should be replaced with less toxic solvent system.

6.5. Concentration of Omega-3 Fatty Acids

In this study, the optimum conditions for extractions were a temperature of 35°C, a hydrolysis time of 20 h and an enzyme concentration of 800 Units which as recommended by Wanasundara and Shahidi (1998). The fish oil used for enrichment of omega-3 FAs was chemically extracted oil (Bligh and Dyer method). Optimization was carried out with four different stirring levels: constant fast stirring (200 rpm), constant slow stirring (50 rpm), intermediate stirring (100 rpm with 4 h interval) and no stirring. The constant fast stirring gave the best yield of 17.6%. The lipase *Candida rugosa* helped in the enrichment of DHA compared to EPA. Tanaka et al. (1992) stated that high speed shaking will help mix oil and enzymatic solution well which in turn increases the reaction rate. Ingesson et al. (2001) reported that in enzymatic hydrolysis, the effect of shaking speed is minimum compared to other factors (enzyme concentration, hydrolysis time).

Alonso et al. (2005) reported that the optimum stirring speed to be 200 rpm. Kahveci and Xu (2011) studied enzymatic hydrolysis of salmon oil using candida rugosa with stirring speed of 300 rpm and found that the total omega-3 PUFA was 38.7%. Wanasundara and Shahidi (1998) used different enzymes (*Aspergillus niger, Candida cylindracea (CC), Chromobacterium viscosum, Geotrichum candidum, Mucor miehei, Pseudomonas sp., Rhizopus oryzae, and Rhizopus niveus*) with stirring speed of 200 rpm and found *Candida cylindracea* lipase to be the most active in increasing the contents of total n-3 fatty acids SBO by 43% and MHO by 44%. Carvalho et al. (2002) reported on the enzymatic hydrolysis of sardine oil with stirring speed of 200 rpm using four microbial lipases (*Candida cylindracea, Rhizopus delemar, Aspergillus oryzae, Chromobacterium viscosum*) among which *Candida cylindracea* was the most effective for the production of oil with high concentration of DHA. It was however, less effective in the enrichment of EPA of unhydrolysed acylglycerol.

The effect of lipase in concentrating EPA and DHA varies for different oils. For example lipase from *A. niger* increased DHA concentration in cod liver oil (Hoshino et al., 1990) whereas similar lipase did not show increase of DHA in menhaden oil (Wanasundara and Shahidi, 1998) or tuna oil (Tanaka et al., 1992). *Candida rugosa* increased the EPA+DHA concentration in salmon viscera oil in the first 12h and then decreased (Sun et al., 2002).

Sirin et al. (2005) studied the enrichment of DHA using *Candida rugosa* from cod liver oil and observed that the optimum condition (a pH of 5.0, a temperature of 35°C and a hydrolysis time of 12 h with 2000 U/g enzyme concentration) gave a yield of 17.6% whereas 1000 U/g enzyme concentration with a pH of 5.0, a temperature of 35°C and a hydrolysis time of 24 h resulted in 1.45 fold increase in the amount of DHA in cod liver oil. Sun et al. (2002) reported that the highest concentration of EPA was obtained at a temperature of 50°C and the highest DHA concentration was obtained at a temperature of 35°C. Mbatia et al. (2010) stated that temperature change did not significantly affect substrate specificity for *Candida rugosa*.

In this study, the fish oil obtained from chemical extraction method had initial EPA and DHA content as 4.46 and 5.92%, respectively. However, after enzymatic hydrolysis of fish oil by *Candida rugosa,* the EPA and DHA content were increased to 6.49 and 11.17%, respectively. Kahveci et al. (2010) reported that the initial EPA and DHA content of salmon oil to be 4.8 and 6.93%. However, after hydrolysis of salmon oil by lipase *Candida rugosa* the amount of enrichment of EPA and DHA increased to 5.9 and 19.39%. Wanasundara and Shahidi (1998) observed that the initial DHA content of menhaden oil to be 10.06% and after the hydrolysis by *Candida rugosa* the DHA content increased to 17.3%. In present study, the amount of omega-3 enriched oil obtained was too small to carry out the quality analysis (FFA, POV, pAV).

CHAPTER 7. CONCLUSIONS

The effects of pH (7.0, 7.5 and 8.0), enzyme load (0.5, 1.0 and 2.0%) and temperature (55 and 70°C) on the enzymatic hydrolysis of fish oil were studied using the enzyme alcalase. A comparison between solvent extraction and enzymatic extraction for fish oil was carried out. The effect of stirring level (constant fast stirring, constant slow stirring, intermediate stirring and no stirring) on the concentration of omega-3 fatty acids from herring fish was studied. The following are the conclusions drawn from the study.

1. The temperature, pH and enzyme load had significant effects on the oil recovery yield at the 0.001 level. There appeared to be significant interaction between the three parameters.

   (a) Increasing the temperature from 55°C to 70°C decreased the oil recovery yield.
   (b) For most of the combinations the oil recovery yield increased when the pH was increased from 7.0 to 7.5 and then decreased with further increased in pH to 8.0.
   (c) Increasing the enzyme load from 0.5% to 2.0% showed increase in the oil recovery yield.
   (d) The highest oil recovery yield was achieved at a temperature of 55°C, a pH of 7.5 and an enzyme load of 2.0%

2. The temperature, pH and enzyme load had significant effects on the peroxide value at the 0.001 level. Also, the two way and three way interactions among these parameters were highly significant.

   (a) Increasing the temperature from 55°C to 70°C increased the peroxide value.
   (b) Most of the peroxide values first slightly decreased when the pH was increased from 7.0 to pH 7.5 and then increased when the pH was further increased to pH 8.0.
   (c) The peroxide values of some samples increased slightly when the enzyme load was increased from 0.5% to 2.0%. Other samples showed an initial decrease in the

peroxide value when the enzyme load was increased from 0.5% to 1.0% followed by an increase with further increases in the enzyme load up to 2.0%.

(d) The lowest peroxide value was achieved at a temperature of 55°C, a pH of 7.0 and an enzyme load of 0.5%.

(e) The proxide value obtained in this study was above the recommended range (3-20 Meq/kg) due to lipid autoxidation in the initial propagation stage which led to increases in peroxide formation.

3. The temperature, pH and enzyme load had significant effects on the acid value at the 0.001 level. All the interactions among these factors were also highly significant.

(a) The acid values increased when the temperature was increased from 55°C to 70°C.

(b) The acid value increased when the pH was increased from 7.0 to 8.0 with the higher temperature. However, with the lower temperature, there was an increase in acid value when the pH was increased from 7.0 to 7.5 which then decreased when the pH was further increased to 8.0.

(c) Increasing the enzyme load from 0.5% to 2.0% increased in acid value.

(d) The lowest acid value was achieved at a temperature of 55°C, a pH of 7.0 and an enzyme load of 0.5%.

(e) The acid value obtained in this study was higher than the recommended range (<5 mg KOH/g) due to lipid oxidation as triacylglyceride (TAG) were converted to fatty acid (FA) and glycerol which increased the acid number.

4. The temperature, pH and enzyme load had significant effects on the *p*-anisidine value at the 0.001 level. The interactions among these factors were also highly significant.

(a) Increasing the temperature from 55°C to 70°C slightly increased the *p*-anisidine value.

(b) For most of the combinations, increasing the pH (from 7.0 to 8.0) resulted in a slight increase in the *p*-anisidine value.

(c) For most of the combinations, increasing the enzyme load (from 0.5% to 2.0%) resulted in a slight increase in the *p*-anisidine value.

(d) The lowest *p*-anisidine value was achieved at a temperature of 55°C, a pH of 7.0 and an enzyme load of 0.5%.

(e) The p-anisidine value obtained in this study at the optimum condition was within the recommended range (4-60).

5. The temperature had significant effect on the saturated fatty acids at the 0.001 level whereas the effects of pH and enzyme load were insignificant. There were no significant interactions among the parameters.

(a) The highest saturated fatty acids were achieved at a temperature of 70°C, a pH of 7.5 and an enzyme load of 2.0%.

(b) The fatty acid with highest concentration was palmitic acid (17.03%) followed by myristic acid (7.65%) and stearic acid (1.91%).

6. The effects of temperature, pH and enzyme load on the monounsaturated fatty acids (MUFA) were insignificant at the 0.001 level. The interactions among these parameters were also insignificant.

(a) The highest monounsaturated fatty acids were achieved at a temperature of 55°C, a pH of 8.0 and an enzyme load of 2.0%.

(b) The fatty acid with highest concentration was oleic acid (23.82%) followed by palmitoleic acid (8.86%) and vaccenic acid (4.45%).

7. The effect of temperature on most of the polyunsaturated fatty acids (PUFA) was insignificant at the 0.001 level. The pH and enzyme load had no significant effects on the PUFA. There were no interactions among these parameters.

(a) The highest PUFA were achieved at a temperature of 70°C, a pH of 7.0 and an enzyme load of 2.0%.

(b) The fatty acid with highest concentration was docosahexaenoic acid (8.39%) followed by eicosapentaenoic acid (7.02%), linoleic acid (1.67%), alpha-linolenic acid (0.80%), docosapentaenoic acid (0.74%) and arachidonic (0.51%).

8. The chemical extraction method was superior to the enzymatic extraction method for fish oil.
   (a) The chemical extraction method gave higher oil recovery yield than that of the enzymatic extraction method.
   (b) The chemical extraction method gave lower peroxide value (12.04 Meq/g) than that of the enzymatic extraction method (31.44 Meq/g).
   (c) The chemical extraction method gave lower acid value (5.11 mg KOH/g) than that of the enzymatic extraction method (11.13 mg KOH/g).
   (d) The chemical extraction method gave lower p-anisidine value (9.33) than that of the enzymatic extraction method (58.27).
   (e) The difference in oil quality between the two methods was due to initial heating of raw material in enzymatic hydrolysis which had a significant effect on yield and quality.

9. Stirring had a significant effect on EPA and DHA.
   (a) There was an increase in the amount of fatty acids composition with increase in the stirring speed.
   (b) The concentration of omega-3 FAs by *Candida rugosa* showed enrichment of EPA from 4.46% to 6.46%, DPA from 0.44% to 0.82% and DHA from 5.92% to 11.17%. Also there was an enrichment of omega-6 and omega-7 FAs.
   (c) The highest EPA, DPA and DHA were achieved with constant fast stirring (200 rpm).
   (d) The higher FAs composition is due to mixing allow good contact between enzyme and substrate and speeds up the enzymatic reaction rate.

7.1. Future Work

1. Fish processing waste should be used for extracting fish oil and omega-3 fatty acids to enhance the economics of fish processing industry and to minimize problems associated with disposal of fish waste.

2. Extraction of fish oil using solvent system can be used but choloroform:methanol should be replaced with hexane:isopropanol and the optimum level of solvent ratio should be determined.

3. Higher centrifugal speed should be used to separate the oil after enzymatic hydrolysis and the optimum centrifugal force should be determined.

4. Concentration of omega-3 fatty acid should be carried out with immobilized enzymes and the efficiency of the immobilized process and optimum extraction conditions should be investigated.

5. The discarded lower phase in the extraction of omega-3 fatty acid should be investigated for fatty acid content.

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APPENDIX A: Enzymatic Hydrolysis Data

Table A1. Recovery of fish oil extracted using enzymatic hydrolysis at 55°C.

pH	Replicates	Enzyme load (%)	Oil recovered (g)
7.0	1	0.5	0.97
	2		0.91
	3		1.02
	Average		0.96
	1	1.0	1.12
	2		1.15
	3		1.08
	Average		1.11
	1	2.0	1.45
	2		1.39
	3		1.48
	Average		1.44
7.5	1	0.5	1.13
	2		1.21
	3		1.24
	Average		1.19
	1	1.0	1.65
	2		1.59
	3		1.56
	Average		1.60
	1	2.0	1.80
	2		1.74
	3		1.81
	Average		1.78
8.0	1	0.5	0.98
	2		1.02
	3		1.01
	Average		1.003
	1	1.0	1.38
	2		1.30
	3		1.32
	Average		1.33
	1	2.0	1.43
	2		1.46
	3		1.51
	Average		1.46

Table A2. Recovery of fish oil extracted using enzymatic hydrolysis at 70°C.

pH	Replicates	Enzyme load (%)	Oil recovered (g)
7.0	1	0.5	0.42
	2		0.37
	3		0.43
	Average		0.40
	1	1.0	0.55
	2		0.62
	3		0.59
	Average		0.58
	1	2.0	0.69
	2		0.68
	3		0.66
	Average		0.67
7.5	1	0.5	0.72
	2		0.61
	3		0.60
	Average		0.64
	1	1.0	0.76
	2		0.82
	3		0.85
	Average		0.81
	1	2.0	1.41
	2		1.45
	3		1.30
	Average		1.38
8.0	1	0.5	0.48
	2		0.50
	3		0.57
	Average		0.51
	1	1.0	0.83
	2		0.85
	3		0.80
	Average		0.82
	1	2.0	0.98
	2		0.90
	3		0.88
	Average		0.92

Table A3. Chemical analysis of fish oil obtained using enzymatic hydrolysis at 55°C.

pH	Replicates	Enzyme load (%)	Peroxide value (Meq/g)	Acid value (mg KOH/g)	*p*-Anisidine value
7.0	1	0.5	26.98	7.33	56.21
	2		26.23	8.05	57.22
	3		27.14	7.92	57.37
	Average		26.78	7.76	56.93
	1	1.0	27.75	9.26	60.65
	2		28.07	9.04	59.49
	3		28.62	9.51	59.76
	Average		28.14	9.27	59.96
	1	2.0	32.33	9.89	63.51
	2		31.71	9.95	64.44
	3		32.56	10.20	64.08
	Average		32.20	10.01	64.01
7.5	1	0.5	36.63	8.45	58.48
	2		35.80	8.73	59.32
	3		35.95	8.01	58.75
	Average		36.12	8.39	58.85
	1	1.0	28.02	10.18	58.96
	2		27.53	10.11	59.53
	3		28.88	10.06	59.61
	Average		28.14	10.11	59.36
	1	2.0	31.11	11.39	58.40
	2		31.78	11.42	58.05
	3		31.44	10.60	58.38
	Average		31.44	11.13	58.27
8.0	1	0.5	37.16	7.72	64.26
	2		38.05	8.85	64.74
	3		37.53	8.26	63.90
	Average		37.58	8.27	64.30
	1	1.0	35.24	8.90	59.81
	2		35.77	7.67	60.49
	3		35.12	8.73	59.77
	Average		35.37	8.43	60.02
	1	2.0	39.01	8.10	62.10
	2		39.00	9.17	62.53
	3		38.87	8.66	62.36
	Average		38.96	8.64	62.33

Table A4. Chemical analysis of fish oil obtained using enzymatic hydrolysis at 70°C.

pH	Replicates	Enzyme load (%)	Peroxide value (Meq/g)	Acid value (mg KOH/g)	*p*-anisidine value
7.0	1	0.5	37.25	9.21	61.36
	2		37.31	8.64	61.68
	3		36.92	9.83	60.24
	Average		37.16	9.22	61.09
	1	1.0	38.76	12.34	64.30
	2		39.04	12.70	64.51
	3		39.60	12.56	64.36
	Average		39.13	12.53	64.39
	1	2.0	40.48	15.75	65.93
	2		40.91	15.22	66.52
	3		40.72	14.89	65.99
	Average		40.70	15.28	66.14
7.5	1	0.5	36.55	13.11	62.76
	2		37.26	13.04	62.04
	3		36.19	12.62	62.09
	Average		36.66	12.92	62.29
	1	1.0	38.80	15.66	64.94
	2		38.33	14.00	65.21
	3		38.57	14.53	65.47
	Average		38.56	14.73	65.20
	1	2.0	39.45	17.59	66.72
	2		38.22	16.65	67.58
	3		39.18	17.28	67.30
	Average		38.95	17.17	67.20
8.0	1	0.5	40.27	16.02	68.44
	2		40.99	16.41	68.06
	3		41.20	16.35	69.71
	Average		40.82	16.26	68.73
	1	1.0	37.88	18.38	65.68
	2		37.31	17.71	64.97
	3		36.79	18.07	65.11
	Average		37.32	18.05	65.25
	1	2.0	41.50	19.50	69.61
	2		42.14	20.05	70.04
	3		41.75	19.89	69.84
	Average		41.79	19.81	69.83

Table A5. Fatty acid composition of fish oil obtained using enzymatic hydrolysis at 55°C and pH 7.0.

R	EL (%)	Saturated fatty acid (wt %)			Monounsaturated fatty acid (wt %)			Polyunsaturated fatty acid (wt %)					
		Myristic acid (14:0)	Palmitic acid (16:0)	Stearic acid (18:0)	Palmitoleic acid (16:1n-7)	Oleic acid (18:1n-9)	Vaccenic acid (18:1n-7)	Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)	Arachidonic (20:4n-6)	EPA (20:5n-3)	DPA (22:5n-3)	DHA (22:6n-3)
1	0.5	5.93	14.43	1.64	7.20	21.56	3.43	1.37	0.50	0.37	4.76	0.50	5.40
2	0.5	6.29	15.27	1.73	7.62	22.99	3.63	1.45	0.55	0.39	5.03	0.54	5.71
Avg		6.11	14.85	1.69	7.41	22.28	3.53	1.41	0.53	0.38	4.90	0.52	5.56
1	1.0	6.95	16.47	0.10	8.29	17.99	4.00	1.65	0.62	0.49	6.87	0.70	8.13
2	1.0	5.64	13.36	1.61	6.73	19.56	3.25	1.33	0.47	0.39	5.60	0.58	6.76
Avg		6.30	14.92	0.86	7.51	18.77	3.62	1.49	0.54	0.44	6.24	0.64	7.45
1	2.0	5.39	12.83	1.60	6.86	19.92	3.28	1.36	0.51	0.41	5.82	0.60	7.19
2	2.0	5.39	12.87	1.57	6.88	20.03	3.28	1.35	0.49	0.43	5.87	0.59	7.20
Avg		5.39	12.85	1.58	6.87	19.98	3.28	1.36	0.50	0.42	5.84	0.59	7.20

R = Replicate

EL= Enzyme load

Table A6. Fatty acid composition of fish oil obtained using enzymatic hydrolysis at 55°C and pH 7.5.

R	EL (%)	Saturated fatty acid (wt %)			Monounsaturated fatty acid (wt %)			Polyunsaturated fatty acid (wt %)					
		Myristic acid (14:0)	Palmitic acid (16:0)	Stearic acid (18:0)	Palmitoleic acid (16:1n-7)	Oleic acid (18:1n-9)	Vaccenic acid (18:1n-7)	Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)	Arachidonic (20:4n-6)	EPA (20:5n-3)	DPA (22:5n-3)	DHA (22:6n-3)
1	0.5	6.58	15.13	1.70	7.76	22.35	3.65	1.54	0.59	0.43	6.00	0.72	6.89
2	0.5	6.62	15.11	1.67	7.75	23.07	3.63	1.54	0.60	0.42	5.93	0.69	6.80
Avg		6.60	15.12	1.68	7.76	22.71	3.64	1.54	0.60	0.42	5.97	0.71	6.84
1	1.0	6.66	15.14	0.04	8.00	23.16	3.76	1.57	0.57	0.43	6.17	0.72	7.03
2	1.0	5.77	13.09	1.45	6.92	20.05	3.25	1.36	0.56	0.36	5.33	0.56	6.17
Avg		6.22	14.11	0.74	7.46	21.61	3.51	1.46	0.57	0.39	5.75	0.64	6.60
1	2.0	5.74	12.94	1.42	6.80	19.58	3.21	1.36	0.41	0.37	5.43	0.58	6.32
2	2.0	5.89	13.27	0.03	6.97	20.09	3.26	1.40	0.30	0.38	5.56	0.58	6.51
Avg		5.81	13.10	0.73	6.88	19.84	3.23	1.38	0.36	0.38	5.50	0.58	6.41

R = Replicate

EL= Enzyme load

Table A7. Fatty acid composition of fish oil obtained using enzymatic hydrolysis at 55°C and pH 8.0.

R	EL (%)	Saturated fatty acid (wt %)			Monounsaturated fatty acid (wt %)			Polyunsaturated fatty acid (wt %)					
		Myristic acid (14:0)	Palmitic acid (16:0)	Stearic acid (18:0)	Palmitoleic acid (16:1n-7)	Oleic acid (18:1n-9)	Vaccenic acid (18:1n-7)	Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)	Arachidonic (20:4n-6)	EPA (20:5n-3)	DPA (22:5n-3)	DHA (22:6n-3)
1	0.5	6.39	15.31	0.05	7.99	23.96	3.84	1.57	0.49	0.43	6.17	0.52	7.17
2	0.5	6.19	14.94	1.67	7.79	23.47	3.78	1.52	0.62	0.45	6.14	0.63	7.04
Avg		6.29	15.12	0.86	7.89	23.72	3.81	1.55	0.56	0.44	6.15	057	7.11
1	1.0	6.22	15.13	1.70	7.83	23.67	3.83	1.52	0.57	0.43	6.11	0.63	7.12
2	1.0	6.19	15.12	1.69	7.85	23.60	3.81	1.51	0.62	0.44	6.06	0.63	7.10
Avg		6.20	15.13	1.69	7.84	23.64	3.82	1.51	0.60	0.43	6.08	0.63	7.11
1	2.0	6.36	15.55	1.65	7.94	23.91	3.80	1.51	0.55	0.41	6.12	0.63	7.10
2	2.0	6.25	15.29	1.70	7.81	23.73	3.82	1.51	0.62	0.42	6.11	0.62	7.18
Avg		6.30	15.42	1.67	7.88	23.82	3.81	1.51	0.59	0.42	6.11	0.63	7.14

R = Replicate

EL= Enzyme load

Table A8. Fatty acid composition of fish oil obtained using enzymatic hydrolysis at 70°C and pH 7.0.

R	EL (%)	Saturated fatty acid (wt %)			Monounsaturated fatty acid (wt %)			Polyunsaturated fatty acid (wt %)					
		Myristic acid (14:0)	Palmitic acid (16:0)	Stearic acid (18:0)	Palmitoleic acid (16:1n-7)	Oleic acid (18:1n-9)	Vaccenic acid (18:1n-7)	Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)	Arachidonic (20:4n-6)	EPA (20:5n-3)	DPA (22:5n-3)	DHA (22:6n-3)
1	0.5	5.56	13.26	1.50	7.10	20.67	3.57	1.35	0.53	0.42	5.37	0.52	6.76
2	0.5	7.00	16.70	1.95	8.94	20.71	4.51	1.72	0.71	0.52	6.78	0.66	8.57
Avg		6.28	14.98	1.73	8.02	20.69	4.04	1.53	0.62	0.47	6.07	0.59	7.66
1	1.0	5.62	13.27	1.51	7.05	21.40	3.42	1.36	0.57	0.39	5.39	0.55	6.47
2	1.0	5.75	13.58	1.55	7.21	21.91	3.51	1.40	0.59	0.40	5.52	0.56	6.59
Avg		5.68	13.42	1.53	7.13	21.65	3.47	1.38	0.58	0.39	5.46	0.56	6.53
1	2.0	6.91	16.22	1.85	9.06	21.75	4.44	1.76	0.73	0.51	7.02	0.72	8.38
2	2.0	6.91	16.19	1.85	9.07	21.63	4.47	1.76	0.73	0.52	7.02	0.71	8.39
Avg		6.91	16.21	1.85	9.06	21.69	4.45	1.76	0.73	0.51	7.02	0.72	8.39

R = Replicate

EL= Enzyme load

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Table A9. Fatty acid composition of fish oil obtained using enzymatic hydrolysis at 70°C and pH 7.5.

R	EL (%)	Saturated fatty acid (wt %)			Monounsaturated fatty acid (wt %)			Polyunsaturated fatty acid (wt %)					
		Myristic acid (14:0)	Palmitic acid (16:0)	Stearic acid (18:0)	Palmitoleic acid (16:1n-7)	Oleic acid (18:1n-9)	Vaccenic acid (18:1n-7)	Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)	Arachidonic (20:4n-6)	EPA (20:5n-3)	DPA (22:5n-3)	DHA (22:6n-3)
1	0.5	6.88	14.53	1.62	7.47	21.35	3.47	1.48	0.71	0.45	5.96	0.67	6.94
2	0.5	7.90	16.72	1.85	8.52	19.22	3.99	1.68	0.80	0.47	6.86	0.75	7.96
Avg		7.39	15.63	1.74	8.00	20.30	3.73	1.58	0.75	0.46	6.41	0.71	7.45
1	1.0	6.84	14.21	1.54	7.39	21.10	3.34	1.47	0.71	0.43	6.15	0.66	7.17
2	1.0	7.48	15.51	1.70	7.94	23.14	3.66	1.61	0.78	0.47	6.80	0.76	7.93
Avg		7.16	14.87	1.62	7.67	22.13	3.50	1.54	0.75	0.45	6.48	0.71	7.55
1	2.0	7.78	17.41	1.95	6.98	19.63	4.12	1.67	0.78	0.48	6.71	0.72	6.91
2	2.0	7.44	16.63	1.86	5.83	20.27	3.98	1.59	0.75	0.47	6.41	0.72	5.15
Avg		7.61	17.03	1.91	6.41	19.96	4.05	1.63	0.77	0.47	6.57	0.72	6.03

R = Replicate

EL= Enzyme load

Table A10. Fatty acid composition of fish oil obtained using enzymatic hydrolysis at 70°C and pH 8.0.

R	EL (%)	Saturated fatty acid (wt %)			Monounsaturated fatty acid (wt %)			Polyunsaturated fatty acid (wt %)					
		Myristic acid (14:0)	Palmitic acid (16:0)	Stearic acid (18:0)	Palmitoleic acid (16:1n-7)	Oleic acid (18:1n-9)	Vaccenic acid (18:1n-7)	Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)	Arachidonic (20:4n-6)	EPA (20:5n-3)	DPA (22:5n-3)	DHA (22:6n-3)
1	0.5	7.01	15.81	1.07	7.87	15.58	3.88	1.61	0.77	0.47	6.54	0.72	7.69
2	0.5	6.23	14.03	1.60	6.79	22.16	3.48	1.45	0.70	0.45	5.89	0.65	6.92
Avg		6.63	14.93	1.34	7.34	18.89	3.68	1.53	0.74	0.46	6.22	0.69	7.31
1	1.0	6.60	14.70	1.68	7.89	17.75	3.80	1.42	0.60	0.42	5.04	0.57	5.91
2	1.0	6.77	15.01	1.71	8.22	20.82	4.00	1.43	0.57	0.38	5.15	0.57	6.08
Avg		6.69	14.85	1.70	8.06	19.30	3.90	1.43	0.59	0.40	5.10	0.57	6.00
1	2.0	7.51	16.48	1.90	8.73	16.66	4.12	1.66	0.80	0.56	6.40	0.78	7.58
2	2.0	7.77	17.10	1.93	8.97	11.66	4.34	1.68	0.79	0.47	6.57	0.71	7.78
Avg		7.65	16.81	1.91	8.86	14.18	4.23	1.67	0.80	0.51	6.49	0.74	7.69

R = Replicate

EL= Enzyme load

APPENDIX B: Chemical Extraction Data

Table B1. Recovery and chemical analysis of fish oil extracted using the Bligh and Dyer method.

Replicates	Oil recovered (g/100g)	Recovery oil yield (%)	Solvent recovery (%)	Peroxide value (Meq/g)	Acid value (mg KOH/g)	*p*-anisidine value
1	8.80	99.77	91.0	12.054	5.149	9.066
2	8.23	93.31	89.0	12.041	5.033	9.704
3	8.68	98.41	91.5	12.034	5.142	9.227
Average	8.57	97.16	90.5	12.043	5.108	9.332

Table B2. Fatty acid composition of fish oil obtained using the Bligh and Dyer method.

R	Saturated fatty acid (wt %)			Monounsaturated fatty acid (wt %)			Polyunsaturated fatty acid (wt %)					
	Myristic acid (14:0)	Palmitic acid (16:0)	Stearic acid (18:0)	Palmitoleic acid (16:1n-7)	Oleic acid (18:1n-9)	Vaccenic acid (18:1n-7)	Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)	Arachidonic (20:4n-6)	EPA (20:5n-3)	DPA (22:5n-3)	DHA (22:6n-3)
1	5.446	14.979	1.760	6.780	19.564	3.407	1.170	0.485	0.383	4.458	0.438	5.910
2	5.481	14.307	1.762	6.590	19.955	3.407	1.171	0.463	0.370	4.453	0.447	5.933
A	5.464	14.643	1.761	6.685	19.760	3.407	1.171	0.474	0.377	4.456	0.443	5.922

R= Replicate
A= Average

APPENDIX C: Enzymatic Extraction of Omega-3 Fatty Acid Data

Table C1. Fatty acid composition of omega-3 fatty acids recovered using enzymatic hydrolysis (wt %).

Sample	Replicates	EPA	DPA	DHA
CFS	1	6.226	1.361	11.012
	2	6.907	0.720	11.297
	3	6.365	0.651	11.222
	Average	6.499	0.910	11.177
NS	1	5.008	0.567	9.927
	2	4.565	0.397	8.510
	3	4.367	0.374	8.296
	Average	4.646	0.446	8.911
CSS	1	6.213	0.740	10.958
	2	6.305	0.606	11.273
	3	6.107	0.572	9.849
	Average	6.208	0.639	10.693
IS	1	5.483	0.783	9.994
	2	6.202	0.670	9.793
	3	5.429	0.646	11.518
	Average	5.704	0.699	10.435

CFS= Constant fast stirring

NS= No stirring

CSS= Constant fast stirring

IS= Intermediate stirring