CHAPTER II– Materials and methods

Materials

Commonly consumed and commercial varieties of five freshwater fishes viz., Rohu (Labeo rohita), Catla (Catla catla), Comman carp (Cyprinus carpio), Mrigal (Cirrhinus mrigala) and Tilapia (Oreochromis niloticus) and five marine fishes viz., pink perch (Nemipterus japonicus), Indian mackerel (Rastrelliger kanagurta) and Indian Oil Sardine (Sardinella longiceps), Tuna (Thunnus albacares) and Sear fish (Scomberomororus commerson) were chosen for byproduct collection to characterize their lipids. For fermentation and enzymatic hydrolysis experiments, visceral mass obtained by dressing of freshly harvested Indian major carps (rohu and catla) was procured from local market and transported to laboratory under iced conditions.

Chemicals

Microbiological media were procured from Hi-Media (Hi-Media, Mumbai, India). Solvents and chemicals were of analytical grade, unless otherwise mentioned. The LAB isolates were maintained on Mann–Rogosa–Sharpe (MRS) agar (Hi-Media, Mumbai, India) slants, stored at 4°C and sub-cultured periodically. Alpha cellulose, thiobarbituric acid, ascorbic acid, adenosine diphosphate, dipalmitoylphosphatidylcholine, triolein, HMG CoA, NADPH, 5,5’-dithiobis (2-nitrobenzoic acid), collagen, heparin, xanthine oxidase, glutathione (reduced and oxidized), glutathione reductase, hydrogen peroxide, 1 chloro 2,4-dinitrobenzene, dithiothreitol and bovine serum albumin, were purchased from Sigma Chemical Co., St. Louis, USA. 6-keto-prostaglandin F_1α (PGF_1α) and thromboxane B_2 (TXB_2) were bought from Cayman Chemical Co., Ann Arbor, MI, U.S.A. Ferrous sulphate, ferric chloride, ammonium thiocyanate were purchased from Qualigens, Mumbai, India. Cytochrome C, calcium gluconate, xanthine and EDTA were purchased from Sisco Research Laboratory, Mumbai, India. HPLC grade hexane, isopropanol, methanol, butanol, acetonitrile, benzene were obtained from E. Merck, Mumbai, India. Choline chloride, DL-methionine and tocopherol acetate were purchased from Hi Media Lab, Mumbai, India. Casein was purchased from Nimesh Corporation (Mumbai, India). Fatty acid standards were obtained from Nu Chek Prep, Elysian, MN,
USA. Groundnut oil was purchased from local market. All the other chemicals and solvents used were of analytical grade.

**Methodology**

**Total lipids and their classes**

Head, meat and waste (visceral) were and minced separately in a wearing blender (Stephen Mill, Stephan UM, Germany); and, were used for analysis of lipids and their fatty acids. Total lipids (TL) were extracted according to the method of Bligh and Dyer (1959). Briefly, TL from samples of different body components were obtained by homogenizing (Polytron PT3100, Kinematica AG, Switzerland) the samples with solvent mixture (chloroform : methanol; 2:1 v/v) at a sample to solvent ratio of 1:3 (w/v). The homogenized samples were kept for overnight extraction followed by filtration to collect the filtrate. The chloroform layer, (which contains dissolved lipids) was collected, washed with 0.88% potassium chloride, and removed completely using a rotary flash evaporator. Traces of moisture in the lipid extract were removed by drying it over anhydrous sodium sulphate and solvent was evaporated using a rotary flash evaporator (Superfit, Bangalore, India) to obtain to TL extract and estimated gravimetrically.

Different lipid classes in the TL were separated by open column chromatography (OCC) on silica gel 60-120 mesh size (1:30 w/w of lipid) (Dreyfus et al. 1997). Successive elution of TL on OCC with chloroform (1:100 w/v), acetone-methanol (9:1 v/v) mixture (1:150 w/v) and methanol (1:100 w/v) resulted in elution of neutral lipids (NL), glycolipids (GL) and phospholipids (PL), respectively (Dreyfus et al. 1997). These lipid fractions were evaporated and were expressed as % of TL (Figure 2.1). The neutral, glyco-, and phospholipid fractions, purified by OCC and confirmed by Thin layer chromatography. As neutral lipid was found to be the major class of TL in all the fishes, it was further separated by OCC into different sub-classes (Figure 2.1) namely hydrocarbons (HC), sterolesters, triacyl glycerol (TAG), free fatty acids (FFA), diacyl glycerol (DAG), monoacyl glycerol (MAG) by eluting with varying ratios (1:99 to 15:85 v/v) of hexane and diethyl ether on silica gel column (60-120 mesh size; 1:30 w/w of lipid). These subclasses were collected, evaporated, weighed and was expressed as % of NL.
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Separation of different lipid classes

Total lipid (Fish oil)
Silica gel, Column chromatography
Elute with 1:100 (w/v) Chloroform

Neutral Lipids (NL)
Elute with 1:150 (w/v) Acetone - Methanol (9:1 v/v) mixture

Glycolipids (GL)
Elute with 1:100 (w/v) Methanol
Phospholipids (PL)

Fractionation of Neutral Lipids

Neutral Lipids
Silica gel, Column chromatography
Elute with 1:30 (w/v) hexane

Hydrocarbons
Elute with 1: 60 (w/v) 1% Diethyl ether in hexane (1:99 v/v)

Sterolesters
Elute with 1: 50 (w/v) 5% Diethyl ether in hexane (1:95 v/v)

TAG/Tocopherol
Elute with 1: 50 (w/v) 8% Diethyl ether in hexane (1:92 v/v)

FFA
Elute with 1: 80 (w/v) 15% Diethyl ether in hexane (1:85 v/v)

DAG/Cholesterol
Elute with 1: 50 (w/v) Diethyl ether
MAG/remaining DAG

Figure 1. Scheme for separation of total lipids into different classes and fractionation of neutral lipids into subclasses.
Fatty acid composition of TL, NL, GL and PL

TL, NL, GL and PL methyl esters were prepared by transesterification using 0.5M sodium methoxide in methanol to obtain fatty acid methyl esters (FAME) as per the procedure of Horii et al (2007). FAME was analyzed by gas chromatography (Shimadzu GC 2014; M/s Shimadzu, Kyoto, Japan) fitted with a flame ionization detector for identifying the individual fatty acids. FAME dissolved in hexane was analyzed on a fused silica capillary column (30 m x 0.32 mm x 0.25 μm) (Omegawax™ 320; M/s Supelco, Bellefonte, USA). The temperatures of injector, column and detector were set at 250, 200 and 260°C, respectively. Fatty acids were identified by comparing with authentic fatty acids standard. Fatty acid concentrations above 0.1% of total fatty acids were only considered for calculation fatty acids composition.

Lactic acid fermentation of fish visceral waste (FVW)

Lactic acid fermentation of FVW was carried out using four native LAB (Enterococcus faecalis NCIM5367, Pediococcus acidilactici NCIM5368, Pediococcus acidilactici FM37; Pediococcus acidilactici MW2) and a standard proteolytic LAB having wide spectrum of antimicrobial properties (Enterococcus faecium NCIM5335) (Amit et al. 2009). The native LAB selected were isolated from fresh water fish processing waste based on their proteolytic (to hydrolyze protein and recover lipids) and antibacterial activity (probiotic properties). The selected LAB are the best among the 100 LAB isolated during the screening for a starter culture to ferment fish waste.

The lactic acid bacterial cultures were grown in 100 ml of MRS-Broth (Hi-Media, India) for 24 h at 37 ± 1°C in a shaking (100 rpm) incubator (Technico Ltd., India). The cells were harvested by centrifuging (C31 Cooling centrifuge, Remi-India, Mumbai, India) at 5000xg for 10 min. The harvested cell pellets were washed twice with sterile physiological saline and resuspended in physiological saline (100 ml). The viable cells, after serial dilution, were assayed by counting colony forming units (CFU) on MRS agar (Hi-Media, India) plates. The counts in the inoculum were in the range of 8.5 to 9 log CFU/ml.

As fresh water FVW had higher lipid content compared to marine fishes they were selected for further studies for simultaneous recovery of lipids and protein by fermentation and enzymatic hydrolysis. Among fresh water carps, rohu (highest lipid
content) and catla (higher EPA and DHA content), commercially the most dominant species were selected in this study. FVW of catla and rohu were mixed in the ratio of 1:1 (w/w) and this mixed waste was used in the fermentation experiments. Mixed FVW was minced in a Waring blender (Stephen Mill, UM5 Universal, Hong Kong) followed by heating at 85°C for 10 min to inactivate the endogenous enzymes and native microflora (Bhaskar et al., 2007). The homogenized and cooked FVW was mixed with 10% dextrose and 2% salt (both w/w) by continuous stirring followed by the addition of 24 h old LAB inoculum at 10% (v/w). The mix ~ 500gm was placed in a sterile airtight container (1 liter bottle) and incubated for 72 h at 37±1°C.

![Diagram](image.png)

**Figure 2.2.** Schematic diagram showing methodology used for simultaneous recovery of lipids and proteins from fish visceral waste on lactic acid fermentation. “*” - *Enterococcus faecium* NCIM5335, *Pediococcus acidilactici* NCIM5368, *Enterococcus faecalis* NCIM5367, *Pediococcus acidilactici* MW2, *Pediococcus acidilactici* FM37. FO-LAF – fish oil recovered by lactic acid fermentation.
After incubation, the slurry was centrifuged at 6000×g for 20 min to separate the mass into three phases viz., an upper layer of oil, middle layer of fermentation liquor with hydrolyzed proteins and a bottom residue rich in collagen. Oil recovery upon fermentation was measured (described later in this chapter). The middle and bottom layer were collected together and extracted with demineralized water (DMW; 1:1, w/v; 2X) to obtain fractions – the hydrolysed protein rich liquid portion which was labeled as fermented waste protein hydrolysate (FWPH) and the solid residue rich in collagen. The FWPH was analyzed for extractability, degree of hydrolysis and antioxidant activities; and the residue was estimated for collagen content.

**pH and total titrable acidity**

pH of samples was measured using pH meter (Cyberscan 1000, Eutech, Singapore) by directly immersing the combined glass calomel electrode in the sample. Total titrable acidity was estimated by determining the volume (ml) of 0.1N NaOH required to increase the pH of one gram of the fermented waste to 8.

**Yield, quality and fatty acid composition of lipids recovered on fermentation**

Fish oil (FO-LAF) was recovered on centrifugation of fermented FVW (100gm). The volume of FO-LAF recovered was determined as % of total lipid content in FVW. Total lipid content in the FVW was determined by the method of Bligh and Dyer (1959) considered as 100%; and, the yield of FO-LAF on fermentation was computed as -

\[
\text{Yield (\%) = Volume of FO-LAF recovered/Total lipid content × 100}
\]

Fatty acid composition of FO-LAF was determined by preparing the fatty acid methyl esters (FAME) of the recovered oil. FAME was analyzed by using a gas chromatography (GC) (Shimadzu GC 2014; M/s Shimadzu, Kyoto, Japan) fitted with a flame ionization detector (FID) for identifying the individual fatty acids. Conditions of Gas chromatography have been described earlier in this chapter.

**Total lipid extraction**

Lipid extract from fresh mixed waste was obtained by homogenizing the samples in a homogenizer (Polytron PT3100, Kinematica AG, Switzerland) with solvent mixture (choloroform : methanol; 2:1 v/v) at a sample to solvent ratio of 1:3 (w/v). The homogenized samples were kept for overnight extraction followed by filtration to collect
the filtrate. The chloroform layer, (which contains dissolved lipids) was collected, washed with 0.88% potassium chloride, and removed completely using a rotary flash evaporator. Traces of moisture in the lipid extract were removed by drying it over anhydrous sodium sulphate and solvent was evaporated using a rotary flash evaporator.

**Acid and peroxide value**

Acid and peroxide values of the FO-LAF were estimated as per AOAC (2000). Acid value (mg KOH/gm oil) was expressed as the number of milligram of KOH required to neutralize the free fatty acids in 1gm of oil. Briefly, 1 gm of FO-LAF was dissolved in 100 ml of neutralized alcohol and titrated with standard alkali (0.05N KOH) for permanent pink color (the color developed must persist for 30 sec). Peroxide value is used to assess the extent of oxidation of oil during fermentation. Briefly, 2gm of FO-LAF was dissolved in 20 ml of chloroform in a clean 250ml stopper flask. To the content, 30ml of glacial acetic acid (30ml) and 1ml of saturated potassium iodide was added, mixed properly and incubated in dark for 30 min. After incubation, 50 ml of distilled water was added through the sides of the stopper opening slowly. The final content was titrated against 0.01 N Na$_2$S$_2$O$_3$, with starch as the indicator till the blue color disappears. Peroxide value was calculated as follows:

$$\text{Peroxide value} = \frac{(S-B) \times \text{Normality of Na}_2\text{S}_2\text{O}_3 \times 1000}{\text{Weight (gm) of the oil}}$$

Where, $S =$ volume (ml) of titrant for oil; $B =$ volume (ml) of titrant for blank

**Protein hydrolysis**

Degree of protein hydrolysis (DH; %), extractability of protein (%) and collagen content were determined to find out the effect of fermentation on protein of the FVW. Degree of protein hydrolysis (DH) was estimated as per the methodology described by Amit et al. (2009) and was computed as -

$$\text{DH} (%) = \frac{[10\% \text{TCA soluble N}_2 \text{ in the sample} \div \text{Total N}_2 \text{ in the sample}]}{\times 100}$$

Extractability was calculated as -

Extractability (%) = (Water soluble protein on hydrolysis ÷ total protein in sample) × 100

Protein and nitrogen measurement in the samples were carried out by Kjeldahl method (AOAC, 2000) using Kjeltec protein analyzer (Foss Analytica AB, Sweden).
Collagen content in the residue of hydrolyzed fish viscera using different LAB was estimated by spectroscopic determination of hydroxyproline content as per the method of AOAC (2000). In brief, this data was used to compute the collagen recovery in the residue as compared to the total collagen in the FVW. Collagen recovery was estimated by the following equation -

\[
\text{Collagen recovery (\%)} = \left( \frac{\text{Collagen content in residue}}{\text{total collagen}} \right) \times 100
\]

**Preparation of sample for in-vitro antioxidant and antibacterial assays**

The FWPH obtained was filtered using muslin cloth (0.5 mm) to obtain filtered fermented liquor (FFL). FFL was further centrifuged at 5000×g for 20 min to obtain a sediment-free supernatant. This supernatant was referred to as centrifuged FFL (CFFL). CFFL was used for determining the in-vitro antioxidant and antibacterial properties. The in-vitro antioxidant assays included total antioxidant activity, scavenging of DPPH and superoxide radicals.

**Total antioxidant activity**

Total antioxidant activity (TAO) of CFFL was carried out as per the method described by Prieto et al. (1999). Sample was mixed with 3 ml of reagent solution [3.0 ml; 0.6 M sulfuric acid: 28 mM sodium phosphate: 4 mM ammonium molybdate (1:1:1 v/v/v)]. Reaction mixture was incubated in a water bath at 95°C for 90 min, cooled to room temperature and the absorbance was measured at 695 nm. Total antioxidant activity was expressed as of µg of ascorbic acid equivalents (AAE) per mg of protein in fermented hydrolysed sample.

**Diphenylpicrylhydrazyl (DPPH) radical scavenging activity**

The DPPH radical scavenging capacity of CFFL was determined by the method previously described in Duan et al. (2006). Briefly, 2.0 ml of 0.16 mM DPPH solution (in methanol) was added to the test tube containing 100 l of sample and made up to 2 ml with distilled water. The mixture was vortexed for 15 sec and kept at room temperature for 30 min in the dark. Sample blank was prepared by replacing DPPH with methanol. The absorbance of all the sample solutions was measured spectrophotometrically at 517 nm. Methanol along with DPPH served as the control. The scavenging activity (%) was calculated by using the formula:
Scavenging activity (%) = \[1-\{(A_{\text{sample}}-A_{\text{sample blank}})/A_{\text{control}}\}\] x100

**Super oxide scavenging activity**

Super oxide scavenging property of CFFL was determined by the method explained by Heo et al (2005). Sample (250 l) was made up to 3ml with distilled water followed by 2.6 ml of 50 mM phosphate buffer (pH 8.2) was added and to this 90 µl of freshly prepared 3 mM pyrogallol dissolved in 10 mM HCl. Then the absorbance was measured spectrophotometrically from 0 min and 10\(^{th}\) min at 325 nm. Distilled water (250 l) along with 2.6 ml phosphate buffer (50 mM and pH 8.2) served as blank.

**Antibacterial properties**

The antibacterial activity of CFFL was assayed by the agar-well diffusion method (Geis et al. 1983) under aerobic conditions. Pre-poured agar (nutrient agar) media plates were overlaid with Brain Heart Infusion soft agar and inoculated with freshly grown pathogenic bacteria. In the agar plates, wells of 5 mm diameter were cut using a sterile cork borer. The wells were filled with 50 l of each of samples at different concentrations and incubated for 18 – 24 h at 37\(^{\circ}\)C. The antagonistic zones were detected at the end of incubation period and diameter of zone of inhibition was measured in mm.

**Optimization of fermentation conditions by response surface methodology**

Fermentation of FVW and preparation of inoculum (\(P\) acidilactici NCIM5368) was carried out according to the method described in the earlier section. The independent factors considered for optimization included inoculum level (X1; % v/w), glucose (X2; % w/w) and incubation time (X3; h). Full factorial design was employed for the study. The design consisted of three factors (X1, X2 and X3) at three equidistant levels (5%, 10% and 15% for X1; 7.5%, 12.5% and 17.5% for X2; and 24, 48 and 72 h for X3). In the optimization experiments, pH, TTA, oil recovery, degree of protein hydrolysis and antioxidant activity of protein fraction was recorded as the additional response (dependent) variables. Each run comprised 100 g of homogenized cooked FVW mixed with NaCl (2%, w/w) along with respective levels of glucose (% w/w) and inoculum (% v/w). The inoculum prepared had a LAB count of 8.1 – 8.4 log CFU/ml depending on the culture. Ingredients were mixed according to the predetermined levels in 250 ml container and incubated at 37 ± 1\(^{\circ}\)C in an incubator for specified time.
incubation, the slurry was centrifuged at 5000×g for 20 min to separate the mass into three phases viz., an upper layer of oil, middle layer of fermentation liquor with hydrolyzed proteins and a bottom residue rich in collagen. Oil recovery upon fermentation was calculated, as earlier described. Both the middle and bottom layer were collected together and extracted with demineralized water (1:1, w/v; 2X) to obtain two fractions – the hydrolysed protein rich liquid portion (FWPH - fermented waste protein hydrolysate) and the solid residue rich in collagen. The FWPH was analysed for extractability, degree of hydrolys and antioxidant activities; and the residue was estimated for collagen recovery. The designed model was further validated using random combinations of the independent variables. All the results were analyzed by employing STATISTICA software, to determine the optimum conditions for fermentation.

**Large-scale fermentation for simultaneous recovery of lipids and protein**

Ten kilogram of cooked homogenized FVW (5 batches each) was mixed with NaCl (2%, w/w) along with optimized levels of glucose (12%, w/w) and inoculum (15%, v/w). The content was mixed well and incubated for 48 h at 37 ± 2°C. After the incubation time, oil recovered was decanted and the protein fraction was extracted with water (1:1, w/v; 2X). All the extracts were pooled freeze dried to obtain lyophilized fermented liquor. Freeze drying was accomplished by a pilot scale lyophilized (Lyophilization Inc, USA). The recovered lipid was used for animal feeding studies.

**Enzymatic hydrolysis of FVW for simultaneous recovery of lipids and protein**

Four food grade enzymes employed in the study include Alcalase® (Novo Industry, Denmark; alkaline enzyme; declared activity of 0.6 Anson-U/g), Neutrase® (Novo Industry, Denmark; microgranulated; declared activity of 1.5 Anson-U/g), Protex 7L® (Genencor Intl., USA; 580,000 DU/g) and Protease-P-amano® (P Amano6; 60,000 U/g). The former 3 were from bacterial sources while the 4th one is a fungal protease.

The FVW was minced in a Waring blender (Stephen Mill, UM5 Universal, Hong Kong) followed by steam cooking the visceral mass at 85°C for 20 min to inactivate the endogenous enzymes (Guerard et al. 2001). Enzymatic hydrolysis was carried out using different commercial proteases. Minced FVW (200 gm) was mixed with water (1:1w/v) and the content was homogenized after addition of enzymes (0.5% w/w of FVW). The
content was incubated for 2 h at 40°C with shaking after every 10 min. The resulting sample was centrifuged at 8000 x g for 20 min and the upper oil layer (FO-EH) was collected. The lower aqueous phase (protein hydrolysate) was extracted with demineralized water (1:1, w/v; 2X) and all the extractants were pooled. The pooled extract was analyzed for protein extractability, degree of protein hydrolysis and antioxidant activities as described earlier. The residue was estimated for collagen recovery. The oil recovery and its quality with respect to acid value, peroxide value and fatty acid composition were determined as described earlier in this chapter.

**Optimization of enzymatic hydrolysis conditions for simultaneous recovery of lipids and protein by response surface methodology for Fungal Protease**

In preliminary experiments on screening of commercial protease for simultaneous recovery of lipids and protein, it was found that fungal protease resulted in higher recovery of lipids and protein. Hence, conditions for hydrolysis of FVW by Fungal protease were optimized for simultaneous recovery of lipids and protein. The independent factors considered for optimization included enzyme concentration (X1; %w/w), time, (X2; min) and substrate: water ratio (X3, w/v). Full factorial design was employed for the study. The design consisted of three factors (X1, X2 and X3) at three equidistant levels (0.5%, 1.0% and 1.5% for X1; 60, 120 and 180 min for X2; and 1:1, 1:2, 1:3 for X3). In the optimization experiments, oil recovery, degree of protein hydrolysis and antioxidant activities (DPPH radical scavenging and total antioxidant activity as described earlier) of protein fraction were evaluated as the additional response (dependent) variables. Each run comprised 100 g of homogenized cooked FVW mixed with respective levels of water and enzyme (% w/w). The ingredients were mixed according to the predetermined levels in 250 ml container and incubated at 37 ± 1°C in a incubator for specified time. The designed model was further validated using random combinations of the independent variables. All the results were analyzed by employing STATISTICA software, to determine the optimum conditions for enzymatic hydrolysis.

**Large-scale enzymatic hydrolysis under optimized condition for simultaneous recovery of lipids and protein**

Ten kilogram of cooked homogenized FVW (5 batches each) was mixed with water (at optimized level) along with optimized levels of enzyme concentration (0.5%, w/w).
The content was mixed well and incubated for 2 h at 37±2°C. After the incubation time, oil recovered was decanted and the protein fraction was extracted with demineralised water (1:1, w/v; 2X). The protein extracts were pooled and concentrated to minimum volume and freeze dried to obtain lyophilized enzymatic hydrolysed liquor. Freeze drying was accomplished by a pilot scale lyophilizer (Lyophilization Inc, USA). Lipid recovered was used for animal feeding experiments.

**Enrichment of PUFA in FVW-FO by lipase aided hydrolysis during fermentation of fish visceral waste with *Pediococcus acidilactici* NCIM5368**

Five commercial lipases were used for this study: *Candida rugosa* (CR) lipase, *Aspergillus niger* (AN) lipase, *Mucor javanicus* (MM) lipase, *Thermommyces lanuginococa* (TL) lipase and *Candida cylindrica* (CC) lipase. The characteristics of the lipase used are given in Table 2.1.

**Table 2.1. Lipases used for enrichment experiment and their properties**

<table>
<thead>
<tr>
<th>Source of lipase</th>
<th>Specificity</th>
<th>Activity (U/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em> (AN)</td>
<td>1-3,3-&gt;&gt;2-</td>
<td>3391</td>
</tr>
<tr>
<td><em>Candida rugosa</em> (CR)</td>
<td>None</td>
<td>10355</td>
</tr>
<tr>
<td><em>Candida cylindrica</em> (CC)</td>
<td>None</td>
<td>21740</td>
</tr>
<tr>
<td><em>Thermommyces lanuginococa</em> (TL)</td>
<td>1-3,3-&gt;&gt;2-</td>
<td>4460</td>
</tr>
<tr>
<td><em>Mucor javanicus</em> (MJ)</td>
<td>1-3,3-&gt;&gt;2-</td>
<td>3478</td>
</tr>
</tbody>
</table>

CR and CC were non-specific lipases and MM, TL and AR were sn-1,3 specific lipases. Enzyme activities were determined by the Japanese industrial standard method substituting Sardine oil for olive oil. (JIS, 1995). Briefly, Free fatty acids released by hydrolysis reaction (20 min) were titrated against 0.5 N sodium hydroxide and the pH change was monitored by adding 1% methonoloc phenolphthalein solution. One unit of enzyme activity (U) was defined as the amount of enzyme that liberated 1µmol of fatty acid per min at 37°C.

**Lactic acid fermentation**

Fermentation of FVW was carried out as described earlier [Inoculum – 10% (v/w), sugar – 15% (w/w) and salt 2% (w/w)] under shaking condition. Five different
commercial lipases (800 U/gm) from fungal source (*Aspergillus niger*, *Candida rugosa*, *Candida cylindrcal*, *Thermomyces lanuginose* and *Mucor javanicus*) were analyzed for their efficiency to hydrolyze SFA and MUFA and enrich PUFA content in FO-LAF.

Ideally, enzymatic hydrolysis using lipases followed by removal FFA increase EPA and DHA concentrations by reducing the SFA and MUFA. sn-1,3 specific lipases hydrolyze fatty acids present in sn-1 and sn-3 position of triglyceride and leaves the second position of the glycerol moiety which is usually more enriched with n-3 PUFAs (Bornscheuer, 2000) in case of fish oil. Non-specific lipases to discriminate SFAs and MUFAs from n-3 PUFA in FO-FVW, most likely due to the reduced steric hindrance observed with SFAs and MUFAs when linked to a glycerol backbone (Gamez-Meza et al. 2003). Protein hydrolysate after 24h of lactic acid fermentation acted as an emulsifier as protein has shown to be a better emulsifier for lipase hydrolysis (Byun et al. 2007). Lipases were added after 24 h of lactic acid fermentation (Figure 2.3). The content was incubated for next 24 h at 37 ± 2°C with shaking condition (200 rpm). The fermented mass was centrifuged at 7000 x g and the upper oil layer was collected. The collected oil layer was analyzed for % of oil yield (considering solvent extracted oil as 100%), acid value, peroxide value and triglyceride fatty acid composition as described earlier in this chapter.

*Aspergillus niger* sn-1,3-non specific lipase showed better hydrolysis which resulted in highest PUFA content in triglyceride. Effect of time (2, 4, 8, 16, 24 h) for hydrolysis, was done at 40°C to find out the optimum time for concentrating the oil rich in PUFA. After hydrolysis of oil, the content was centrifuged at 7000 x g for 20 min and the oil layer was collected by decanting. Free fatty acids released during hydrolysis were determined as acid value by adding an amount of KOH (mg) required to neutralize the released FFA. Aliquots (100 ml) of hexane and (50 ml) of deionized water were added to the collected oil (FO-LAF) to remove the FFA with two additional water extractions. The hexane layer including mono-, di- and triglyceride (MG, DG and TG, respectively) with n-3 PUFAs were collected and filtered through a bed of anhydrous sodium sulfate. Hexane was removed by a rotary evaporator at 45°C, and the final oil with the n-3 PUFA concentrate was flushed with N₂ and analyzed for fatty acid composition.
Fish visceral waste (FVW)

Homogenized

Cooked (85°C, 10min), cooled to room temperature

sugar (10% w/w), salt (2% w/w), Inoculum* (10% v/w)

Waste : water (1:3 w/v), enzyme® (0.5%)

**Fermentation (24 h at 37±2°C)**

50% of FWPH was removed + Addition of Lipases#

Fermentation (48 h)#

Centrifugation (7000xg, 20min)

Collected upper oil layer

Analyzed for acid value, PUFA content, triglyceride fatty acid composition

*Figure 2.3. Flowchart showing the procedure adopted for enrichment of PUFA in triglyceride during fermentation and enzymatic hydrolysis of fish visceral waste with commercial lipases, ‘*’ – Pediococcus acidilactici NCIM5368, “#” – Enzymes mentioned in Table 1; “#” – Shaking at 200 rpm; FWPH – fish waste protein hydrolysate; Protein hydrolysate also acts as an emulsifier

**Enzymatic hydrolysis**

Enzymatic hydrolysis (Fungal protease) of cooked FVW for recovery of lipids was carried out at optimized condition [Enzyme concentration – [0.5% (v/w) and waste : water ratio (1:3)] as described earlier. Five different commercial lipases (0.1% w/w) from fungal source (Aspergillus niger, Candida rugosa, Candida cylindrica, Thermomyces lanuginose and Mucor javanicus) were analyzed for their efficiency to enrich PUFA content in recovered oil. Fungal protease aid simultaneous recovery of lipids and protein whereas lipase hydrolyse the lipids to remove SFA and MUFA thereby concentrate the PUFA in FO-EH. Lipases were added after 1 h of fungal protease hydrolysis of FVW and
lipase hydrolysis was carried out for another 1 h at 37 ± 2°C with shaking (200 rpm). The enzymatic hydrolysate mass was centrifuged at 6000 rpm and the upper oil layer was collected. The collected oil layer was analyzed to find out % of oil yield (considering solvent extracted oil as 100%), acid value and triglyceride fatty acid composition (as described above).

**Stabilization of oil during lactic acid fermentation and enzymatic hydrolysis**

Lactic acid fermentation (*Pediococcus acidilactici* NCIM5368) and fungal protease hydrolysis of FVW was carried out in an optimized conditions as described earlier. Fermentation and proteolytic hydrolysis of homogenized viscera was carried out after adding antioxidants [α-tocopherol, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ)] at 100ppm (w/w) level to evaluate their effect on quality characteristics of lipids recovered from the fish visceral waste (Figure 2.4).

**Fish visceral waste (FVW)**

- **Homogenized**

  **Cooked (85°C, 10min), allowed to cool, Added 100 ppm antioxidant**

  **Sugar (10% w/w), salt (2%w/w),**

  **inoculum** (10% v/w)

  **Fermentation (24 h at 37±2°C)**

  **Addition of protease (0.05v/w)**

  **Centrifugation (6000rpm, 20min)**

  **Collected upper oil layer**

  **Analyzed for acid value, triglyceride fatty acid composition**

Figure 2.4. Schematic diagram showing the steps in stabilization of PUFA during lactic acid fermentation and enzymatic hydrolysis of FVW. “*” – *Pediococcus acidilactici* NCIM5368, ‘#’ - TBHQ, BHT, BHA and α-tocopherol
These antioxidants were added after homogenization of FVW and before the addition of enzymes/LAB so as to prevent the oxidation of lipids during processing. The recovered lipid was characterized for acid value, peroxide value and fatty acid composition as described earlier.

As TBHQ and α-tocopherol showed better effect on preventing lipid oxidation during lactic acid fermentation and enzymatic hydrolysis in recovered oil, their effect at different concentration (50ppm, 100ppm, 150ppm, 200ppm) was evaluated so as to find out the optimum concentration of them to recover quality oil. In case of α-tocopherol different concentration of α-tocopherol was first prepared in hexane, evaporated under nitrogen and then homogenized FVW was added to the container having α-tocopherol. Other antioxidants were weighed and added directly to the cooked fish visceral waste. The content was mixed thoroughly and used for lactic acid fermentation or proteolytic hydrolysis (Figure 2.4).

**Dietary studies with fish oil recovered from fish visceral waste**

**Experimental animals**

Male Wistar rats [OUTB-Wistar, IND-cft (2c)] (*Rattus norvegicus*) weighing 50 ± 3.0g was obtained from the institutes animal house facility. They were housed in individual cages with 12h light and dark cycles with temperature 25 ± 2ºC and fed fresh powder diets daily. The experimental protocol was approved by the Institute’s animal ethical committee.

**Diet composition**

Diets were prepared according to AIN-76 (American Institute of Nutrition) (Anon. 1977). The basal composition of the purified diets used for both the experiments is given in Table 2.2. Fatty acid composition (% of total fatty acids) of FO recovered by LAF consisted of 14:0 (3.32), 16:0 (24.22), 16:1 (11.98), 18:0 (5.60), 18:1n-9 (15.94), 18:1n-7 (3.01), 20:0 (0.96), 20:1n-9 (0.78), 18:2n-6 (13.3), 18:3n-3 (8.2), 20:4n-6 (4.2), 20:5n-3 (2.1) and 22:6n-3 (3.2). FO recovered by enzymatic hydrolysis had 14:0 (3.66), 16:0 (23.92), 16:1 (11.85), 18:0 (5.30), 18:1n-9 (15.3), 18:1n-7 (3.32), 20:0 (0.92), 20:1n-9 (0.80), 18:2n-6 (13.7), 18:3n-3 (8.9), 20:4n-6 (4.3), 20:5n-3 (2.2) and 22:6n-3 (3.3).
Table 2.2. Composition (g/kg) of AIN-76 purified diets used for feeding experiments

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>FO-LAF</th>
<th>FO-EH</th>
<th>CLO</th>
<th>GNO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.25#</td>
<td>2.5#</td>
<td>5.0#</td>
<td>1.25#</td>
</tr>
<tr>
<td>Sucrose</td>
<td>597</td>
<td>597</td>
<td>597</td>
<td>597</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Fish oil</td>
<td>24</td>
<td>48</td>
<td>96</td>
<td>24</td>
</tr>
<tr>
<td>GNO</td>
<td>76</td>
<td>52</td>
<td>4</td>
<td>76</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>AIN-76 vitamin mix&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>AIN-76 mineral mix&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Methionine</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> – composition given in Table 2.3; <sup>b</sup> – composition given in Table 2.4; FO-LAF – fish oil recovered by lactic acid fermentation; FO-EH – fish oil recovered by enzymatic hydrolysis; CLO – cod liver oil; GNO – ground nut oil; ‘#’ – concentration of EPA+DHA in diet.
Table 2.3. Composition of vitamin mixture used to prepare animal diets.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>mg/100g</th>
<th>Vitamin</th>
<th>mg/100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine hydrochloride</td>
<td>60</td>
<td>D-biotin</td>
<td>2</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>60</td>
<td>Cyanocobalamine</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>70</td>
<td>Vitamin E (α-tocopherol acetate, 5000 IU)</td>
<td>335.5</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>300</td>
<td>Cholecalciferol</td>
<td>0.25</td>
</tr>
<tr>
<td>D-calcium pantothenate</td>
<td>160</td>
<td>Menadione</td>
<td>5</td>
</tr>
<tr>
<td>Folic acid</td>
<td>20</td>
<td>Vitamin A (retinyl acetate)</td>
<td>40000/IU</td>
</tr>
<tr>
<td>Sucrose (fine powder, to make to 100 g)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: AIN, 1976

Table 2.4. Composition of mineral mixture used to prepare animal diets.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>g/100g</th>
<th>Mineral</th>
<th>g/100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium phosphate</td>
<td>50</td>
<td>Ferric citrate</td>
<td>0.6</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>7.4</td>
<td>Sodium selenate</td>
<td>0.001</td>
</tr>
<tr>
<td>Potassium citrate monohydrate</td>
<td>22</td>
<td>Zinc carbonate</td>
<td>0.16</td>
</tr>
<tr>
<td>Potassium sulphate</td>
<td>5.2</td>
<td>Cupric carbonate (55% Cu)</td>
<td>0.03</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>2.4</td>
<td>Potassium iodate</td>
<td>0.001</td>
</tr>
<tr>
<td>Manganese carbonate</td>
<td>0.35</td>
<td>Potassium chromate</td>
<td>0.0213</td>
</tr>
<tr>
<td>Sucrose (fine powder, to make to 100 g)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: AIN, 1976

**Effect of fish oil recovered from fish visceral waste on biochemical profile and growth parameters of experimental animals**

In this study, after 7 days of acclimatization, rats were randomly assigned to 7 groups (n=6 per group) and fed on experimental diet with FO recovered by LAF (3 groups) and EH (3 groups) contributing 1.25, 2.5, 5% EPA+DHA (made isocaloric with groundnut oil) for 8 weeks (Figure 2.5). Rats received diet containing groundnut oil
(GNO) as the sole source of fat served as control. The animals had free access to food and water *ad libitum*. The food intake (daily) and growth (once in 8 days) of the animals were monitored at regular intervals.

Figure 2.5. Schematic representation of experimental plan adopted to study the effect FO recovered from FVW by LAF and EH on biochemical changes in rats; FO–LAF – fish oil recovered by lactic acid fermentation; FO-EH – fish oil recovered by enzymatic hydrolysis (Fungal protease).

**Bioefficacy of fish oil recovered from fish visceral waste in comparison to cod liver oil**

In the previous experiment (Figure 2.5) rats fed with diet containing incremental levels of EPA+DHA from FVW-FO showed no mortality and clinical sign of toxicity during the treatment period even at a highest dose. No significant difference in growth, organ weight, hematological parameters, serum biochemical parameters except significant decrease in serum cholesterol and triacylglycerols, serum biochemical parameters in serum. Hence, in this study the bioefficacy of fish oil recovered from FVW by LAF and EH in comparison to CLO fed groups was performed (Figure 2.6).
Rats received diet containing groundnut oil (GNO) as the sole source of fat served as negative control. The animals had free access to food and water ad libitum. The food intake (daily) and growth (once in 8 days) of the animals were monitored at regular intervals. The effects of these oils were studied on antioxidant enzymes (Catalase, SOD and GST in liver, brain and heart), anticholesterol properties (HMG CoA reductase) and antithrombic properties (platelets aggregation and eicasanoids levels).

Isolation of serum and analysis of biochemical indices

After feeding for 8 weeks, rats were fasted overnight and sacrificed under ether anesthesia. Blood was drawn by cardiac puncture and kept at 4°C for 2 h. Blood was collected in two different tubes, heparinized tubes for plasma and non-heparinized tubes for serum separation. Serum was separated by centrifugation at 1100 x g in a table top Remi 8 C centrifuge for 20 min and stored at -20°C till analyzed.

Relative organ weights

The animals were autopsied and vital organs of each rat, viz liver, kidney, spleen, heart and brain were excised. Organs were examined for any gross pathological
symptoms. The fresh organ weights were recorded as absolute values and relative values were calculated on the basis of the final body weights of the rats. Liver, heart and brain were removed and rinsed with ice cold saline, blotted, weighed and stored at –20°C until used for biochemical analysis.

**Hematology and serum biochemical indices**

Hematological examination was performed using a K-4500 automated hematology analyzer (Sysmex Corp, Japan). Aliquots of whole blood samples were mixed with a 4-fold volume of buffer containing 0.5% ethylenediaminetetraacetic acid (EDTA.2K) and applied to the analyzer for the following parameters: haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), packed cell volume (PCV), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), platelet count (PC). For measuring differential leukocyte count, blood samples were mixed with ¼ volumes of 5.0% EDTA.2K, analyzed with a Microx HEG-120A (Omron Tateishi Electronics Co., Ltd, Tokyo, Japan). Levels of glucose, total protein, albumin and globulin in the serum were assayed by using respective standard kits mentioned elsewhere in the text and as per manufacturer’s protocol (M/S Agappe).

**Lipid peroxidation estimation**

Lipid peroxides level in serum and tissue (liver, brain and heart) homogenate was estimated using thiobarbituric acid as described by Okhawa et al (1979). Acetic acid (20%, 1.5 ml), 8% sodium dodecyl sulphate (0.2 ml) and 0.8% thiobarbituric acid (1.5 ml) were added to the sample in that order, incubated for 1 hour in a boiling water bath, cooled at room temperature followed by the addition of n-butanol (5 ml) and centrifuged at 3000 g for 15 min. The upper n-butanol phase containing thiobarbituric acid-reactive substances was read spectro-fluorometrically (Hitachi F-2000, Japan) with an excitation at 515 nm and emission at 553 nm. Tetramethoxypropane (TMP) was used as a standard to estimate the thiobarbituric acid -reactive substances. Absorbance of known concentrations of TMP were plotted to obtain a standard linear graph and was used to quantify the concentration of lipid peroxides in the samples.
Assays for antioxidant enzymes

Catalase

Activity of catalase (CAT, E.C. 1.11.1.6) in serum and tissue (liver, brain and heart) homogenates was determined by measuring the decrease in absorption at 240 nm using spectrophotometer (Shimadzu- Japan, 1601) in a reaction mixture containing 950 µL phosphate buffer (0.1 mM, pH 7.0), 50µL of sample containing the enzyme (approx. 10 µg) and 50 µL of hydrogen peroxide (8.8 mM) according to Aebi (1984). One CAT unit is defined as the amount of enzyme required to decompose 1 µM of H$_2$O$_2$/min. Reaction was initiated by the addition of H$_2$O$_2$ and the initial absorbance was 0.5. Reading at every 60 seconds for a period of 300 seconds was noted. Specific activity was calculated using the molar coefficient for H$_2$O$_2$.

Superoxide dismutase

Superoxide dismutase activity (SOD, E.C. 1.15.1.1) in serum and tissue homogenate was measured by the inhibition of cytochrome - C reduction mediated via superoxide ions generated by xanthine-xanthine oxidase system (Flohe and Otting, 1984) and measured using spectrophotometer at 550nm. One SOD unit is defined as the amount-required to inhibit the cytochrome-C reduction by 50%. Solution A was prepared by admixing xanthine (5 µmol) in 0.001N NaOH and cytochrome C (2 µmol) with 100 ml 50 mM phosphate buffer (containing 0.1mM disodium EDTA). The solution is stable for 3 days at 4°C and is used at 25°C. Solution B containing xanthine oxidase in 0.01M disodium EDTA (approximately 0.2units/ml) is freshly prepared and kept on ice. Sufficient enzyme to produce a rate of 0.025 units/min of cytochrome C without SOD is used. For the assay, Solution A (2.9 mL) and enzyme source (approx. 10 µg) are taken in a cuvette and reaction is started by addition of Solution B (50 µl). The contents are mixed well and absorbance was read at every 60 seconds for a period of 300 seconds at 550 nm.

Glutathione-S-transferase

Glutathione-S-transferase (GST, E.C. 2.5.1.18) activity in tissue homogenates was determined following the formation of conjugate of GSH and CDNB at 340 nm in a reaction mixture containing 50 µL GSH (20mM), 50 µL CDNB (20mM), enzyme source (1-20 µg) and 850-895 µl phosphate buffer (0.1mM, pH 6.5) containing 1mM EDTA, at 30°C (Gluthenberg et al., 1985). The increase in absorbance was measured every 60
seconds for a period of 5 min for the enzyme assay. Specific activity was calculated using the molar coefficient for CDNB.

**Assays for membrane bound enzymes in microsomes**

**Preparation of liver, brain and heart microsomes**

Liver, heart and brain microsomes were prepared according to Gutman and Katzper-Shamir (1971) from a 10% homogenate in 0.25M sucrose, 0.03M histidine and 0.001M EDTA (pH 7.4). The homogenate was centrifuged at 10000 g for 30 min in a refrigerated centrifuge (Hermle, Germany) at 4°C. The pellet was discarded and the supernatant was subjected to ultracentrifugation (L-65, Beckman, USA) at 105,000 g for 1 hour at 4°C. The supernatant was discarded and the pellet formed was re-suspended in the sucrose buffer (0.2 times the volume of the homogenate taken) and subjected to analysis or immediately stored at -80°C.

**Na⁺K⁺-ATPase**

Activity of the Na⁺K⁺-ATPases in liver, heart and brain microsomes was estimated by the method of Kaplay (1978) with slight modification. Buffer composition used for the Na⁺K⁺-ATPase assay was as follows - MgCl₂ (3 mM), KCl (14 mM), NaCl (140 mM), EDTA (0.2 mM) and Tris-HCl (20 mM, pH 7.4). Samples were run in two batches, one containing Na⁺K⁺-ATPase inhibitor ouabain (2 mM) and the other without. ATP (3 mM) was used as substrate. The sample blank contained no assay standard and microsomes and was run simultaneously. The reaction was stopped by the addition of trichloroacetic acid (10%). Inorganic phosphate (Pᵢ) liberated was determined in aliquots (0.7 ml) of incubated mixtures by the addition of ascorbic acid-ammonium molybdate solution (0.3 ml) prepared according to the method of Ames (1966). The reaction mixture was mixed well and incubated at 45°C for 20 min. Extinction at 820 nm was measured in by UV-Visible spectrophotometer. Specific activity was expressed as μmol Pᵢ/h/mg protein. Samples that contained ouabain were measured for the Mg²⁺-ATPase activity and this was subtracted from the total ATPase activity to determine the Na⁺K⁺-ATPase activity.
Chapter II- Materials and methods

**Ca\(^{2+}\) Mg\(^{2+}\)-ATPase in microsomes**

Assay for Ca\(^{2+}\) Mg\(^{2+}\)-ATPase activity in liver and heart microsomes was measured as per Kaplay (1978) with slight modification. Buffer composition was as follows - MgCl\(_2\) (3mM), KCl (14mM), CaCl\(_2\) (140mM), EDTA (0.2mM) and Tris-HCl (20mM, pH 7.4). ATP (3 mM) was used as substrate. Samples were run in two batches, one containing Ca\(^{2+}\)-ATPase inhibitor ethylene glycol tetra-acetic acid (EGTA, 0.5 mM) and the other without. The sample blank contained no assay standard and microsomes and was run simultaneously. The reaction was stopped by the addition of trichloroacetic acid (10%). Specific activity was expressed as µmol P/h/mg protein. Samples that contained EGTA were measured for the Mg\(^{2+}\)-ATPase activity and this was subtracted from the total ATPase activity to determine the Ca\(^{2+}\)-ATPase activity.

**Acetylcholine esterase activity in brain microsomes**

Acetylcholine esterase (AchE) activity in brain microsomes was determined according to the method described by Ellman et al. (1961). A cocktail containing 13 ml of 1M NaCl, 2 ml of 1M MgCl\(_2\), 10 ml of 0.5M Tris-HCl (pH 7.5) and 10 ml of 0.2M EDTA was prepared. Other reagents included 1mM DTNB and the substrate 0.1M acetylthiocholine chloride. The reaction mixture which contained 10.5 ml of the cocktail, 3 ml DTNB and 6.5 ml double distilled water. 2 ml of the reaction mixture, 30 µl of 0.1 M acetylthiocholine chloride and brain microsomes containing the enzyme (10 µg) were taken and the change in optical density was measured at 412 nm over a period of 5 min. Specific activity was calculated using the molar coefficient for –SH group of DTNB.

**Effect of feeding FVW- FO on lipid profile**

**Extraction of lipids from serum, tissue homogenate and microsomes**

Total lipid in serum, tissue (liver, brain and heart) homogenates (in 0.74% KCl) and microsomes was extracted by the method of Folch et al. (1957) by adding 10 ml chloroform: methanol (2:1) and left overnight. Thereafter, it was filtered through Whatman No. 1 filter paper and washed with 2 ml 0.74% KCl, followed by washing with 1.5 ml chloroform: methanol: water (3: 48: 47) two times. The filtrate containing the lipid was made to a single phase with methanol and then made up to a known volume with chloroform: methanol (2:1) and considered as crude or total lipid extract.
Fatty acid analysis

Fatty acid methyl esters were prepared from the lipid extracted from plasma and microsomes using boron trifluoride in methanol (Morrison and Smith, 1965) and analyzed by gas chromatography (Shimadzu 14B, Shimadzu, Kyoto, Japan) fitted with flame ionization detector and a fused silica capillary column (25 m × 0.25 mm; Konik Tech, Barcelona, Spain). The lipid extracted from tissue samples was incubated at 65°C for 1 h with 1 ml of 0.5 M methanolic KOH. Thereafter, the unsaponified layer matter was drawn by addition of 2 ml hexane, which was allowed to stand for 15 min, followed by centrifugation for 15 min. The upper layer was discarded. To the lower phase, 1 ml of 0.7 N HCl and 2 ml hexane was added and allowed to stand for 15 min and centrifuged for 15 min. The hexane layer was collected. This process was repeated twice with addition of 2 ml hexane. The hexane layers were pooled and evaporated under a stream of nitrogen gas. Benzene (0.2 ml) and boron trifluoride in methanol (0.5 ml) were then added to it and incubated at 65°C for 45 min. After cooling, 1 ml water was added and extraction performed thrice with 2 ml hexane. Double distilled water (5 ml) was added to the pooled hexane layers and centrifuged at 1500xg for 10 min. The hexane layer was carefully separated, evaporated under a stream of nitrogen and re-dissolved in 25-100 µl of hexane. The hexane containing the fatty acid methyl esters was injected to the gas chromatograph. The injector, column and detector temperatures were 220, 230 and 240°C respectively with nitrogen as carrier gas at 1 ml/min. Individual fatty acids were identified by comparing their retention time with those of standards and were quantified using Clarity Lite 420 integrator.

Estimation of triglycerides

Triglycerides were measured by the method of Fletcher (1968). Isopropanol (3 ml) and alumina (2 g) were added to the sample, vortexed and centrifuged at 4000 g for 5 min. To the supernatant (2 ml), 5% KOH (0.6 ml) was added and incubated at 60-70°C for 15 min. Working solution (12 ml stock and 20 ml isopropanol made to 100 ml with 1N acetic acid) of sodium metaperiodate stock (0.025M in 1N acetic acid) was prepared fresh. After cooling samples, 1 ml of working sodium metaperiodate was added, mixed well followed by addition of 0.5 ml of acetyl acetone reagent (0.75 ml acetyl acetone and...
2.5 ml isopropanol made to 100 ml with 2M ammonium acetate (pH 6.0). The reaction mixture was mixed and incubation at 50°C for 30 min. The colour that developed was read at 405nm. Assay with standard triolein was simultaneously run for plotting the standard graph to calculate triglyceride content.

**Estimation of phospholipids**

Phospholipids were determined by the method of Stewart (1980). To the dry sample, 2 ml chloroform and 2 ml ammonium ferrothiocyanate reagent (2.7 g hexahydrate ferric chloride and 3.24 g ammonium thiocyanate in 100 ml deionized water) were added, gently mixed, allowed to stand for 5 min and centrifuged at 3000 rpm. The absorbance of the lower chloroform layer was read at 488 nm. Assay with standard dipalmitoyl phosphatidylcholine (DPPC) was run simultaneously for plotting the standard graph and calculations of phospholipid content.

**Estimation of cholesterol**

Cholesterol was estimated according to the method of Zlatkis et al (1963). The ferric chloride acetic acid reagent stock was prepared by dissolving 840 mg of hexahydrate ferric chloride in 10 ml of acetic acid. Lipid previously extracted from tissue was evaporated and 1.5 ml of working solution of ferric chloride acetic acid (1 ml stock made to 100 ml with glacial acetic acid) was added. The mixture was mixed well by vortexing and allowed to stand for 10 min, thereafter, 1 ml sulphuric acid was added, mixed well, and allowed to stand for 45 min in the dark. The absorbance of the colour developed was read at 540 nm in a spectrophotometer. Assay with standard cholesterol was simultaneously run for plotting of standard graph and calculations of cholesterol content in samples.

**Estimation of LDL and HDL-Cholesterol**

HDL and LDL were separated by the method of Warnick and Albers (1978). Serum (0.5 ml), heparin (5000 U/ml, 25 µl) and 2M manganese chloride (25 µl) were mixed well by vortexing and allowed to stand for 30 min at 4°C. The mixture was then centrifuged at 1600 g for 20 min. HDL in the supernatant was extracted using 3 ml acetone: ethanol (1:1 v/v) while the LDL and VLDL rich precipitate was dissolved in 0.5 ml saline and the cholesterol in both fractions was estimated by the method of Zlatkis et al. (1963), as described earlier.
Chapter II - Materials and methods

Assay of HMG CoA reductase activity

Preparation of rat liver microsomes for HMG-CoA reductase

Rats were sacrificed by stunning between 9.00 to 10.00 p.m. The liver was removed and kept in ice-cold isotonic saline, blotted and weighed. Liver (1 gm) was homogenized with 4mL of 0.1M triethanolamine hydrochloride containing 0.02M EDTA and 2mM DTT pH 7.4, and centrifuged at 1,00,000 g in Beckman LS-50B ultracentrifuge using type 65 rotor for 1hr. The microsomal pellet was washed with 1mL of triethanolamine hydrochloride buffer and homogenized with 2mL of same buffer and used immediately for enzyme activity. The microsomes were also used for estimation of cholesterol, phospholipid and fatty acid analysis.

Assay for HMG-CoA reductase

HMG-CoA reductase activity was assayed by the method of Hulcher and Oleson (1973) by measuring the monothiols with 5,5’ dithiobis (2-nitrobenzoic acid). To the 0.4mL HMG CoA (6.486 mg / 6mL), 0.2 mL of dithiothreitol (DTT) (3.086mg/ 2mL) and NADPH 0.2 mL (50mg / 6mL) prepared in 0.1M triethanolamine buffer containing 0.02M EDTA and 2mM DTT pH 7.4 was added to microsomes (≈200μg protein). The mixture was incubated at 37°C for 30 min. Twenty μL of sodium arsenite (0.01M) was added and after 1 min, the reaction was terminated by the addition of 0.01mL of 2M citrate buffer pH (3.5) containing 3% sodium tungstate to precipitate microsomal proteins. The mixture was again incubated at 37°C for 10 min. The solution was transferred to plastic tubes and centrifuged at 10,000 rpm (6000 g) for 15 min to remove proteins.

Five minutes before assaying the enzyme, 0.8mL of the supernatant was mixed with 0.2mL of tris buffer (pH 10.6), 0.1mL of 2M tris buffer (pH 8.0) and 50μL of 5 5’-dithiobis nitrobenzoic acid (DTNB) (3mM), mixed thoroughly and absorbance was measured for 5 min at 412 nm (Shimadzu 160A spectrophotometer). The absorbance due to monothiols was determined by extrapolating the linear portion of the curve after the addition of DTNB. The difference in absorbance between the complete reaction and that of all the components except NADPH represented the activity due to HMG CoA reductase.
Effect of FVW-FO on Platelet Aggregation

Preparation of platelets

Blood was collected by cardiac puncture from anesthetized rats in heparinized (50 IU/mL, blood) tubes, centrifuged at 150g for 20 min at room temperature to separate platelets from erythrocytes and leukocytes. The platelet rich plasma thus obtained was then centrifuged at 150g for 20 min at room temperature to remove any residual erythrocytes and leukocytes and used to study platelets aggregation. Platelet poor plasma (PPP) was prepared by centrifugation of anticoagulated blood or PRP at 5000g for 10 min at 4±1°C (Gerrard, 1982). The platelet count was adjusted to 400,000/mL.

Platelets aggregation

The platelet aggregation experiments were performed within 2 h of blood collection. Platelet poor plasma was used as the blank. Platelet aggregation was performed by addition of 20 µL of 25 µM adenosine diphosphate or 20 µL of arachidonic acid (1mM) to 450 µl of PRP and PPP (control) in a Chronolog Dual Channel platelet aggregometer (Denmark) at 37±1°C with constant stirring at 1000 rpm. Aggregation was followed for at least 5 min. The platelet aggregation was quantitated as the maximum change in light transmittance through PRP expressed as a percentage of the light transmittance through the blank (Niranjan and Krishnakanta, 2000). Rate of platelets aggregation (aggregation/min) was calculated for the initial rate of aggregation till 3 mins (Niranjan and Krishnakanta, 2000).

Fatty acid composition of platelets

Platelets were isolated and washed as described by Brunauer and Huestis (1993) with slight modification. The platelets were separated from PRP by centrifugation at 700g for 10 min. The cells were washed twice in Tyrodes buffer (pH 7.4, NaCl 137 mM, KCl 2.7 mM, NaHCO₃ 12 mM, EDTA 1 mM, NaH₂PO₄ 0.4 mM, MgCl 1 mM, glucose 5.6 mM). The final platelet pellet was suspended in the tyrodes buffer and lipid was extracted as described by Bligh and Dyer method (1959). Fatty acid compositions were analyzed as methyl esters (Morrison and Smith, 1963) by gas chromatography system as described earlier. Phospholipids were measured by the method of Stewart (1980) using dipalmitoylphosphatidyl choline as reference standard.
Chapter II- Materials and methods

Estimation of malondialdehyde (MDA) in agonist challenged platelets

After aggregation, platelet suspension containing PRP (450 µL), which had been challenged by agonist, were transferred to an eppendorf tube containing, 1% butylatedhydroxyltoluene in ethanol (20 µL). To this 0.1mL of 100% TCA in 3N HCl was added, mixed well and centrifuged at 12,000g for 20 min. The supernatant was treated with 0.1mL TBA reagent (0.12 M TBA in 0.26M Tris– HCl), mixed well and incubated in a boiling waterbath for 30min. The color developed was measured at 532nm (Maguire and Csona-Khalifah, 1987). The amount of malondialdehyde (MDA) formed was calculated using molar extinction coefficient of 1.56 x 10^{-5} M^{-1} cm^{-1}.

Analysis of serum prostaglandins

Analysis of serum prostaglandins was done according to Rajakrishnan et al (2000). 6-Keto-PGF_{1α} and TXB_{2} were extracted from serum after acidification with 100 µL of 3.0% formic acid to pH 3.0. The extract was purified on Sep Pak C-18 column and eluted into ethyl acetate (Waters, Millipore Corp., Milford, MA, USA). The combined ethyl acetate extracts were evaporated under the stream of nitrogen and loaded on a RP-C-18 column (Phenomenex, Torrance, California, USA 250 mm x 4.5 mm) of pore size 5 mm fitted to a SPD-20A HPLC (Shimadzu Corp., Tokyo, Japan). The prostaglandins were eluted with potassium dihydrogen phosphate (0.0174 M pH 3.5): acetonitrile (62.5:37.5, v/v) at a flow rate of 1 mL/min and monitored at 196 nm. The prostaglandins were identified and quantified using authentic standards.

Statistical analysis

Design and data obtained through Response Surface Methodology, to optimize hydrolysis conditions as well as data obtained from validation runs were analyzed by STATISTICA (Statsoft, 1999). Desirability function to get optimum condition to obtain maximum degree of protein hydrolysis and oil recovery was fitted by the least square method using same software. Wherever differences were significant, mean separation was accomplished by Duncan’s multiple range tests.