Fish is an important source of highly nutritive proteins, vitamins, minerals and lipids that have several health benefits. In the present study, lipids from different body component (head meat and waste) of commercial fresh water and marine fishes were analyzed for lipids (TL, NL, GL and PL) and their fatty acid profile. The lipid content and fatty acid composition of fish processing byproduct/ waste highlights the importance for an alternative source of fish oil. Lipid content in waste was higher in case of fresh water fish visceral waste compared to marine fishes analyzed. Ackman et al. (1994) have also reported higher lipid content in livers of carps compared to muscle. Based on the lipid content in the muscle, fishes are classified into the following 4 categories: very low fat (<2% fat), low fat (2–4%), medium fat (4–8%), and high fat (>8%) fishes (Ackman 1994). According to this classification, rohu and common carp can be categorized as low fat fishes, while, tilapia, mrigal and catla as very low fat fishes. Sen (2005) also categorized carps under low fat fishes. Taking viscera in consideration, except Tilapia, other fresh water fishes evaluated in this study are high fat fish. This reflects that visceral waste can be a good source of lipids and sustainable alternative source of fish oil. Among marine fishes studied, mackerel (8.79%) and sardine (10.97%) fall into the high fat fishes category while pink perch (2.53%) is a very low fat fish. Technologically, total lipid or fat content of a species decides its storage life under different temperature conditions and suitability of a species for extraction of oil and fish meal production (Sen. 2005).

Fatty acids in fishes are derived from two main sources namely, biosynthesis and diet; and, the chain length of fatty acids vary between 14 and 24 carbon atoms with varying degree of unsaturation (Morris et al. 1995; Kamler et al. 2001). Fatty acid composition of total lipids and its lipid classes from meat, head and waste of fresh water and marine fishes revealed palmitic acid (16:0) as the major fatty acid among all the fatty acids observed. Myristic (14:0) and stearic acid (18:0) were the other main saturated fatty acids (SFA) observed. In case of mono unsaturated fatty acids (MUFA), oleic acid (18:1 n-9) was the dominant MUFA followed by palmitoleic acid (C16:1). Among the two forms of oleic acid observed i.e., 18:1n-9 and C18:1n-7, 18:1n-9 was the dominant form.
Similarly, Andrade et al (1996) have reported palmitic acid as the dominant SFA accounting for 7-76% of total SFA with oleic acid as the most abundant MUFA in 10 marine fishes from Brazil. It has also been reported that edible portion of both tropical and non-tropical aquatic organisms including fish ranges from 14 to 22 carbon atoms (Sen. 2005). Most of the fatty acids observed in total lipid and lipid classes from different body components of marine fishes analyzed in this study conform to this range.

Studies on lipid class and fatty acid composition of byproduct from various marine and fresh water fishes clearly reveals the value of byproducts as an alternative source of fish oil. As the present study reveals the value of these processing waste, development of methodology/techniques to recover lipid without affecting the other biomolecules specially protein is the need of the hour. Cleaner methods covering biotechnological approaches can make these biomolecules fit for human consumption as well as, which maximize the nutritive benefits

**Biotechnological approaches for simultaneous recovery of lipids and proteins from fish industry waste**

As fresh water fish visceral waste found to have higher lipid content compared to marine processing waste, they were processed for the recovery of lipids and protein simultaneously by fermentation and enzymatic hydrolysis. Among fresh water carps, rohu (highest lipid content) and catla are the commercially most dominant species. Also, the lipid content was higher in rohu followed by catla and mrigal. As EPA+DHA content was higher in catla (8.2) compared to mrigal (2.3%), it was used in combination with rohu for the entire experiments. Hence, hereforth rohu and catla visceral waste that was used for detail studies is referred as byproduct.

Lactic acid fermentation (LAF) and enzymatic hydrolysis (EH) was carried out for the simultaneous recovery of lipids and protein from the fish byproducts. Although, natural fermentation by *in-situ* LAB (without inactivating indigenous microflora and enzymes) can alone could be a better method for the utilisation of fish processing waste as compared to conventional acid silaging (Amit et al. 2009, Ganesan et al. 2009), it has the disadvantage that LAB composition in fish viscera varies seasonally, depending on the diet of fish apart from being influenced by fluctuations in temperature and pH of the
aquatic environment (Hagi et al. 2004). In addition, studies demonstrate the usefulness of LAB isolated from the same source serve as a better starter culture for fermentation of byproducts when compared to LAB from other sources (Amit et al. 2009, Ndaw et al. 2008). Hence, in this study, native proteolytic LAB isolated from FVW itself was employed as starter cultures for fermentation of FVW. LAF was carried out using native isolates (Pediococcus sp FM37, P. acidilactici NCIM5368, Enterococcus sp MW2, E. fecalis NCIM5367) in comparison to Enterococcus faecium NCIM5335.

There was rapid decrease in pH of fermented FVW with LAB compared to fresh (0 h) and control (no LAB). Reduction in pH of fermented FVW signifies the obvious utilization of sugar and subsequent production of organic acids leading to lowering of pH, which in turn helps in preventing the growth of spoilage organisms. LAB when added as inoculum for fermentation may dominate other microorganisms present in the material and that may result in quick reduction of pH (Amit et al. 2009, Ennouali et al. 2006). Decrease in pH from 8 to 6 during fermentation of crab waste by Lactobacillus paracasei subsp. tolerans KCTC-3074 has been reported by Jung et al (2005). Adams et al. (1987), using a minced fish-salt-glucose model, suggested that a pH of 4.5 should be reached within the first 48 h of fermentation. The most important factor in controlling the fermentation is to decrease pH (Faid et al. 1997), which must be achieved as quickly as possible in order to inhibit the growth of spoilage microorganisms in the final product.

In the present study, higher recovery of oil from FVW fermented with P acidilactici NCIM5368 and E faecium NCIM5367, which could probably because of their proteolytic nature (Jini et al. 2010). These proteolytic LAB presumably hydrolyze the protein-lipid complex, releasing in phase separation of oil and protein. The protein released from complex matrix probably is further hydrolyzed by the proteolytic action of LAB as well as the lactic acid produced therein (Vidotti et al. 2003; Nilsang et al. 2005). Relatively lower degree of protein hydrolysis, extractability and oil recovery observed in this study in case of control sample is presumably due to the effect of heat treatment prior to fermentation process and partly may be due to the activity of endogenous enzymes or microflora before and/or after and to some extent during heat treatment, as fish are known to harbor some heat resistant proteases (Klomklao et al. 2004). This demonstrates
that the higher recovery of oil and degree of protein hydrolysis is due to the LAB used in the study.

In case of EH, four different commercial proteases - Alcalase, Neutrase, Protex 7L and Protease-P-amano were evaluated for recovering lipids and proteins simultaneously by hydrolysis. Homogenized FVW was cooked to inactivate the indigenous enzymes (proteases, lipases etc) and microflora present in the fish waste. Although, enzymatic hydrolysis of fish waste has been reported for preparation of protein hydrolysate (Bhaskar et al. 2008a,b), lipids were discarded in that process. Hence in this study enzymatic hydrolysis was studied for the simultaneous recovery of lipids and proteins. FVW hydrolysed with fungal protease resulted in higher recovery of lipids as well as higher degree of protein hydrolysis when compared to other enzymes used. Similar degree of protein hydrolysis has also been reported in case of hydrolysis of visceral wastes of catla (Bhaskar et al. 2008). The least recovery of lipids and lowest degree of protein hydrolysis was in control, where no enzyme was added. Extractability of the protein also followed the similar pattern wherein protein hydrolysate of fungal protease was found to be higher. The degree of protein hydrolysis and oil recovery observed in case of control samples could possibly be due to the heat treatment; and, partly due to the activity of endogenous heat resistant enzymes before or after and to some extent during heat treatment, as fish are known to contain some heat activated proteases (Bhaskar et al. 2007). Hydrolysis of protein involves major structural changes where the protein molecule is gradually cleaved into smaller peptide units and thereby increasing the solubility of hydrolysed protein with increased hydrolysis (Kristinssons and Rasco, 2000). The collagen content (% of total collagen) recovered in the residue on enzymatic hydrolysis was lowest in case of control than the enzymatic hydrolyzed samples. This again clearly shows there was least hydrolysis in case of control and also that part of the collagen could be hydrolyzed by the proteases used.

The fatty acid profile of lipids of FVW was not affected by either LAF or EH process. Among SFA, palmitic acid (16:0) was the major fatty acid whereas in case of USFA, oleic acid (C18:1n-9) was dominant one followed by linoleic acid (C18:2n-6). Irrespective of the type of LAB used for fermentation of FVW, no change in
eicosapentaenoic acid (EPA) (C20:5n-3) and docosahexaenoic acid (DHA) (C22:6) content was observed in oil recovered as compared to oil from fresh FVW. Further, no change in n-3 PUFA content of oil recovered by LAF and EH to that of oil extracted by solvent extraction. Higher n-3 PUFA has been reported in cod viscera (Falch et al. 2006), liver oil of Himantura bleekeri (Nechet et al. 2007) and Northern pink shrimp byproduct (Hue et al. 2003). The increased acid value in case of oil recovered on fermentation could presumably be due to the release of organic acids during fermentation process (Healy et al. 2003). Visceral wastes from fish are known to be rich source of lipases (Shahidi and Kamil, 2002; Nayak et al. 2003). Hence, any delay in processing of wastes results in increased acid value and oil recovered from such wastes need further refining.

The protein hydrolysate obtained either by LAF exhibited antioxidant and antibacterial properties. The in-vitro antioxidant properties of protein hydrolysate analyzed included DPPH radical scavenging, superoxide radical scavenging activity and total antioxidant activity. Protein hydrolysates resulted from LAB fermentation of tannery waste (Amit et al. 2009), shrimp industry waste (Sachindra and Bhaskar, 2008) and marine fishes (Rajaram and Nazeer, 2010) have shown to exhibit antioxidant activities. The exact mechanism by which protein hydrolysate (peptides) display an antioxidant activities is not fully understood. However, studies have shown that protein hydrolysate and peptides can act as a radical scavengers, transition metal chelators and exerts antioxidant activities against enzymatic (lipoxygenase-mediated) and non-enzymatic peroxidation of lipid and fats (Erdmann et al. 2008; Sarmadi and Ismail, 2010).

Pediococcus acidilactici NCIM5368 and fungal protease were the best LAB and proteolytic enzyme respectively, for the simultaneous recovery of lipids and proteins. Comparative study of LAB fermentation and EH at optimized condition for the best LAB and proteolytic study is shown in Table 7.1. Oil and protein recovery was higher by LAB fermentation compared to EH process. However, protein hydrolysate by both the process showed higher antioxidant activity while, the antibacterial properties was observed only in protein hydrolysate by LAB. The antibacterial activity exhibited by the protein hydrolysate could possibly be because of the presence of bacteriocin as well as
hydrolysed FVWP produced during LAB fermentation (Amit et al. 2009a). This fraction may have potential application as an ingredient in livestock, aquaculture feeds and can be used as a probiotic. The use of probiotic as feed additives is been preferred over that of antibiotics as they do not exhibit any of the undesirable effect viz, toxicity, allergy, residues in food, bacterial drug resists (Ajitha et al., 2004). Moreover, these protein hydrolysates may also serve as an alternative therapy against bacterial infections to conventional antibiotics in an age of increasing occurrence of antibiotic resistant bacteria.

Table 7.1. Comparison between lactic acid fermentation and enzymatic hydrolysis on recovery of lipids and protein from fish visceral waste

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Lactic acid Fermentation</th>
<th>Enzymatic hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best LAB/ Enzyme</td>
<td><em>P. acidilactici</em> NCIM5368</td>
<td>Fungal protease</td>
</tr>
<tr>
<td>Optimized conditions</td>
<td>Sugar – 12% (w/w),</td>
<td>Enzyme conc. - 0.5%(w/w),</td>
</tr>
<tr>
<td></td>
<td>Inoculum – 15% (v/w),</td>
<td>Time – 120 min,</td>
</tr>
<tr>
<td></td>
<td>Time – 48 hours</td>
<td>Substrate-water ratio – 1:3</td>
</tr>
<tr>
<td>Total oil yield</td>
<td>94%</td>
<td>84%</td>
</tr>
<tr>
<td>Oil quality</td>
<td>No significant change in fatty acid composition</td>
<td></td>
</tr>
<tr>
<td>Protein recovery</td>
<td>74 %</td>
<td>64%</td>
</tr>
<tr>
<td>Antioxidant activity</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Antibacterial activity</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

LAB – lactic acid bacteria

**Enrichment and stabilization of PUFA in recovered from FVW**

*Enrichment/concentration of PUFA using lipases in recovered oil*

In the present study (chapter III) we have standardized lactic acid fermentation and enzymatic hydrolysis for the recovery of lipids from FVW. Fatty acid composition of FO-LAF and FO-EH showed almost similar levels of SFA, MUFA and PUFA. PUFA concentration in triglycerides, devoid of more saturated fatty acids, is much better than
original oil themselves because they allowed daily intake of total lipid to be as low as possible.

Lipase added during recovery of FVW-FO by lactic acid fermentation and enzymatic hydrolysis resulted in alteration in acid value and fatty acid composition in varying levels. Higher concentration of PUFA in triglycerides was observed in case of lipase added during lactic acid fermentation compared to enzymatic hydrolysis. Results show that, lipase catalyzed hydrolysis, especially with AN and TL sn-1,3-specific lipases resulted in concentration of both EPA and DHA with different efficiencies. Ideally, enzymatic hydrolysis using lipases followed by removal FFA will increase EPA and DHA concentrations while reducing SFA and MUFA. Aspergillus niger sn-1,3-specific lipase used in the present study, significantly improved the PUFA levels compared to other lipases during the recovery process. Morrissey and Okada (2007) have reported that non-specific lipases are better than sn-1, 3-specific lipase in concentrating PUFA in sardine oil. In triglycerides of fish oil, the second position of the glycerol moiety is usually more enriched with n-3 PUFA (Bornscheuer, 2000), although this vary depending on species (Gamez-Meza et al. 1999). The results on concentration of PUFA by sn-1,3 specific lipase also highlights the presence of n-3 PUFA on the sn-2 position in FVW-FO.

Hydrolysis with non-specific lipase CC also resulted in concentration of PUFA. This indicates the ability of CC 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). The molecular conformation of cis carbon–carbon double bonds in PUFAs, particularly EPA and DHA, causes steric hindrance and subsequent bending of the fatty acid chains, bringing the terminal methyl groups very close to the ester bonds (Chakraborty et al. 2010). Because of this steric hindrance effect, enzymatic active sites cannot reach the ester-linkages of these fatty acids with their glycerol backbones, thereby protecting EPA and DHA from lipase-catalyzed hydrolysis. However, this does not occur with the relatively straight chains of SFAs and MUFAs, and therefore hydrolysis is not hindered (Shahidi and Wanasundara, 1998; Carvalho et al. 2002). Also, it has been suggested that TGs without
EPA and DHA are hydrolyzed in the first phase, and TGs with EPA and DHA are hydrolyzed later, indicating that the lipase recognizes the whole molecular structure, not only its ester bonds (Hoshino et al. 1990). This may be true in the present study too that lipase aided hydrolysis of lipids recovered resulted in higher PUFA content compared to control.

Therefore, lipase catalyzed hydrolysis is demonstrated to be a feasible method for concentration of n-3 PUFAs during the recovery of lipids by lactic acid fermentation and enzymatic hydrolysis. Use of lipase to produce n-3 PUFA concentrate has an advantage over traditional methods such as chromatographic separation, molecular distillation etc. because such procedures involves extreme pH and high temperature which may affect the quality of oil. Therefore, a mild condition using Lipase hydrolysis adopted in this study provide a promising alternative that could also save energy and increase product selectivity. In addition, the lipase hydrolysis method produces n-3 fatty acids in the glycerol form, which is considered nutritionally favorable.

**Stabilization of PUFA during fermentation and enzymatic hydrolysis**

Fish lipids are rich in higher content of polyunsaturated fatty acids with multiple double bonds. Due to the presence of multiple double bonds in PUFAs, they are highly susceptible to oxidation and the oxidation products can have adverse health effects due to their cytotoxic and genotoxic potential (Esterbauer et al, 1990, Fang et al, 1996). Peroxide value (PV) is a simple measure of an extent of oxidation of a lipid system either during processing or storage. PV indicates the quantity of oxidized substances, normally hydroperoxides, which liberate iodine from potassium iodide under specified conditions (Rogers et al. 2001; Yanishlieva and Marinova, 2001). Fish oil during lactic acid fermentation and enzymatic hydrolysis may decompose readily which is measured by an increase in PV. In the present study, antioxidants were used to assess their ability in protecting PUFA from oxidation during the recovery of FVW-FO. The study has shown that 100 ppm of TBHQ and 150 ppm of α-tocopherol are sufficient to prevent auto-oxidation of fish oil during its recovery of oil by lactic acid fermentation and enzymatic hydrolysis.
Antioxidant inhibits or interrupts a free radical reaction of lipid auto-oxidation. Antioxidants function as free radical acceptors, thereby terminate at the initial step and also scavenge radicals formed later, in the oxidation process. The antioxidant free radical that forms is stable and does not split into other compounds that provide off-flavor and odors, nor does it propagate further oxidation of the lipid (O’Brien, 1998). In the present study, TBHQ and α-tocopherol are found to be the best antioxidant compared to other in reducing peroxide value. Previous studied by Haung et al (1994) with corn oil and by Kulas and Ackman (2001) the fish oil showed 100 ppm α-tocopherol as a concentration for maximal antioxidant activity in those oil.

It is concluded from the stabilization experiment that low concentration of TBHQ (100ppm) and α-tocopherol (150ppm) minimizes oxidation of oil during fermentation and enzymatic hydrolysis. These antioxidants can be added to homogenized FVW before lactic acid fermentation/enzymatic hydrolysis to recover good quality (unoxidized) lipids. In case of enrichment/ concentration of PUFA by lipase treatment sn-1,3 specific AN lipase was found to be the best among the lipase used in the study for both LAF and EH.

**Effect of FVW-FO on biochemical profile and growth parameters of experimental animals**

Fish oil is the major source of PUFA especially EPA and DHA, which has significant effect on biochemical and physiological parameters in the body. As fish oil was recovered from fresh water fish visceral waste by lactic acid fermentation and enzymatic hydrolysis (protease), its effect with respect to safety (growth parameters, relative organ weight, hematological and serum biochemical parameters) on feeding to rats was studied. Results showed that feeding diet containing incremental levels of FVW-FO as a source of EPA+DHA from FVW, no significant difference was observed in food intake and growth characteristics compared to control group. Changes in the body and vital organ weight have been used as one of the indicator of adverse effect of any chemical/toxic compound (Fedorova-Dahms et al. 2011). The present study suggests that feeding lipids recovered from FVW is non-toxic to rats. In addition, there was no evidence of any adverse effect of feeding dietary FVW-FO behavior and liver histology of rats. Hematological studies generally reveal anomalies in the body metabolic process.
and the blood profile usually furnishes vital information on the response of the body to injury, deprivation and/or stress (Amlashi et al. 2011). There was no significant difference in the blood parameters were observed between rats fed with FVW-FO and control groups. Earlier reports on safety of recommended pharmaceutical dose or dietary feeding of n-3 PUFA from different source also revealed no adverse effect on such hematological parameters (Liu et al. 2004; Kawashima et al. 2009). Ideally, all the values remain within the normal limits and do not show any toxic effect as a result of feeding fish oil recovered from FVW.

Triglyceride and total cholesterol are the two well recognized risk factors for cardiovascular diseases (Das, 2009). The primary objective of this investigation was to analyze the effect of FO recovered from FPW through fermentation and enzymatic hydrolysis in reducing serum cholesterol and triglycerides levels. The lipids recovered from FVW had significant (p>0.05) lowering effects on serum total cholesterol and triacylglycerol, lowered in rats fed diet containing EPA+DHA at incremental levels compared to control. Human and animal studies have shown that SFA rich diet raises serum cholesterol level whereas MUFA and PUFA rich diet reduces cholesterol and plasma triglyceride (Martin et al. 1986; Mensink and Katan, 1989). In the present study, similar to serum lipids, cholesterol and triglyceride levels in liver lipids were also decreased on feeding EPA+DHA containing diets. Present investigation also showed that FO diet containing 5% n-3 PUFA as EPA and DHA recovered by different methods had similar effect in modulating serum lipids in beneficial manner. With incremental levels of EPA+DHA in diet, there was progressive accumulation of these fatty acids in serum and liver which may be in the expense of linoleic and arachidonic acid. This may indicate that n-6 fatty acids are displaced by long chain n-3 PUFA in serum and the liver. Similar results have also been reported in rats fed with dietary cod liver oil at same concentration of EPA+DHA (Ramaprasad et al. 2006).

**Effect of FVW-FO on membrane bound enzymes and microsomal lipid composition**

The present results clearly show that dietary lipids influence both the fatty acids composition of microsomes and the activity of some of the major membrane bound enzymes. The Na\(^+\)K\(^+\) ATPase is an ubiquitous membrane-bound enzyme complex that
plays a fundamental role in cellular function. Na$^+$K$^+$ ATPase activity have been shown to decrease in rats brain fed with n-3 PUFA deficient diet (Gerbi et al. 1999), which is also proven in long term deficiency of n-3 PUFA at optimal ATP concentrations (Bourre et al. 1984). Supplementation of fish oil in diet normalized the synaptosomal Na$^+$ ATPase activity (Horrocks and Farooqui, 2004) which may be due to stabilization of neural membranes by DHA. Feeding of DHA has shown to increase membrane fluidity and activity of Na$^+$K$^+$ ATPase (Hashimoto et al. 2001). Supplementation of cod liver oil and DHA has been reported to increase acetylcholinesterase and Na$^+$K$^+$ ATPase activities in different parts of the brain (Kumosani and Moselhy, 2011). Studies have reported that, the altered learning and memory abilities in rats and mice fed low LNA diet may be due to lower Na$^+$K$^+$ ATPase suggesting be a biochemical basis for a altered brain functions (Carrie et al. 1999; 2000). In the present study, we observed a significant increase in Na$^+$K$^+$ ATPase activity in liver and brain microsomes and Ca$^+$Mg$^+$ ATPases in heart microsomes of rats fed with diet containing 5% EPA+DHA compared to control. The increase in the activity of acetylcholine esterase was also found with the increase in DHA levels in microsomes. This indicates that these enzymes show higher activity on feeding fish oil recovered from fishery byproducts indicating maintanance of membrane function.

PUFA and cholesterol levels in membrane also play important role in proper functioning of membrane proteins. Animals fed on a fish oil diet over a 4 week period have shown an increase in the DHA level and decreases in the arachidonic acid levels of brain phospholipids (Galli et al. 1971; Eddy and Harman, 1977; Suzuki et al. 1989; Bourre et al., 1990). The synapase plays an important role in communication between brain nerve cells and the membrane contains high levels of DHA. Moreover, the learning ability in young second generation animals bred by animals fed on the n-3 PUFA rich diet is higher than that in the young bred fed on the fatty acid deficient diets (Lamptey and Walker, 1976; Yamamoto et al. 1987; Yonekubo et al. 1994). These studies assume to clarify the effect of n-3 PUFAs on the learning ability and the development of intelligence in children. Many studies using PUFA deficient diets have shown reductions in the level of DHA in brain and loss of cognitive functions (Greiner et al. 1999; Ahmed et al. 2002). DHA level in liver, brain and heart microsomes increased significantly with
incremental level of EPA+DHA in the diet from FO-LAF and FO-EH. It is also evident from the results that there was a marked increase in the proportion of total n-3 fatty acids (mainly 20:5 and 22:6), and a concomitant decrease in n-6 (20:4 and 18:2) fatty acids in the microsomes of rats fed on FVW-FO. As a consequence, there is a significant reduction in the ratio n-6: n-3 in fish oil fed groups compared to control.

Altered cholesterol to phospholipid ratio decreases membrane fluidity, as the cholesterol sterically prevents the large motion of phospholipid fatty acyl chains (Yeagle et al. 1990). Oxidative stress reported to raise the level of brain cholesterol to the level of aged rats (Denisova et al. 2001). Feeding rats with diet containing incremental levels of EPA+DHA from FVW-FO showed reduction in cholesterol level and cholesterol/phospholipid ratio in microsomes of liver, brain and heart suggesting the beneficial effect of FVW-FO. The present results, supports the view that feeding FVW-FO affects activities of membrane bound enzymes and microsomal lipid profile in a beneficial manner suggesting that FVW-FO can be a sustainable alternative to fish oil.

**Bioefficacy of lipids recovered from FVW by fermentation and enzymatic hydrolysis**

Utilization of lipids rich in n-3 PUFA from FVW with potential biological activities may provide a means for value addition to the FVW. The primary objective of this investigation was to find out the effect of FVW-FO recovered by biotechnological approaches in reducing cholesterol and triacylglycerols content as they are the two well recognized risk factors for cardiovascular diseases (Das, 2008). The FVW-FO recovered by LAF and EH had significant and similar effect to CLO on serum and liver lipids. The serum and liver total cholesterol, LDL and triacylglycerols lowered significantly (p<0.05) in rats fed with diet containing FO-LAF and FO-EH in a dose dependent manner compared to GNO fed group. With incremental levels of EPA+DHA in diet there was progressive accumulation of these fatty acids in serum and liver which may be at the expense of LA and ARA. This may indicate that n-6 fatty acids are displaced by long chain n-3 PUFA in serum and liver, which is previously reported in rats fed with incremental level of CLO (Ramaprasad et al. 2010). FVW-FO also showed similar effects in comparison to CLO by replacing the n-6 PUFA in liver, brain and heart. The present
study clearly reveals the value of n-3 PUFA from byproducts/wastes generated from fresh water fish processing as an alternative sources of commercial fish oils.

Liver is a major site of lipid metabolism and sensitive to alteration in the dietary fatty acids. As FVW-FO showed cholesterol lowering properties in serum and liver, this study investigated the mechanism with respect to down regulation of HMG CoA reductase activity in comparison with CLO. The biosynthetic pathway for cholesterol is regulated by the activity of HMG-CoA reductase (Dietschy et al. 1993). The activity of this enzyme in liver microsomes was reduced significantly when EPA+DHA containing diet was fed to rats. Results indicate that FO-LAF and FO-EH affects cholesterol levels by reducing the activity of HMG-CoA reductase. Le Jossic et al (2005) have shown that feeding tuna fish oil reduced cholesterol levels in rodents by down-regulating hepatic cholesterol synthesis through inhibition of HMG-CoA reductase. Similarly, Castillo et al (1999) observed lower activity of HMG-CoA reductase in chicken which were fed with menhaden fish oil. FVW-FO in this study can be a sustainable alternative to commercial FO as they have similar to that of CLO in reducing cholesterol and triacylglycerols levels and HMG-CoA reductase activity.

PUFA also plays an important role in maintaining heart and brain functions. Depending on the extent of incorporation of EPA and DHA recovered from FVW in these tissues they will have health benefits like those of CLO. Studies using PUFA deficient diets have shown reduction in the level of DHA in brain and loss of cognitive functions (Greiner et al. 1999; Ahmed et al. 2002). Results of the present study showed that DHA levels in heart tissue increased by 70 - 220% and 74-204% with incremental level of EPA+DHA in diet with FO-LAF and FO-EH, respectively. Incorporation of DHA in heart and brain lipids was similar between the groups fed with FO-LAF and FO-EH and comparable with CLO fed group. There are reports on very high content of DHA in heart and skeletal tissue on high dose of DHA in the diet by Atkinson et al (1997). In this study, we have found a decrease in ARA in heart tissue of rats fed with FO-LAF (6.7 - 17.8%) and FO-EH (5.92 – 16.45%), with increased concentration of EPA and DHA. Similarly, higher uptake of DHA and reduction in ARA has also been reported in rat’s heart (O’shea et al. 2009; Ramaprasad et al 2010). Increases in n-3 fatty acids are
compensated by decreased ARA, primarily in heart, liver and kidney while in other tissues the content of lauric and myristic acids are also reduced (Otten et al. 1993). The higher levels of DHA in the brain play a crucial role in the nervous system (Connor and Neuringer, 1988). Feeding incremental levels of FO-LAF and FO-EH as a source of EPA+DHA progressively increased DHA levels in brain by 47.3 - 153% and 53–140% compared to that of control. The level of incorporation of DHA was found to be tissue specific and it was higher in heart (70-220%) than that in brain (47.3-153%). Animals fed with higher level of n-3 fatty acids have been reported to increase its content by 40-165% compared to the control (Otten et al. 1993). Similarly, Ramaprasad et al (2010) also observed an increase in DHA levels in brain by 37-104% when rats were fed with diet containing 1-5 % of EPA+DHA over that found in GNO fed group. The present study demonstrates that the effect of FVW-FO obtained through solvent free biotechnological approaches is as effective as CLO in reducing cholesterol, HMG-CoA reductase activity.

**Lipid peroxidation and antioxidant enzymes**

Fish oil is the major source of PUFA especially EPA and DHA, which has significant beneficial effect on biochemical and physiological changes in the body. Higher levels of PUFA in tissue increase the susceptibility to oxidation and, as a result, may increase lipid peroxides concentration in the cellular systems (Shireen et al. 2008). Under such conditions, the endogenous antioxidant defense molecule should scavenge the superoxide and peroxides before they react with metal catalyst which leads to oxidative stress. A decrease in activity of antioxidant enzymes may predispose cells to free radical damage (Huang and Fwu, 1993). Hence, in the present study bioefficacy of FVW-FO recovered by LAF and EH was examined with respect to antioxidant potential.

Feeding diet incorporated with FVW-FO at 1.25, 2.5 and 5.0% of EPA+DHA resulted in elevation of the levels of lipid peroxides in serum, liver, brain and heart. Further the antioxidative defenses (CAT and SOD activity) increased significantly; this compensates the peroxides level in different tissues. Earlier reports have shown that n-3 PUFA can elevate the mRNA expression of CAT and strengthens the antioxidant status in mice (Venkatraman et al. 1994; Aguilera et al. 2003). The inability of the antioxidant enzymes to prevent against oxidative damage in tissues may affect the cellular function
such as membrane permeability. In this context, plasma membrane fluidity and activity of enzymes can get affected because lipid peroxidation rigidifies the membrane by extensive cross-linking of the membrane constituents. Levin et al (1990) have proposed that the oxidation of membrane lipids results in the formation of peroxidation degradation products (Malondialdehyde), which leads to the crosslinking reactions of the lipid-lipid and lipid-protein type thereby making the membrane more rigid and hence less fluid.

**Platelets aggregation**

Blood platelets function is thought to be critical in mechanism involved in atherosclerosis and arterial thrombosis. It is reported that dietary PUFA influences platelet aggregation in beneficial manner (Martins et al., 2007). The present investigation was undertaken to assess the influence of dietary FVW-FO recovered through biotechnological approaches on platelets aggregation and eicosanoids levels in serum. It was found that rats fed with FO-LAF and FO-EH showed reduction in both ADP and AA induced platelet aggregation compared to rats fed with GNO. FVW-FO recovered in this study and the commercial CLO showed similar effect in reducing platelets aggregation. The present study clearly reflects the value of byproducts/wastes generated from fresh water fish processing industries as an alternative source of FO.

Changes in dietary lipids provide a non pharmacological means of altering eicosanoids synthesis that may in turn influence the aggregability of platelets (Ramaprasad et al. 2005; Reena et al. 2010). With the incremental level of EPA+DHA in diet, there was progressive accumulation of these fatty acids in platelets which may be at the expense of linoleic (LA) and arachidonic acid (AA). This indicates that n-6 PUFA may be displaced by a long chain n-3 PUFA in platelets, which increases the n-3/n-6 ratio. LA is the precursor of AA, which in turn is a substrate for the generation of eicosanoids synthesized by the action of cyclooxygenase (Bunting et al. 1983). Ratio of prostacyclin to thromboxane is considered to be an indicator of thrombosis (Bunting et al. 1983). FO enriched diet also lowers the LA and AA levels in platelets that are the substrate for thromboxane production. This resulted in reduction of thromboxane production in rats fed with FO-LAF and FO-EH compared to GNO fed rats. The reduction of thromboxane was higher (21.2 – 40.6%) compared to prostacyclins (12.6 –
22.4 %). The higher percentage of reduction of thromboxane compared to prostacyclin tilted the balance for prostacyclin, thromboxane ratio favorably towards reducing the aggregation of platelets. This was reflected on reduction of platelets aggregation in rats fed with FO-LAF and FO-EH compared to those fed with GNO.

Reduction in cholesterol levels and cholesterol/phospholipid ratio also affects the platelets aggregation in a beneficial manner (Osamah et al. 1997; Bagger et al. 1996). Higher cholesterol content has shown to influence AA metabolism by increasing the conversion of AA to thromboxane B\(_2\) (the stable end product of thromboxane A\(_2\)) (Stuart et al. 1980). Feeding rats with FO-LAF and FO-EH reduces the cholesterol and cholesterol/phospholipid ratio as compared to rats fed with GNO. This suggested that reduction of cholesterol in platelets of FVW-FO fed groups also may be contributed in reducing platelet aggregation.

Platelet aggregation is a complex process, where a cascade of reaction occurs that are influenced by number of factors such as dietary fatty acid composition, platelets fatty acid composition, cholesterol/phospholipids ratio and types of eicosanoids synthesized. Earlier studies have shown that dietary lipids influences fatty acid composition of platelets and also affect release of thromboxane from platelets (Reena et al. 2010). The present study analysed the correlation between platelet aggregation and variable factors, which influence the process of platelet aggregation. It was found that platelet aggregation is negatively well correlated with n-3/n-6 ratio, DHA/ARA ratio and PGF\(_{1\alpha}/\)TBXB\(_2\).
Summery

1. Fish processing waste is a potential source of lipids rich in polyunsaturated fatty acids. Lipid content in processing waste (on wet weight basis) was higher in fresh water fishes (4.3 – 27.8 %) compared to marine fishes (2.7 – 15.1%).
2. In case of fresh water fishes lipid content in higher in visceral waste (4.3 – 27.8 %) compared to meat portion (0.8 – 3.8%). Among fresh water fishes lipid content was found to be highest in rohu visceral waste (27.8 %). The presence of high lipid content in waste reflects to their potential as a source of fish oil.
3. Lactic acid fermentation and enzymatic hydrolysis were standardized for simultaneous recovery of lipids and protein from fish visceral waste (FVW).
4. Lactic acid fermentation of FVW using a native lactic acid bacteria *P. acidilactici* NCIM5368 resulted in higher lipid (93.57%) and protein (74.59%) recovery whereas, enzymatic hydrolysis of FVW using Fungal protease resulted in lipid (80.33 %) and protein (62.5%) recovery indicating the potential application of lactic acid fermentation.
5. *Aspergillus niger* was the best lipase for concentration of PUFA during Lactic acid fermentation and enzymatic hydrolysis.
6. Addition of TBHQ (100ppm) or α-tocopherol (150ppm) can be added to homogenized fish visceral waste to prevent oxidation of PUFA during lactic acid fermentation and enzymatic hydrolysis.
7. Studies on effect of feeding fish oil recovered from FVW by lactic acid fermentation (FO-LAF) and enzymatic hydrolysis (FO-EH) to rats showed no adverse effect in growth and biochemical parameters even at oil containing 5% EPA+DHA.
8. Feeding FO-LAF and FO-EH improved the activity of membrane bound enzymes in a tissue specific manner and improved the fatty acid composition in microsomes demonstrating incorporation of EPA and DHA.
9. FO-LAF and FO-EH showed similar hypocholestermic properties to cod liver oil (CLO).
10. Compared to GNO fed group, LAF-FO and EH-FO fed groups showed a decrease in platelet aggregation by 12.8 - 23.3% and 11.8 - 21.7%, respectively.
11. FO-LAF and FO-EH enhanced the activity of antioxidant enzymes in tissues, and improved the fatty acid composition (incorporation of EPA and DHA) in serum, brain, liver and heart similar to CLO.

Conclusions

Studies on lipid class and fatty acid composition of waste from various marine and fresh water fishes clearly reveals the value of byproducts/ waste as an alternative source of fish oil. Approaches like lactic acid fermentation and enzymatic hydrolysis can be effectively used to recover biomolecules like lipids, protein hydrolysate, and collagen from fish processing wastes. These approaches may help in reducing the organic load caused by the fish processing industry. Lipids recovered are rich sources of PUFA (EPA+DHA) which have several health benefits that can be a better alternative to regular fish oil. The protein hydrolysates obtained which are rich in low molecular weight peptides exhibit various biofunctionallities like antioxidant activity. Collagen recovered as a residue in these processes may finds its way in diverse fields like cosmetic and biomedical industries. The recovered biomolecules may have potential application as a feed ingredient and/or as a nutraceutical in animal/livestock feeds.

Lipids recovered from fish processing byproducts (FVW-FO) of commercial fresh water fishes by eco-friendly biotechnological approaches can be better alternatives to commercial fish oil as they don’t have any undesirable effect on growth and biochemical properties. The present investigation shows that rats fed with fish oil recovered through biotechnological approach showed similar anticholesterolemic properties as compared to CLO. FVW-FO beneficially modulated serum prostaglandins and lowered platelets aggregation in beneficial manner as in case of CLO and thereby reduces the risk of thrombosis. FVW-FO recovered from FVW can effectively contribute towards sufficing the demand for fish oils in the aqua- or livestock feed industries and nutraceuticals. In addition, utilization of these processing wastes for production of valuable biofunctional products can reduce the mounting economic values of fish oil and benefit the processing industry economically and by minimizing the environmental pollution problems.