Valorisation of fish waste biomass through recovery of nutritional lipids and biogas

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2011

LUND
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Valorisation of fish waste biomass through recovery of nutritional lipids and biogas

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Department of Biotechnology

Doctoral Thesis

April 2011

Academic thesis which, by due permission of the Faculty of Engineering of Lund University will be publicly defended on Wednesday, April 13 at 10.30 a.m. in Lecture Hall B, at the Centre for Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of philosophy in Engineering.

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Doctoral thesis, 2011

Doctoral thesis, 2011

Department of Biotechnology

Lund University

P.O. Box 124, SE-221 00

Sweden

ISBN 978-91-89627-72-7

ISRN LUTKDH/TKBT--11/1126—SE

Printed by Media-Tryck, Lund University, Sweden, 2011

Organization LUND UNIVERSITY Department of Biotechnology Center for Chemistry and Chemical Engineering P.O. Box 124 SE-221 00 Lund, Sweden	**Document name** DOCTORAL DISSERTATION **Date of issue** April 13, 2011
Author(s) Betty Nyambura Mbatia	**Sponsoring organization** Sida/SAREC

Title and subtitle
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Abstract

Commercial fish catch in Eastern Africa is dominated by Nile Perch. Of the fish that is processed for human consumption, 30-40 % is wasted. Currently, these wastes are not fully utilized; they are sold off at low price, converted to low valued products or left to decompose leading to environmental pollution and wastage of bioresource. This biomass has however a potential to generate considerable revenue and can be turned into a commercially viable business. It can be used in production of fish oils, bio-energy, proteins, enzymes, organic fertilizers among others. Fish oils are a source of n-3 polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) associated with positive effects on human health.

The current thesis focuses on extraction and enrichment of PUFAs and production of biogas from fish by-products hence value addition. An enzymatic pre-treatment step was used to hydrolyse fish by-products resulting in recovery of three intermediate products (i) fish oil, (ii) fish protein hydrolysate and (iii) insoluble fraction (sludge + emulsion). The three fractions can be upgraded to products of more value.

By use of lipases, the PUFA content in the extracted oil was enriched in three forms, (i) acylglycerols, (ii) free fatty acids, (iii) ethyl esters hence adding value to the intermediate oil fraction. Best EPA and DHA recovery was attained when PUFAs were enriched as ethyl esters. Consumption of PUFAs devoid of saturated and monounsaturated fatty acids is preferred for preventive purposes. Due to their high susceptibility to oxidation, PUFAs were esterified to natural phenolics with antioxidant properties to stabilise the PUFAs against oxidation and also improve hydrophobic character of the phenolics.

To ensure maximum utilisation of the fish by-products, the insoluble fraction achieved after proteases hydrolysis was digested under anaerobic conditions for biogas production. Due to its high content in plant nutrients such as nitrogen, phosphorous and potassium, the digestate is a good biofertilizer.

Key words
by-products, value addition, EPA, DHA, PUFA, biogas

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language English

ISSN and key title

Recipient's notes

ISBN
ISBN 978-91-89627-72-7

Number of pages 137

Price

Sesurity classification

Distribution by (name and address)

Mbatia B. N., Department of Biotechnology, Lund University P.O. Box 124 SE-221 00 Lund, Sweden

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Dedicated to dad, mum and my twin sister

Abstract

Commercial fish catch in Eastern Africa is dominated by Nile Perch. Of the fish that is processed for human consumption, 30-40% is wasted. Currently, these wastes are not fully utilized; they are sold off at low price, converted to low valued products or left to decompose leading to environmental pollution and wastage of bioresource. This biomass has however a potential to generate considerable revenue and can be turned into a commercially viable business. It can be used in production of fish oils, bio-energy, proteins and organic fertilizers. Fish oils are a source of n-3 polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) associated with positive effect on human health.

In this study, proteases were used to liberate oil from Nile perch (*Lates niloticus*) and salmon (*Salmon salar*) by-products. An oil yield of 11.2% and 15.7% of wet weight was obtained from warm water Nile perch and cold water salmon heads respectively, compared to 13.8% and 17.6% respectively, using solvents. Addition of water during the enzymatic hydrolysis decreased the oil yield. The DHA and EPA contents of oil extracted from Nile perch were 9 and 3 mol%, respectively. To further enrich DHA and EPA contents in Nile perch oil, use of lipases from *Candida rugosa*, *Thermomyces lanuginosus* and *Pseudomanas cepacia* were investigated. In the first case, the lipases were used to hydrolyse the natural oil. Non-regiospecific lipase from *C. rugosa* gave the best combined enrichment of EPA and DHA with EPA and DHA being enriched to 6 and 23 mol%, respectively. On the contrary, lipase from *T. lanuginosus* enriched DHA to 38 mol% but was ineffective in enriching EPA. Being a 1,3-specific lipase, the level of enrichment attained with *T. lanuginous* lipase was to a large extent influenced by the positional distribution of fatty acids within the triglyceride molecule. EPA was mainly in *sn* 1,3 positions while DHA was equally distributed in the 3 positions.

To avoid complications associated with non-homogenous distribution of PUFAs in triglyceride molecules, free fatty acids (FFA) or fatty acid ethyl esters (FA-EE) derived from the natural oil were used as substrates in another study. In this case, lipase from *T. lanuginosus* was able to enrich both DHA and EPA. Evaluated lipases showed lowest specificity to EPA and DHA when present as ethyl esters and better recoveries of EPA and DHA were achieved when they were present as ethyl esters than when present as FFA or in glycerides. Both esterification of FFA and hydrolysis of FA-EE were more effective at enriching PUFAs than hydrolysis of the natural oil.

In an attempt to add more value to fish oil, PUFA concentrate obtained from salmon heads by urea fractionation was used to lipophilize hydrophilic phenolic derivatives (vanillyl alcohol or rutin) which are natural antioxidants. Lipase from *Candida antarctica* was used to catalyse the esterification reaction. The synthesized lipophilic derivatives showed antioxidant activities with rutin esters showing more activity in the 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical assay than the vanillyl esters and on the contrary in the lipophilic medium, the vanillyl esters were found to be superior to the rutin esters. In bulk oil system, the antioxidant activities of rutin and vanillyl derivatives was lower than that of BHT and α-tocopherol but in emulsion, they showed better activity than α-tocopherol. The PUFA-phenolic molecules carry combined health beneficial properties associated with PUFAs and phenolics. In addition, the PUFAs are protected against oxidation by the phenolic moiety while PUFA makes the antioxidant more lipophilic which may enhance its function in lipid systems.

To make maximum utilisation of the fish by-products, the insoluble fraction that remained after oil extraction was used for biogas production through anaerobic digestion. Methane yields before and after oil extractions were 828 and 742 m^3 CH_4/ton of volatile solids (VS) added, respectively. Despite the high methane yields, fish sludge/fish waste cannot be digested alone in a continuous anaerobic digester due to high content of proteins, lipids and light metals (sodium, potassium and calcium) that are inhibitory to methanogenesis. Co-digestion of the sludge with residues from crop cultivation was thus evaluated. Methane yields were 531 and 403 m^3 CH_4/ton of VS added when the ratio of Jerusalem artichoke residues: sludge was 1:1 or 3:1, respectively while that of JA alone was 283 m^3 CH_4/ton of VS.

In conclusion, enzyme technology represents valuable tools that can be used in fish processing industries to convert fish waste into products with a higher market value. The use of proteases for the hydrolysis of the by-products results in maximum utilisation of the by-products since the intermediate hydrolysis products can be processed further for valorisation. Lipid fraction can be used for recovery of omega-3 fatty acids and biodiesel. The soluble protein fraction has several applications e.g. in food industries or in microbiological media and the sludge fraction can be used in anaerobic digestion for biogas production. Due to its high protein content, sludge fraction can also be used as animal feed or as biofertilizer due to high content of plant nutrients such as nitrogen, phosphorous and potassium.

1. List of publications

This thesis is based on the work contained in the following papers, referred to by their Roman numerals in the text. The papers are attached as appendices at the end of the thesis.

Paper I. Betty Mbatia, Dietlind Adlercreutz, Patrick Adlercreutz, Ally Mahadhy, Francis Mulaa, Bo Mattiasson. Enzymatic oil extraction and positional analysis of ω-3 fatty acids in Nile perch and salmon heads (2010). *Process Biochemistry 45(5)815-819.*

Paper II. Betty Mbatia, Patrick Adlercreutz, Francis Mulaa, Bo Mattiasson. Enzymatic enrichment of n-3 polyunsaturated fatty acids in Nile perch (*Lates niloticus*) viscera oil. *European journal of lipid science and technology* (2010), 112, (9), 977-984.

Paper III. Betty Mbatia, Patrick Adlercreutz, Francis Mulaa, Bo Mattiasson. Strategies for the enzymatic enrichment of polyunsaturated fatty acids from fish oil. *Accepted for publication in European journal of lipid science and technology.* DOI: 10.1002/ejlt.201000560.

Paper IV. Betty Mbatia, K. Shiva Shanker, B. Mattiasson, F. Mulaa, P. Adlercreutz. Enzymatic Synthesis of Lipophilic Rutin and Vanillyl Esters From Fish By Products. *Manuscript*.

Paper V. Nges Ivo Achu, **Betty Mbatia** and Lovisa Björnsson. Improved utilization of fish waste by anaerobic digestion following omega-3 fatty acids extraction. *Submitted.*

2. My contribution to the papers

All the work presented in this thesis was performed under the supervision of Professors Bo Mattiasson and Patrick Adlercreutz. For work presented in **Paper I**, Dr. Ditelind Adlercreutz was also a supervisor.

Paper I: I performed the experimental work with Ally and wrote the paper along with the co-authors.

Paper II: I performed the experimental work and wrote the first draft of the manuscript. I was responsible for correcting it after comments from the co-authors

Paper III: I performed the experimental work and wrote the first draft of the manuscript. I was responsible for correcting it after comments from the co-authors.

Paper IV: I performed the experimental work with Shiva. I wrote the first draft of the manuscript and was responsible for correcting it after comments from the co-authors.

Paper V: I performed the experimental work with Ivo. I contributed to the manuscript.

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3.0 Introduction

3.1 Fish by-products

The amount of by-products from fish varies with fish species, size, season and fishing ground [1]. The by-products are generated when the fish is gutted, headed and further processed into fillets. Depending on the efficiency of the production process, only 50-60% of total fish catch goes for human consumption [2]. The by-products include heads, viscera, skin, trimmings and fish rejects [3]. They are often dumped, used as animal feed or as fertilizer [2, 4]. Due to the worldwide decline of fish stocks, a better use of by-catch and by-products is important. These biomasses have great potential as a source of high valued products due to their high protein content, high levels of essential nutrients such as vitamins, minerals, and in particular, fish oils which are the subject of investigation in the current thesis.

By-products from farmed salmon are used to produce fish oil of a quality that is well suited for human consumption [5]. Besides salmon, by-products from other fish species such as herring, cod, mackerel and sardine are used [6, 7]. By-products from warm water fatty fish such as Nile perch, present in East African Lakes and rivers could also be used as a source of raw material for marine oils, enzymes, proteins and other valuable products [8]. Nile perch makes 60% of the total commercial fish catch and it is mainly processed into chilled fish fillet for export [9]. The processing leads to high volume of by-products (**Figure 1**), with an annual solid waste of 36,000 tonnes being generated by fish processing industries along Lake Victoria [10]. A further increase in fish processing wastes is expected with the identification of aquaculture as a means to eradicate poverty and hunger in the region [11].

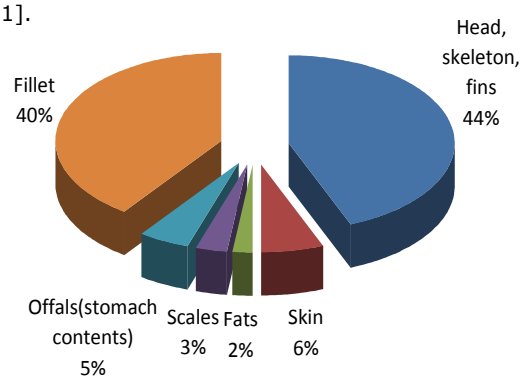


Figure 1. Products and by-products from Nile perch processing.

3.1.1 Social-economic impact on valorisation of fish by-products in Eastern Africa

The current use of by-products from Nile perch processing is shown in **Table 1**. Products obtained from Nile perch processing waste currently have a low market value. For example, 20 litres of unrefined Nile perch oil costs 25 Euro [12], while heads, skin and frames costs 54, 40 and 30 Euro/ton, respectively [10].

Table 1. Current uses of Nile perch processing by-products and alternative more valued products

By-product	**Current use**[10, 12]	**High added value compounds**	**Mean market value of selected high valued products** [4]
Frames (heads, skeleton & fins)	Food , fish meal for animal feed	Fish protein hydrolysate, peptone, amino acids, fish oils, omega-3 PUFAs concentrates, biodiesel	Purified cod liver oil with 23 % omega-3 PUFAs (24 Euro/Kg)
Trimmings	Food	Fish protein hydrolysate, Peptone, amino acids	
Roe/eggs	Food	Phospholipids	
Skin	Fuel, tanned to leather at small scale	Collagen , gelatin, gelatine peptides	Collagen and gelatin (9-14 Euro/Kg)
Fat pads	Unrefined oil for frying other fish products	Fish oils, Omega-3 PUFAs concentrates, fat soluble antioxidants, biodiesel	
Factory rejects and by-catches	Food	Fish protein hydrolysate , peptone, Fish oils,omega-3 PUFAs concentrates, biodiesel	
Viscera	Discarded	Enzymes, fish oils, biodiesel	Cod proteases (14,400 Euro/Kg)

Integration of Nile perch processing with better utilisation of the by-products through recovery of high value end-products presents an opportunity to open new business ventures and thus create new employments in East Africa. Three major groups of products: (1) plant fertilisers, (2) livestock feeds, and (3) human value-added foods and speciality foods can be derived from fish by-products [13]. Conversion of these by-products to fertilisers and animal feeds results in the least value addition. Better profitability is achieved in making useful products for human consumption. The

highest profitability is achieved in extracting and purifying bioactive compounds such as omega-3 PUFAs, enzymes and bioactive peptides for biotechnological or pharmaceutical applications [2]. These products are present in highest concentrations in the by-products [14].

Increase in returns from the sale of value added products would boost national income and enhance food security in the region. Fish proteins would serve as valuable nutritional supplements in diets with low amount of protein in developing countries fighting malnutrition. On the other hand, omega-3 PUFAs are widely accepted as food supplements due to their positive effect on health [15], and could be used to enrich everyday food products like bread and margarine.

Development and establishment of a technology for recovery and purification of high valued end-products from the fish biomass would result in maximum utilization of the by-products thus ensuring sustainability of East Africa fishing activities. It would also reduce environmental pollution resulting from damping of such waste.

3.2 Marine lipids

Lipids found in fish, especially cold water fish, and other marine organisms such as phytoplankton are called marine lipids. High levels of long chain omega-3 polyunsaturated fatty acids (PUFAs); docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), makes marine lipids unique compared to other lipid sources. Due to their well known health benefits, the market for these lipids is expected to grow further [16]. This makes new sources valuable in particular if these sources are presently underutilised. It is reported that oil content of fish waste ranges between 1.4 and 40.1% depending on the species and tissue [17], with PUFAs content up to 30% of total fatty acids [18]. Cold water fish have been reported to have high contents of PUFAs. Due to presence of double bonds, PUFAs remain liquid at low temperatures and thus important in keeping the cell membrane fluid which allows movement of membrane components even at low temperatures. Interestingly, Nile perch, a warm water fish has also been reported to contain considerable amounts of PUFAs [9, 19].

3.2.1 Synthesis of PUFAs in human

Most fatty acids can be synthesised in the body, but humans lack the enzymes required to produce two essential fatty acids (EFA); the n-3 PUFA α-linolenic acid (C18:3, ALA), and the n-6 PUFA linoleic acid (C18:2n-6, LA). These fatty acids must

be acquired from the diet. Linoleic acid is abundant in oils from most vegetable seeds such as corn and safflower while α-linolenic acid is found in the chloroplasts of green leafy vegetables and seed oils such as walnut, rapeseeds and soybeans. The n-3 and n-6 fatty acid families are derived from these EFA through a series of enzyme catalyzed desaturation and elongation reactions that take place in the cell cytosol or in the mitochondria [20]. Docosahexaenoic acid is synthesised from ALA *via* EPA and docosapentanoic acid (DPA; 22:5 n- 3), whereas arachidonic acid (AA; 20:4n-6) is synthesised from LA *via* gamma (γ) linolenic acid (GLA; 18:3n-6) (**Figure 2**).

Since both n-6 and n-3 PUFA are synthesised through the same desaturation/elongation pathway, there exists potential for competition between these two families of fatty acids. The initial conversion of ALA to 18:4n-3 by the action of Δ6-desaturase is the rate limiting reaction of the pathway. Despite the higher affinity of Δ6-desaturase for ALA than for LA [21], the typically higher concentrations of LA than of ALA in cellular pools results in greater conversion of n-6 PUFA. It has also been reported that the pathway for conversion of ALA to EPA and DHA in human is limited and less than 8% of ALA is synthesised to EPA, while only between 0.02% and 4% of ALA is synthesized to DHA, with women having a higher capacity to synthesize DHA than men [22-24]. Since the capacity to synthesize EPA and DHA from the ALA is very limited, direct intake of the former two is the easiest way to increase the amounts of these fatty acids in human tissues. They can be obtained from marine mammals' oils (e.g., seal and whale blubber), fish and fish oils (e.g., menhaden, salmon, tuna and Nile perch), as well as marine algae [25].

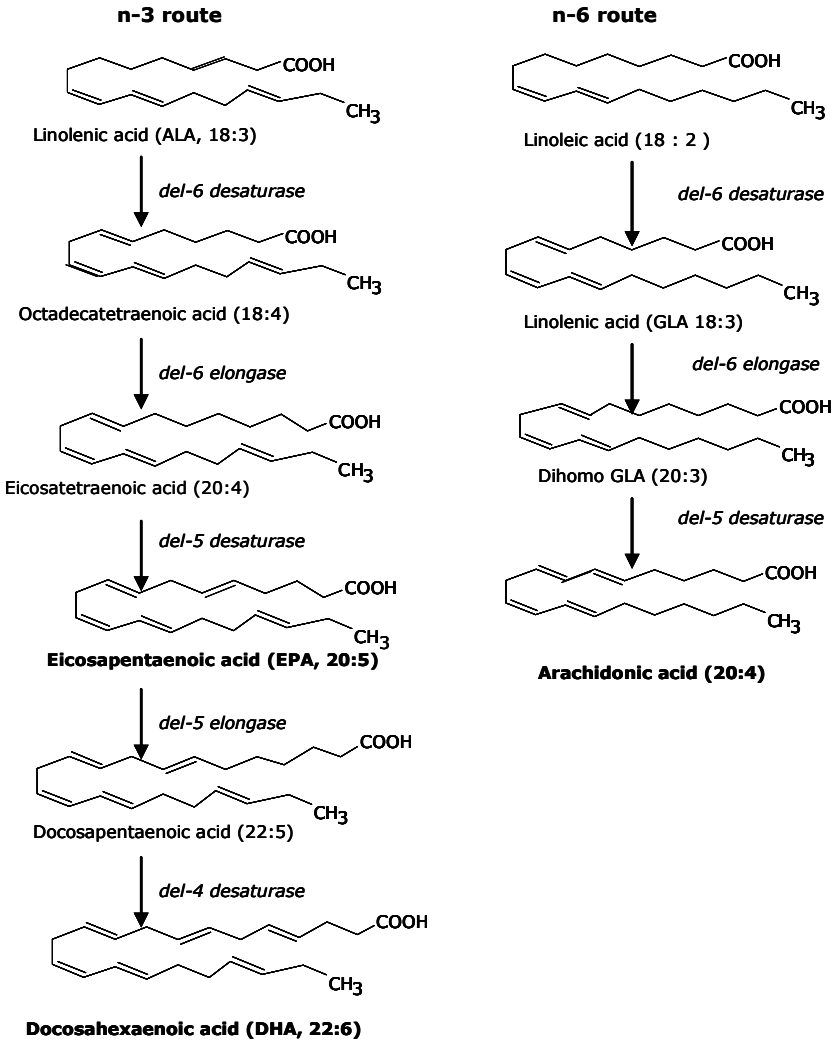


Figure 2. Synthesis of n-3 and n-6 PUFAs in mammals (Adapted from Napier & Sayanova [20].

3.3 Health aspects of marine lipids

The recommendation for dietary consumption of at least two servings of fish per week [26], is based on the many health promoting properties of long chain omega-3 PUFAs. The role of n-3 PUFAs in protecting against atherosclerosis, the pathological process leading to cardiovascular diseases is well documented [27]. The fatty acids favourably affect a number of factors involved in atherosclerosis development for example, they reduce production of inflammatory eicosanoids, lower blood pressure,

serum triacylglycerol and cholesterol levels as well as prevent thrombosis and cardiac arrhythmias [27].

DHA is required in the development of the nervous system, brain and eyesight of both foetus and infant [25, 28], while in adults, it is essential in the maintenance of the normal functions of the nervous system and the brain [28]. Deficiency of DHA is associated with several disorders such as foetal alcohol syndrome, attention deficit/ hyperactivity, depression and Alzheimer disease. EPA is the precursor of eicosanoids that provide anti-inflammatory effect [29].

Eicosanoids are mediators and regulators of inflammation derived from 20 carbon PUFAs, dihomo γ -linolenic acid, AA and EPA [30]. Usually, inflammatory cells contain high proportions of AA and low proportions of other 20-carbon PUFAs thus AA is the major substrate of eicosanoid synthesis [29]. Synthesis begins with release of PUFAs from membrane phospholipids through phospholipase hydrolysis followed by the conversion of PUFA to eicosanoids by the action of cylcooxygenase, lipoxygenase and cytochrome P-450 pathways [31, 32]. Cylooxyganese is responsible for oxidation of AA to prostaglandins (which regulate muscle contraction, reduce blood pressure and inhibit blood clotting) and thromboxane (induce blood clotting) [30], while lipooxygenase catalyses synthesis of leukotrienes that are involved in allergic responses. Omega-3 fatty acids obtained from the diet affect AA metabolism since they displace AA from membranes and also compete with AA for the enzymes that catalyze the biosynthesis of thromboxanes, prostaglandins and leukotrienes [29]. Metabolism of AA produces 2-series prostanoids (prostaglandins and thromboxanes) and 4-series leukotrines while metabolism of EPA induces production of different eicosanoids such as 3-series prostanoids and 5-series leukotrienes, which have lower activity (e.g. less pro-inflammatory) than AA metabolites [33]. Since eicosanoid formation from AA and EPA share a common set of enzymes, the ratio of n-3 to n-6 PUFAs in the diet determines which pathway is most active [31].

3.4 Scope of this work

The purpose of the work presented in this thesis was to valorise waste from fish filleting industries through recovery of valued products. In **Paper I**, oil was extracted from fish by-products using proteolytic enzymes. Effects of hydrolysis time, enzyme concentration and water content on oil yields were evaluated. To add more value to the extracted oils, DHA and EPA contents in the extracted oils were enriched using lipases or urea crystallisation [**Paper II, III** and **IV**]. In **Paper II**, EPA and DHA were enriched in the glyceride fraction using different lipases. The

effect of temperature on level of enrichment was also investigated. In **Paper III**, EPA and DHA were enriched as free fatty acids or fatty acid ethyl esters using lipases. Specificity of lipases towards EPA and DHA was investigated in Paper **II** and **III**. An HPLC-ELSD method for simultaneous analysis and quantification of fatty acids and fatty acid esters from fish oil was also developed [**Paper II**]. The method allowed analysis of most predominant fatty acids and fatty acid esters from fish oil without prior separation and/or derivatization.

Another study was aimed at the synthesis of PUFA-phenolic derivatives which could serve as antioxidants in bulk oil or emulsion systems [**Paper IV**]. PUFA concentrate obtained by urea crystallisation was thus esterified with rutin or vanillyl alcohol using *Candida antarctica* lipase. The potential of the synthesised derivatives to scavenge 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) radical as well as to stabilise fish oil PUFA concentrate against oxidation was evaluated [**Paper IV**]. Finally, anaerobic digestion of the insoluble fraction that remained after recovery of oil and soluble fraction was evaluated in **Paper V** for methane production. Feasibility of co-digestion of this fraction with a carbohydrate-rich leafy biomass was also studied.

4.0 Production of fish oil

Production of fish oil entails the separation of fatty substances (lipids) from other constituents of the fish. Fish oil extraction methods include;

(i) Cooking by steam under pressure with or without the presence of water followed by pressing and centrifugation and/ or filtration to recover the oil is the most common procedure [34]. The drastic conditions of temperature and pressure involved may partially modify the natural all *cis* n-3 PUFAs [35]. Heating also results in protein coagulation.

(ii) Use of solvents for oil extraction. This is a well developed technology. Extraction of oil with organic solvents however causes denaturation of proteins in addition to loss of functional properties [36]. Organic solvents are also harmful to human health as well as the environment.

(iii) Supercritical fluid oil extraction. At or above critical temperature (31.1 $^{\circ}$C) and pressure (7.39 MPa), carbon dioxide turns into a fluid state. This liquid is an inert and safe solvent that has been used in oil extraction [36, 37]. The main advantage with this method is that no solvent remains in the product. At room temperature and pressure, the CO_2 returns to gas phase and evaporates.

Supercritical fluids have lower viscosities and higher diffusivities than conventional solvents which improve mass transfer from solid and liquid matrices and thus decreases overall time needed for extraction [38]. Relatively high capital cost of the high pressure extraction equipment is the major draw back to this technique.

(iv) Enzymatic tissue disruption is another promising alternative technique. A range of products for human and animal consumption can be obtained using this method [39]. Large protein molecules are broken down to a range of smaller peptides and amino acids which facilitates oil release. The main advantage for enzymatic oil extraction is that it can be carried out under mild conditions such as, low temperatures ($\leq 55\ ^{\circ}C$) thus minimising PUFA oxidation, no use of solvents and hydrolysis is performed for a short period of time [35]. In addition, the functional properties of the protein are maintained [40], while the unhydrolysed residue (sludge) present a high quality protein meal. The resulting hydrolysate also provides a good source of soluble fish proteins and phospholipids [41]. Enzymatic hydrolysis could thus result in recovery of multiple products leading to maximum utilisation of waste from fish processing.

4.1 Hydrolysis of fish by-products with commercial enzymes

Solubilisation of fish muscle proteins can be achieved through an autolytic process using endogenous proteolytic enzymes [40]. Autolysis of fish muscle is usually performed to produce fish sauce or fish silage. In the process, an aqueous solution rich in small peptides and free amino acids is formed. The enzymes also promote release of oil [40]. The disadvantage of using endogenous enzymes for the hydrolysis is that the process is time consuming and lasts over several days [40, 42]. In addition, it is difficult to control the process since the presence of digestive enzymes and their concentration may be highly seasonal, gender and age specific, and can vary tremendously within and between species [40].

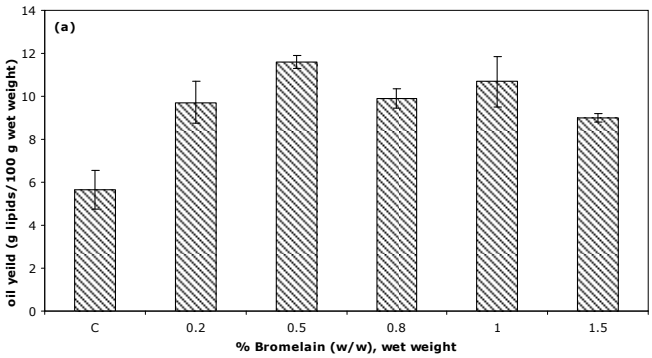
Use of exogenous (added) enzymes makes the hydrolysis process highly controllable and hydrolysis time is also reduced [43]. Use of suitable enzyme/substrate ratios and reaction times permits the production of hydrolysates with different molecular structures and different functional properties that could find applications in various food formulations [44]. In addition, added enzymes have also been reported to improve oil yields [45-47]. Approximately 80% of total lipids can be isolated from fish by-products after enzymatic hydrolysis [42, **Paper I**]. A wide-spectrum of commercial proteases [**Table 2**], have been used for hydrolysis of fish by-products with the aim of recovery of fish oils or proteins.

Table 2. Commercial enzymes used in hydrolysis of fish by-products

Protease used	By-product source	Reference
Protex 30L	Nile perch and salmon	**Paper I** and **II**
Bromelain	Salmon	**Paper I**
Flavourzyme, Protamex, and Alcalase	Sardine	[45]
Alcalase	Cod	[7]
Alcalase, Neutrase, Protamex, Papain, Bromelain, Actinidin and a plant protease mix	Cod	[48]
Protamex	Salmon	[42]
Alcalase, Neutrase, and Flavourzyme	Salmon	[35]

Enzyme concentration has been reported to be the most influential factor for enhanced release of lipids and phospholipids from fish by-products [41]. In **Paper I,** increasing enzyme amount resulted in increased oil yield upto a given concentration beyond which a further increase did not improve the yields (**Figure 3**). An optimum enzyme substrate ratio is thus important in maximising oil yield.

Hydrolysis time is another factor that influences oil yield. According to [35, 41 and **Paper I**], longer hydrolysis time does not result in increased oil yields. Tissue hydrolysis achieved at the initial stage of hydrolysis (1-2 h), could be sufficient enough to release the lipids. This is due to exposure of more hydrophobic amino acids with increased degree of hydrolysis which interact with released lipid molecules forming lipid–proteins complexes.



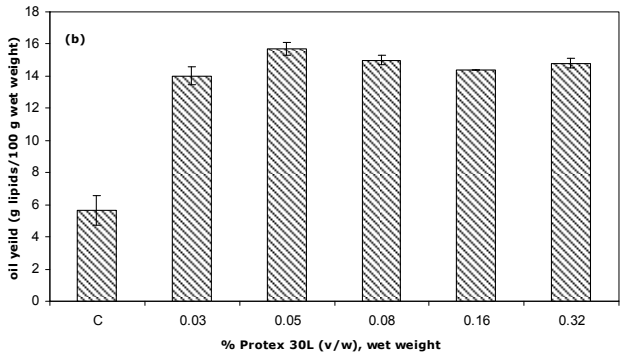


Figure 3. Effect of enzyme concentration: (a) Bromelain, (b) Protex 30L on oil yield on oil yield from 50 g of salmon heads homogenate incubated with different amounts of enzyme at 55 °C without water addition or pH adjustment. C is the oil yield from control (Paper I).

Another critical factor in enzymatic hydrolysis of fish by-products for lipid recovery is the water content. Fish by-products constitute above 60% water [45]. A further dilution of the by-products prior to hydrolysis results in decreased oil yields [7, 49, **Paper I**]. This has been attributed to the formation of lipid-protein emulsion layer that is resistant to enzymatic breakdown and the content of which increases with increasing water content during the hydrolysis [49]. During hydrolysis of cod and salmon by-products, it was observed that maximum oil yields were attained when no water was added to the substrates while a dilution of 1:1 (w/v) resulted in decreased oil yield and large emulsion fraction [49]. Addition of water into the hydrolysis mixture however results in increased recovery of soluble proteins [40, 50]. Therefore, it is difficult to obtain maximum soluble proteins and lipid yields with the same hydrolysis conditions [7, 41].

4.1.1 Up scaled hydrolysis of salmon by-products with Protex 30L

Hydrolysis at large scale was performed using salmon heads (**Figure 4**). Salmon heads (28 kg) were crushed and homogenised using a grinder (GM 200, Retsch GmbH, Germany). Oil was extracted from 23.4 Kg of the homogenate using Protex 30 L as described in **Paper I**. The samples were heated for 30 min prior to enzyme addition, which was then followed by hydrolysis for 1 h with continuous stirring at 400 rpm without water addition. The weight of recovered oil was determined to be 3.9 Kg (16.7% w/w, wet weight). The oil fraction was used for PUFA recovery as described in **Paper IV** and the solid fraction (sludge + emulsion) was used for anaerobic digestion as described in **Paper V**.

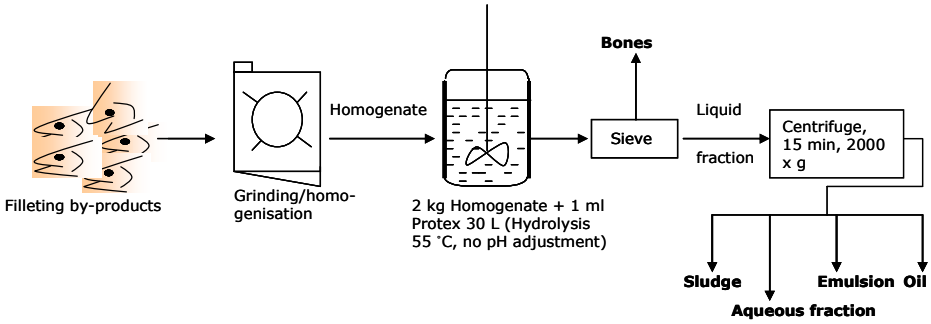


Figure 4. Schematic presentation of enzymatic hydrolysis of salmon by-products using Protex 30L.

5.0 Lipids

The lipids used in the current study were triglycerides (TAG), free fatty acids or fatty acid ethyl esters. As building blocks, glycerides contain a glycerol molecule and one to three fatty acid molecules bound to the glycerol backbone through ester linkages. Rubio et al. [34] defined fatty acids as organic compounds formed by a hydrocarbon chain and a carboxylic group. Fatty acids can be saturated or unsaturated with unsaturated fatty acids having one or several double bonds.

5.1 Determination of fatty acids distribution in triacylglycerols

Due to positional specificity of lipases, during the enzymatic synthesis of structured lipids or enrichment of certain fatty acids such as PUFAs in marine oil triglycerides, it is important to evaluate the distribution of fatty acids in the triglyceride (TAG) molecule. Different fatty acids can be present at any of three positions on the

glycerol molecule (referred as, *sn*-1; *sn*-2 and *sn*-3). Fatty acids distribution in the three positions can be determined using chemical or enzymatic procedures [51-53]. In either case, the fatty acid composition of the original TAG must be determined. The fatty acid composition is usually expressed on a mol% basis since the relative molecular weight contributions of individual fatty acids differ depending on chain length and degree of unsaturation.

In enzymatic procedures, lipases that are specific for fatty acids in *sn*-1 and *sn*-3 positions in the triglyceride molecule are used to hydrolyse TAG to obtain 2-monoacylglycerols. Pancreatic lipase is one of the lipases used to determine the fatty acid composition of *sn*-2 position of triacylglycerols [52]. It is activated by bile salts and calcium ions are essential for the hydrolysis reaction. Other lipases that have been used for positional analysis are lipase from *Rhizopus arrhizus* [53], *Rhizomucor miehei* [54] and *Thermomcyes lanuginosus* [54]. The lipases are specific for positions *sn*-1 and *sn*-3 of the glycerol molecule and are not activated by bile salts. They also do not have an absolute requirement for calcium ion. The resulting 2-monoacylglycerols (2-MAG) are fractionated from free fatty acids, DAG and TAG, transmethylated and analysed by gas chromatography as described in **Papers I** and **II**. The limitation of using lipases to determine fatty acid distribution in fish oil is that most lipases discriminate against long chain PUFAs [52, 54, 55]. However, comparative positional analysis of fatty acid distribution in fish and squid oil using two lipases (*Rhizomucor miehei* and *Thermomcyes lanuginosus)* gave similar results indicating that 1,3 specific lipases can be used to give a rough indication of the fatty acid distribution [54].

Chemical methods provide a suitable alternative to the use of lipases for positional analysis. Triglycerides are usually reacted with Grignard reagent; methyl magnesium bromide, ethyl magnesium bromide or allyl magnesium bromide [56], to produce a representative pool of partial acylglycerols which are separated on thin layer chromatography (TLC) plates impregnated with boric acid. The procedure shows no significant selectivity with respect to fatty acid chain length, degree of unsaturation and double bond configuration [57, 58]. The composition of fatty acids at position 2 is calculated from data for *sn*-1(3),2-DAG minus TAG or from TAG minus 1,3-DAG. Acyl migration between *sn* 1(3) and *sn*-2 positions affects the accuracy of this analysis [59, 60]. The acylglycerols formed from the TAG are usually contaminated by fatty acids that have migrated from the isomeric positions.

6.0 Lipases

Lipases (triacylglycerol ester hydrolase, E.C.3.1.1.3) are ubiquitous enzymes and have been found in most organisms from the microbial, plant and animal kingdom [61]. They catalyze the cleavage of triacylglycerol (TAG) ester bonds to yield diacylglycerols (DAG), monoacylglycerols (MAG), free fatty acids and glycerol. The latter products are taken up by the cells to provide energy and growth intermediates. Hydrolysis reaction can also be oriented towards ester synthesis when working in water poor systems [62]. **Table 3** shows hydrolysis and synthesis reactions catalysed by lipases.

Table 3. Lipase catalysed reactions

Hydrolysis		$RCOOR' + H_2O \longleftrightarrow RCOOH + R`OH$
Synthesis	Esterification	$RCOOH + R`OH \longleftrightarrow RCOOR` + H_2O$
	Interesterification	$RCOOR` + R``COOR* \longleftrightarrow RCOOR* + R``COOR`$
	Alcoholysis	$RCOOR` + R``OH \longleftrightarrow RCOOR`` + ROH$
	Acidolysis	$RCOOR` + R``COOH \longleftrightarrow RCOOR`` + R`COOH$
	Aminolysis	$RCOOR` + R``NH_2 \longleftrightarrow RCONHR`` + R`OH$

The ability of the lipases to catalyse different reactions makes lipases to find applications in the food, detergent, pharmaceutical, leather, textile, cosmetic and paper industries as shown in **Table 4**. Hydrolysis of fish oil triglycerides and fish oil fatty acid ethyl esters is described in **Papers II** and **III.** Esterification of PUFA with alcohol (ethanol) and phenolic derivatives (vanillyl alcohol and rutin) was catalysed by lipases as described in **Papers III** and **IV**.

Table 4. Industrial application of lipases

Industry	Example of lipase used and reaction catalysed	Product	Application	Ref.
Cosmetic	*Rhizomucor miehei* lipase, esterification	Isopropyl myristate, ethylhexyl palmitate	Skin care products	[63]
	Candida rugosa lipase, esterification	Wax esters	Skin care products	[63]
Pharma-ceutical	*Pseudomonas cepacia*, resolution of Racemic acetate, (cis-3-acetyloxy-4-phenyl-2-azetidinone)	Enantiomerically Pure 3R-acetate And (3S-cis)-hydroxy-4-phenyl-2-azetidinone	3R- acetate is intermediate in synthesis of anticancer drug (Paclitaxel) side chain.	[64]
Dairy	*Aspergillus niger* and *Rhizomucor miehei* lipases. Hydrolysis of milk fats	Increased free fatty acids content	Accelerate cheese ripening , flavour and texture enrichment	[65]
Oil and Fat	Lipase from *Mucor miehei*, Interesterification of sunflower oil with behenic acid	Structured lipid	Reduced calorie fats	[66]
	Rhizopus niveus lipase, esterification of stearic and palmitic acid at *sn*-1, 3 positions of sunflower oil	Structured lipid	Cocoa butter substitute	[65]
	Thermomyces lanuginosus lipase, Interesterification of palm stearin and coconut fats	Structured lipid	Margarine fats	[67]
	sn-1, 3–specific *Rhizomucor miehei* lipase interesterification of palm oil with the fatty acids derived from high-oleic sunflower oil.	Structured lipid (Betapol)	Human milk fat substitute	[68]
Detergent	Lipolase from *Thermomyces lanuginosus*	Lipid degradation to release free fatty acids	Stain removal	[63]

6.1 General structure of lipases

Lipases belong to the α/β-hydrolase fold family [69-72]. The α/β-hydrolase fold consists of a central hydrophobic eight stranded β -sheet packed between two layers of amphiphilic α–helices, providing a stable scaffold for the active site [72]. The catalytic triad, Serine-Histidine-Aspartic acid, is similar to that of serine proteases with some variability in the acidic residues which can be aspartic or glutamic acid [73-75]. In contrast to proteases, lipase catalytic site is buried under the head of a helical loop (lid) folded onto the triad and stabilised by electrostatic and hydrophobic interactions. The lid buries non-polar residues underneath and makes the active site inaccessible to solvent and substrate [74, 76]. Activation of the lipase involves displacement of the lid which exposes the hydrophobic residues, and favours interaction with the lipid interface [77, 78]. Displacement of the lid also results in burying of polar residues on the lid helix and the surface adjacent to it. The water molecules which form a well defined network in the nonactivated enzyme are also displaced [79]. Lipase activity is greatly increased at lipid-water interface; this is called interfacial activation [80]. The catalytic reaction in the lipid–water interphase involves at least four steps: (i) binding to the lipid surface, (ii) penetration into the lipid phase, (iii) displacement of the lid (enzyme activation) and (iv) catalytic hydrolysis [81].

6.1.1 Catalytic mechanism of lipases

Catalytic mechanism of lipases involves acylation and deacylation of the active serine residue. Abstraction of hydroxyl proton from the active site serine activates it for a nucleophilic attack on the carbonyl carbon of the ester bond in the acylating agent to form first tetrahedral intermediate and a protonated imidazole (ImH^+) on the histidine residue (**Figure 5**). Proton transfer from ImH^+ to the ester oxygen forms the leaving group H_2O or ROH depending on whether the acylating agent is an acid or an ester yielding the acyl enzyme, (E-O-C(O)R). Deacylation is the hydrolysis or alcoholysis of the acyl enzyme by activation of the incoming nucleophile or acyl acceptor (H_2O/alcohol) by the histidine, thus forming a second tetrahedral intermediate. This intermediate reforms the carbonyl double bond as the serine deprotonates ImH^+ making the whole enzyme the leaving group. Other residues supply necessary stabilization by hydrogen bonding to the oxy-anion of the tetrahedral intermediate carrying much of the negative charge [82, 83].

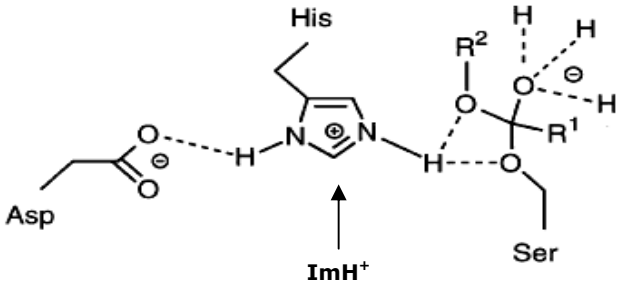


Figure 5. Hydrogen bonding of first tetrahedral intermediate of lipase hydrolysis [82].

6.2 Lipase specificity

Though the catalytic mechanism described above is true for all lipases, the active site of lipases comes in different shapes and sizes [61, 84]. Lipases are thus divided into 3 groups: (1) lipases with a hydrophobic, crevice-like binding site located near the enzyme surface (lipases from *Rhizomucor*, *Rhizopus* and *Thermomyces lanuginosus*); (2) lipases with a funnel-like binding site (lipases from *Candida antarctica*, *Pseudomonas*, mammalian pancreas and cutinase); and (3) lipases with a tunnel-like binding site (lipase from *Candida rugosa*). The shape and micro-environment of the binding site results in lipase preference to react on certain acylglycerols or positions within e.g. triglyceride molecule. This is frequently regarded as the selectivity or specificity of a lipase. Specificities of lipases are divided into four major types [85]: (i) lipid class, (ii) positional, (iii) fatty acid, and (iv) stereochemical. Since few lipases approach absolute specificity within analytical limits in reactions with substrates, the term selectivity is replacing specificity; for example, regioselectivity for positional specificity [86].

Lipid class selectivity is the specificity of lipases towards certain acylglycerols (TAG, DAG or MAG). *Penicillium camembertii* and *Penicillium cyclopium* lipases act upon mono- and diglycerides only [87, 88] wherease other *Penicillium* lipases hydrolyze only triglycerides. Lipases can be non-regiospecific or display one of two kinds of positional specificity: *sn*-l,3 specific or *sn*-2 specific. Nonspecific lipases hydrolyse all three ester bonds of triglycerides at the same rate. *Sn*-l,3 specificity is associated with the preferential release of fatty acid residues from the *sn*-1,3 positions of the glycerol backbone rather than *sn*-2 position, whereas *sn*-2 specificity refers to preferential release from the *sn*-2 position. Lipases from *Candida rugosa* and *Pseudomonas cepacia* used in **Papers II and III** and *Candida antartica* used in Paper **IV** are non-regiospecific whereas lipase from *Thermomyces lanuginosus* used

in **Papers I**, **II** and **III** is *sn*-1,3 specific. Regio-selectivity of lipases makes them effective catalysts for the synthesis of structured lipids that have a predetermined composition and distribution of fatty acids at the glycerol backbone [89]. Stereoselectivity describes the preference of lipases to cleave ester bonds on *sn*-1, or *sn*-3 positions. Many lipases have a preference for the *sn*-1 position, such as the one from *Rhizomucor miehei* [90]. Fatty acid selectivity or typoselectivity refers to lipases that are specific to fatty acids of particular chain length or a particular fatty acid. Enrichment of n-3 PUFA in marine oils depends to a large extent on regio-and fatty acid selectivities of lipases. This was evident in **Paper II** where non regio-specific lipase from *Candida rugosa* enriched both EPA and DHA since it had low selectivity towards these fatty acids (**Figure 6**). On the contrary, *sn* 1,3 specific lipase from *Thermomyces lanuginosus* was ineffective in enriching EPA since a large fraction of this fatty acid was located at *sn* 1,3 position.

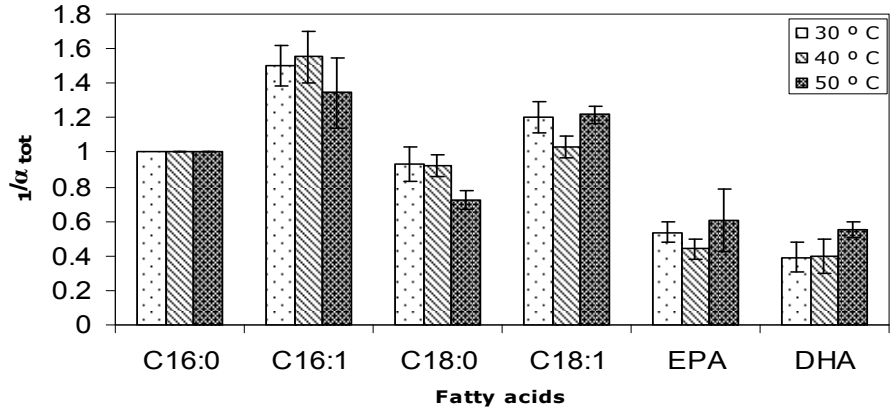


Figure 6. Specificity of *C. rugosa* lipase towards different fatty acids in Nile perch oil at different temperatures (Paper II). 1/α represents fatty acid specificity.

6.2.1 Competitive factor

The term competitive factor (α) has been used to describe the kinetics of the reaction when two substrates are present at the same time in the reaction mixture [91, 92]. α is the ratio of the specificity constants and Rangheard et al. [93] defined 1/ α for a substrate as a convenient measure for the specificity for this substrate. The term specificity refers to enzyme's discrimination between several substrates competing for an enzyme active site [94]. For two substrates competing for the enzyme, the ratio of the reaction rates for each substrate (V_1 and V_2) is given by equation 1 which holds at all concentrations of competing substrates.

$$V_1/V_2 = \alpha\,[Ac_{1,x}]/\,[Ac_{2,x}] \qquad \textit{Equation 1}$$

Where $[Ac_1]$ and $[Ac_2]$ are concentrations of the two substrates at time x and α is the competitive factor defined in equation 2.

$$\alpha = [V_{AC1,x}/K_{AC1,x}]/\,[V_{AC2,x}/K_{AC2,x}] \qquad \textit{Equation 2}$$

where V is the maximal velocity and K is the Michaelis constant.

Practical estimation of the competitive factor is done using equation 3 which is the integrated form of equation 1.

$$\alpha = \log([Ac_{1,x0}]/[Ac_{1,x}])\,/\,\log([Ac_{2,x0}]/[Ac_{2,x}]) \qquad \textit{Equation 3}$$

Usually the best substrate (the highest reaction rate, V*)* is taken as reference and each substrate reactivity towards the enzyme is characterized by a competitive factor calculated using equation 3. The competitive factor for the reference substrate is equal to 1.00. For a known substrate, the higher the α value, the lower the reaction rate of this substrate compared to that of the reference compound, in a mixture of these compounds. In **Papers II** and **III**, specificity factor ($1/\alpha$) was used to compare the reactivity of EPA and DHA towards different lipases. EPA and DHA were present as acylglycerols, free fatty acids or fatty acid ethyl esters. Palmitic acid (C16:0) was taken as the reference fatty acid since it was the predominant fatty acid in the oil and also being a saturated fatty acid, the lipases were expected to have a higher preference towards C16:0 than the PUFAs. Lipase specificity has been shown to decrease with increasing unsaturaration, when dealing with 1, 5 and 6 double bonds [95].

Lipases from *Thermomyces lanuginosus* and *Candida rugosa* showed higher specificity for oleic and palmetoleic acid when compared to EPA and DHA [**Paper II**]. The same lipases showed a clear preference for EPA over DHA in **Paper II** and **III**. This could be explained by the fact that the position of the first double bond relative to the carboxyl group is closer in DHA than in EPA which presumably adds some strain to the active site of these enzymes to accommodate DHA properly, hence making it a poorer substrate [96]. There are however lipases that exhibit a lower specificity towards EPA than DHA. These include lipases from porcine pancrease, *Chromobacterium viscosum, Pseudomonas cepacia* and *Pseudomonas fluorescens* [97].

As discussed in **Paper III**, the lipases had a higher specificity of fatty acids when present as free fatty acids (FFA) than fatty acid ethyl esters (FA-EE). Both lipases from *P. cepacia* and *T. lanuginosus* showed a higher preference of EPA and DHA when present as FFA than FA-EE. The specificity of *T. lanuginosus* lipase towards DHA/ EPA when present as triglyceride, free fatty acid or fatty acid ethyl ester was in the order of TAG hydrolysis > FFA esterification > FFA-EE hydrolysis [**Paper II** & **III**]. This can be explained by the fact that apart from differences in the fatty acid structure, when EPA and DHA are present in triglyceride molecule, factors such as regio, stereo and triglyceride specificity of lipases influence the selectivity of lipases. On the contrary, when present in FFA or FA-EE, problems associated with non homogenous distribution of these fatty acids are eliminated and only the direct effects of fatty acid structure on enzyme activity remain. Fatty acid selectivity/specificity of lipases is exploited in fatty acid enrichments in single-stage reactions such as hydrolysis [**Paper II**] or esterification [**Paper III**], in combination with a physical means for separating the desired end product and the residual unwanted fatty acid fraction.

6.3 Enrichment of long chain polyunsaturated fatty acids (PUFAs) in fish oils

Due to the triglyceride structure of fish oil, the omega-3 PUFAs content (EPA and DHA) in fish oil does not exceed 30% [98]. Higher concentrations are achieved after separation of fatty acids from the triacylglycerol structure using several methods which include; chromatography, distillation, low temperature crystallization, supercritical fluid extraction, urea complexation and enzyme catalysis [17, 99, 100]. Omega-3 PUFAs concentrates can be in the form of free fatty acids, methyl and ethyl esters or acylglycerols [99]. Enzymatic enrichment of PUFAs offers many advantages over other methods due to regio-, stereo-, and substrate specificity of lipases in addition to the mild conditions (e.g. neutral pH and low temperatures) of the process thus producing high quality products [101]. Many lipases have a lower specificity to PUFAs especially EPA and DHA than the saturated and monounsaturated fatty acids which has proven useful in enrichment of PUFAs in fish oils [54, 55, 97 **,Paper II** and **III**].

6.3.1 Enrichment of PUFAs in fish oil glycerides by lipase catalysed hydrolysis

The selectivity of lipases towards some fatty acids in fish oil is used to remove saturated and monounsaturated fatty acids and leave PUFAs attached to the glycerol

backbone. However, prolonged hydrolysis can also results in hydrolysis of PUFAs (**Figure 7**). Therefore, hydrolysis is usually stopped when the desired level of PUFAs enrichment and recovery is attained.

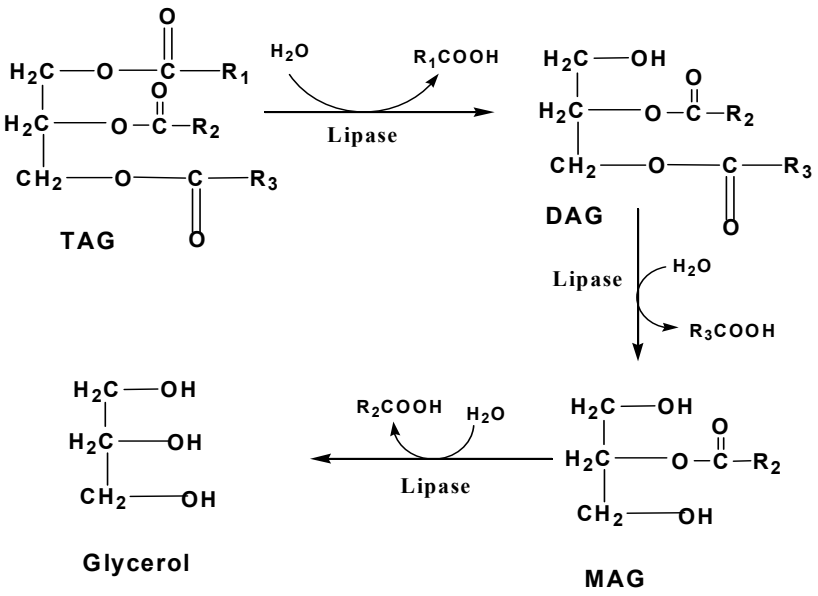


Figure 7. Lipase catalysed hydrolysis of triglyceride (TAG). R_1, R_2 and R_3 are saturated, polyunsaturated (PUFA) and monounsaturated fatty acids, respectively.

After separation of released free fatty acids, a concentrate of PUFAs attached to glycerol molecule in form of triglycerides (TAG), diglycerides (DAG) and monoglycerides (MAG) is obtained. The percentage composition of TAG, DAG and MAG in the concentrate depends on degree of hydrolysis [102]. n-3 PUFAs in the acylglycerol form are considered to be more nutritionally favourable than free fatty acids or ethyl/methyl esters of PUFAs. This is attributed to the high susceptibility of free fatty acids to oxidation while the ethyl/methyl esters are more resistant to hydrolysis by pancreatic lipase in the intestine [103-106].

Lipases from *Candida rugosa*, *Thermomyces lanuginosus* and *Pseudomonas cepacia* have been used to enrich EPA and DHA in the glyceride fraction of fish oil [55]. As described in paper **II**, *C. rugosa* lipase resulted in the best combined EPA and DHA enrichment in Nile perch oil while *T. lanuginosus* lipase resulted in best DHA enrichment but was ineffective in enriching EPA and also resulted in the least decline of palmitic acid (**Figure 8**). This was attributed to the fact that a large fraction of EPA was present in *sn*-1,3 positions while palmitic acid was in *sn*-2 position in the glyceride molecule [**Paper II**]. *Thermomyces lanuginosus* lipase being *sn*-1,3

specific therefore hydrolysed EPA at *sn*-1,3 positions while hydrolysis of palmitic acid in *sn*-2 position was limited.

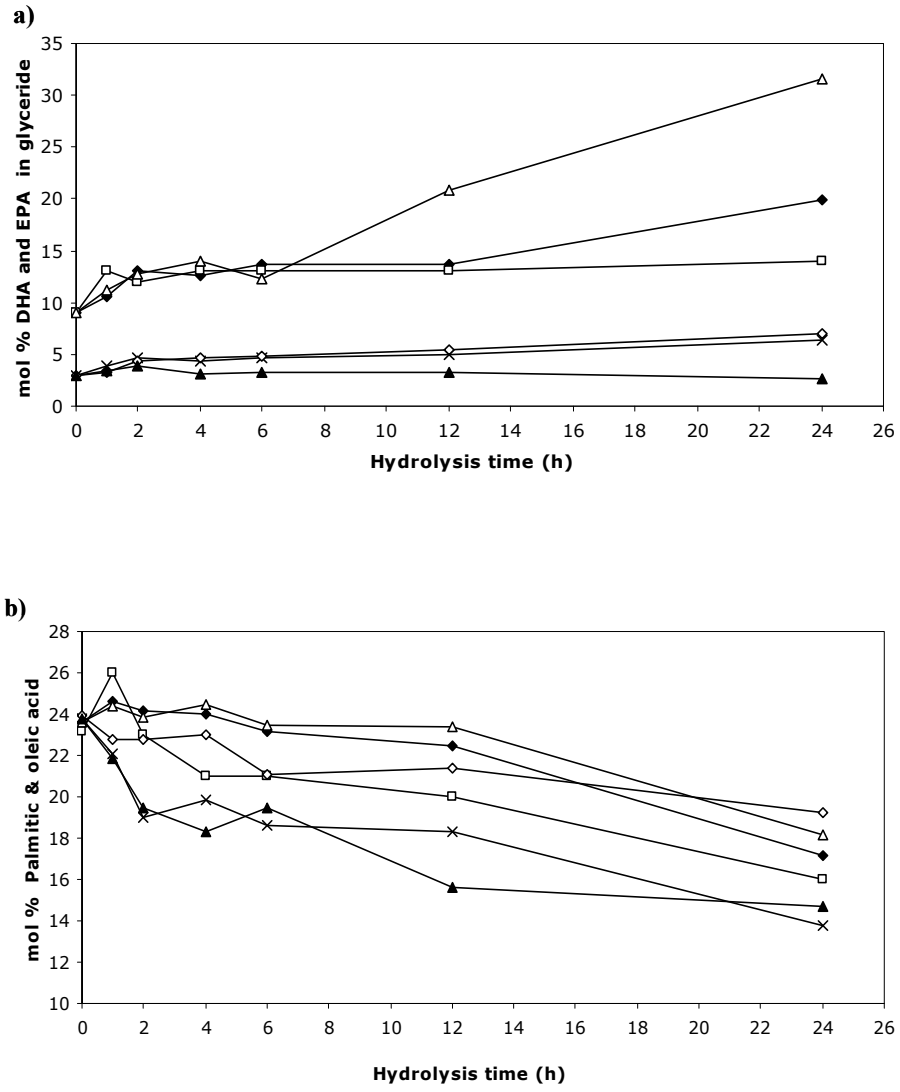


Figure 8. (a) Changes in DHA and EPA mol% in Nile perch viscera oil following hydrolysis at 40 °C. ♦, □, Δ mol% DHA and x, ◇, ▲ mol% EPA, after hydrolysis with *C. rugosa, P. cepacia* and *T. lanuginosus* lipases. (b) Changes in palmitic and oleic acid mol % in Nile perch viscera oil following hydrolysis at 40 °C. ♦, □, Δ mol% palmitic and x, ◇, ▲ mol% oleic acid, after hydrolysis with *C. rugosa*, *P. cepacia* and *T. lanuginosus* lipases (Paper II).

A 1,3-specific *Rhizopus oryzae* lipase has been reported to enrich DHA in seal oil but was ineffective in enriching EPA [107]. Seal oil has n-3 PUFA located mainly in *sn*-1 and *sn*-3 positions of the TAGs with DHA mainly in *sn*-3 position and EPA random in *sn*-1 and *sn*-3 positions [108, 109]. *Candida rugosa* lipase has previously been reported to yield the best DHA enrichment and also combined enrichment of EPA and DHA in the glyceride fraction of fish oils [55, 99, 102, 110]. This could be attributed to the fact that the lipase possesses fatty acid chain length selectivity, showing higher activity with relatively short-chain fatty acids such as C18 or below [111]. Another reason could be its non-regiospecificity and the fatty acid profiles in fish oil [90, 112]. The lipase has been reported to have structural specificity showing low specificity to triglyceride molecules having DHA [113].

Temperature has an effect in the level of PUFA enrichment that is achieved following lipase catalysed hydrolysis. Different levels of PUFAs enrichment were attained when lipases from *C. rugosa* and *T. lanuginosus* were used to catalyse hydrolysis at 30, 40 and 50 °C [**Paper II**]. Maximum EPA and DHA enrichment was attained at 30 °C with *C. rugosa* lipase while *T. lanuginosus* lipase gave best DHA enrichment at 50 °C [**Paper II**]. Denaturation of *C. rugosa* lipase at 50 °C has been reported [114] and this could explain why no enrichment was observed at this temperature. On the contrary, lipase from *T. lanuginosus* is thermostable thus was active at this temperature. Therefore, to obtain maximum enrichment of PUFA using lipases, the reactions should be performed at optimum temperatures of the enzyme of choice. However, the stability of PUFAs at that temperature should be considered**.**

6.3.1.1 Separation of acylglycerols and free fatty acids after enzymatic hydrolysis

To utilise lipase hydrolysis as a means of enriching fish oils, a method to separate free fatty acids (FFA) from mono-, di-, and triglycerides is required. For analytical purposes, the hydrolysis products are separated using thin layer chromatography as described in **Paper I** and **II**. At large scale, glycerides can be separated from FFA by liquid-liquid extraction, alkali neutralisation, molecular distillation, or crystallisation [115]. In this study, alkali neutralisation was used as a means to recover the acylglycerol fraction after hydrolysis of 200 ml of Nile perch viscera oil using 0.3 g of *Candida rugosa* lipase (700 U/mg solid) in 200 ml of 0.2 M sodium phosphate buffer pH 7.0 at 30 °C for 24 h with stirring at 300 rpm. The acid value of hydrolysed oil was 90 mg NaOH /g oil.

Initial attempts to isolate FFA through soap formation with NaOH or KOH solutions resulted in precipitation/solidification of the entire oil fraction probably due to presence of several hydroxyl groups in diglycerides and monoglycerides of the hydrolysed oil. A saponification/extraction technique previously reported was thus used with slight modifications [116]. Hydrolysed oil was neutralised using 1 M KOH in 0.5 M trisodium phosphate buffer (pH 13.3) and fatty acids extracted into ethanolic solution. The ratio of oil/KOH/ethanol was 1:2:1 v/v/v. From 200 ml (179 g) of hydrolysed oil, a concentrate of 26.5 g, (14.8% yield) was achieved. The concentrate contained 24% DHA and 6% EPA. The acylglycerol concentrate was used in **Paper IV** for testing stabilisation of fish oil against oxidation using commercial and synthesised antioxidants.

6.3.2 Enrichment of PUFAs in fish oil as free fatty acids or fatty acid esters

Selective enzymatic esterification of fish oil free fatty acids (FFA) or hydrolysis of the fatty acid methyl/ethyl esters (FA-EE) could be more effective than hydrolysis of fish oil triglycerides in concentrating PUFAs. This is because specificity of lipases towards fatty acids bound to triglyceride molecules is affected by many factors such as regio-selectivity of the lipases toward triglycerides, non-homogeneous distribution of EPA and DHA into various positions of the glycerol backbone, as well as a possible triglyceride selectivity of the lipases. On the contrary, when not attached to glycerol molecule, lipase specificity is influenced by fatty acid structure or alcohol type used during esterification reactions [117, 118].

6.3.2.2 *Enrichment of PUFAs as free fatty acids*

Enrichment of DHA and/or EPA in FFA is achieved by selective esterification of fish oil free fatty acids (FFA) with an alcohol (**Figure 9**). Initially fish oil triglycerides are split into their constituent fatty acids and glycerol by alkaline hydrolysis using alcoholic KOH or NaOH [119, 120] and **Paper III**. Use of lipases to release FFA has also been reported [117, 121, 122]. Due to high level of unsaturation, PUFAs are poorer substrates of the lipases while the saturated (SA) and monounsaturated fatty acids (MUFA) are preferred. Thus most of PUFAs are left as FFA while SA and MUFAs are esterified.

During esterification reaction, the water content should be kept at minimum in order to prevent product hydrolysis but be sufficiently high in order to prevent enzyme deactivation [99]. Thus, water released during the esterification reaction is

continuously removed by use of adsorbents e.g. molecular sieves used in **Paper III** and **IV,** vacuum evaporation or use of salt hydrates.

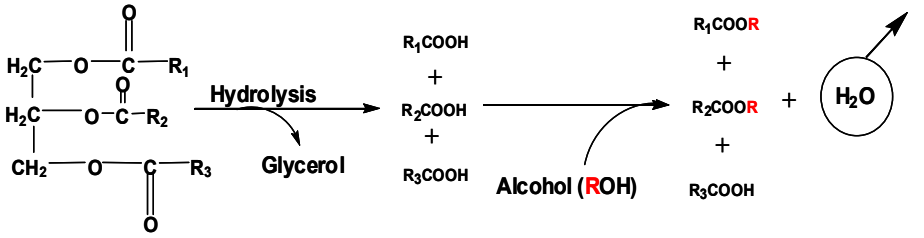


Figure 9. Lipase catalysed esterification of fish oil FFA with alcohol (ethanol). R_1, R_2 and R_3 are saturated, monounsaturated and PUFA fatty acids, respectively.

Lipases from *Rhizopus delemar, Rhizomucor meihei, Pseudomonas fluorescens* have been used to enrich DHA in fish oil by selective esterification of fish oil FFA with lauryl alcohol, glycerol and octanol, respectively [117, 119, 120]. Long chain alcohols have been shown to enhance enzymatic esterification with considerable amount of selectivity towards PUFA [117]. As described in **Paper III**, lipases from *T. lanuginosus* and *P. cepacia* were used to enrich DHA and EPA in Nile perch oil by selective esterification of Nile perch FFA with ethanol. Both lipases enriched DHA and EPA with *T. lanuginosus* lipase giving better DHA enrichment while *P. cepacia* lipase enriched EPA more. It is interesting to note that lipase from *T. lanuginosus* enriched EPA when it was present as FFA or FA-EE [**Paper III**] while in **Paper II**; the same lipase could not enrich EPA in glyceride fraction. EPA and DHA yields were also better than those obtained during enrichment of PUFA as acylglycerols [**Paper II** and **III**]. This indicates that lipase selectivity is more pronounced when fatty acids are present as FFA than when attached to glycerol molecule. It also demonstrates effect of fatty acid distribution in triglyceride molecule on enrichment of PUFAs using regio-specific lipases. Since TAG are considered nutritionally more favourable than FFA, esterification of FFA PUFA concentrate (EPA/DHA), with glycerol is reported [99].

6.3.2.3 *Enrichment of PUFAs as fatty acid methyl/ethyl esters*

PUFAs in fish oil can also be enriched as methyl/ethyl esters. This involves hydrolysis of fish oils to release free fatty acids which are then esterified with methanol or ethanol to obtain FA-ME or FA-EE. Alcoholysis of FA-EE/FA-ME or hydrolysis of such esters results in a fraction enriched in PUFA methyl or ethyl esters. Alcoholysis of tuna oil ethyl esters with lauryl alcohol using *Rhizopus delemar* lipase to enrich DHA-EE has been reported [123]. As described in **Paper III**, lipases from *T. lanuginosus*

and *P. cepacia* were used to enrich DHA-EE and EPA-EE by selective hydrolysis of FA-EE from Nile perch oil. Better yields of DHA-EE and EPA-EE were attained than yields of DHA and EPA in FFA form at the same level of enrichment with both lipases [**Paper III**]. The lipases discriminated DHA and EPA more when they were present as ethyl esters than as free fatty acids (**Figure 10**).

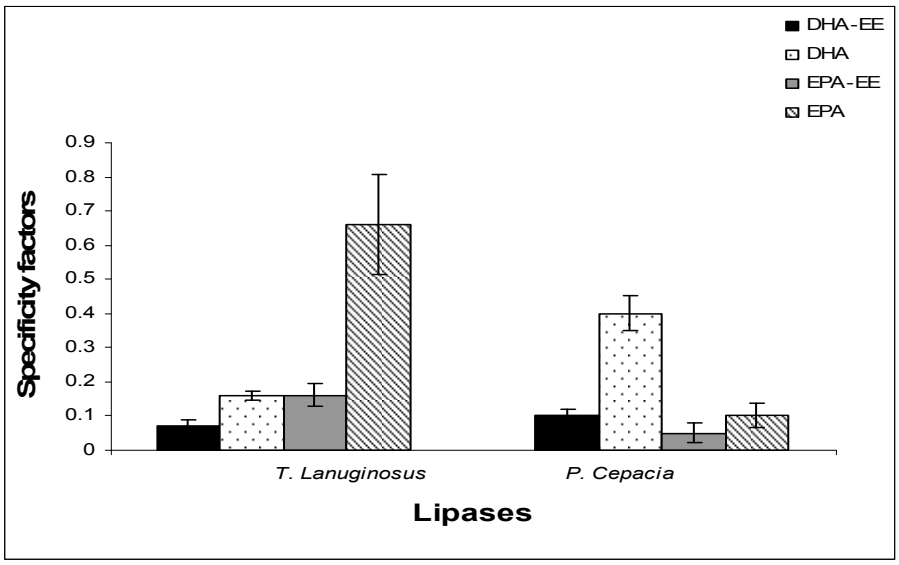


Figure 10. Specificity of lipases from *T. lanuginosus* and *P. cepacia* towards DHA, DHA-ethyl esters, EPA and EPA-ethyl esters. Palmitic acid/Palmitic acid ethyl ester was the reference substrate with a specificity factor of 1.00 (Paper III).

6.3.2.4 Enrichment of PUFAs by urea complexation

Urea complexation is a well established non-enzymatic technique for PUFA enrichment [124, 125]. The process involves formation of a homogeneous 65°C solution of FFA or FFA derivatives such as ethyl esters and urea in ethanol followed by cooling of the resultant urea complex slurry [116, **Paper IV**]. Complexation depends on the fatty acid structure with the saturated and monounsaturated fatty acids complexing more easily with urea than the PUFAs [124]. Strong van der Waals attractions exist between the urea molecules and the included molecules while the urea molecules are bound together *via* hydrogen bonding. Compared to the tetragonal structure of pure urea crystals, most urea complexes have hexagonal crystalline structure [126]. On cooling, the complexes crystallize out and are removed by filtration. The complexes are relatively stable and filtration does not have to be performed at very low temperatures [124]. The liquid or non-urea

complexed fraction is usually enriched in PUFAs. As reported in **Paper IV**, a considerable reduction in saturated FFA content and remarkable increase in PUFA occurs.

7.0 Lipid oxidation

Lipid oxidation is a process by which molecular oxygen reacts with unsaturated lipids to form lipid peroxides. Oxidation of unsaturated fatty acids can be induced through autoxidation by free radical reaction or photo-oxidation.

7.1 Free radical autoxidation

Free radical oxidation occurs in three steps: initiation, propagation and termination [127].

7.1.1 Initiation

In the initiation step, the key event is the formation of a lipid radical $L^{\cdot}$ which can be induced by transition metal ions, hydroperoxide decomposition or heat.

$$LH + In^{\cdot} \longrightarrow L^{\cdot} + InH \qquad \textit{Equation 4}$$

$In^{\cdot}$ is the initiating agent and LH is lipid substrate.

7.1.2 Propagation

Propagation begins with the addition of molecular oxygen to $L^{\cdot}$ to form peroxyl radical ($LOO^{\cdot}$), equation 5. The second step of propagation, the rate-limiting step, is abstraction of a hydrogen atom from LH by $LOO^{\cdot}$ to generate hydroperoxide (LOOH) and another radical ($L^{\cdot}$) which can carry the chain propagation sequence (equation 6).

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \qquad \textit{Equation 5}$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \qquad \textit{Equation 6}$$

The C-H bonds at the bisallylic positions (CH=CH–$\underline{CH_2}$–CH=CH), such as C-7, -10, -13 and -16 for EPA, and C-6, -9, -12, -15 and -18 for DHA are the weakest and the hydrogen atoms at these positions are preferentially abstracted by a peroxyl radical. Resulting pentadienyl radical react (at either end of the radical) with oxygen to form

two kinds of conjugated diene-monohydroperoxides [128]. In the presence of metal ions or at high temperatures, lipid hydroperoxides can undergo decomposition via homolytic cleavage of LOOH to form alkoxyl ($LO^{\cdot}$) and hydroxyl radicals ($^{\cdot}OH$) [127]. Alkoxyl radical undergoes carbon-carbon cleavage to form breakdown products including aldehydes, ketones, alcohols and alkyl radical [129]. Most hydroperoxide decomposition products are responsible for the off-flavor in the oxidized oils.

7.1.3 Termination

This involves coupling of two radicals to form non-radical products. As hydrogen atom abstraction is the rate limiting step, recombination reactions are primarily between two peroxyl radicals. This results in formation of an unstable tetraoxide intermediate that decomposes to give an aldehyde, alcohol, and molecular oxygen [130].

$$L^{\cdot} + L^{\cdot} \longrightarrow L\text{-}L$$

$$LOO^{\cdot} + L^{\cdot} \longrightarrow LOOL$$

$$LOO^{\cdot} + LOO^{\cdot} \longrightarrow \text{non-radical products}$$

7.2 Photosensitized oxidation

Hydroperoxides of unsaturated fatty acids can also be formed following exposure to light in the presence of oxygen and sensitizer that activates ground state oxygen to singlet oxygen. Photosensitizers include chlorophyll, pheophytins, porphyrins, riboflavin and myoglobin. In the presence of light, ground state photosensitizers (^{1}Sen) become excited into triplet state (^{3}Sen*)[131]. Photosensetised oxidation can then occur in two ways: (i) ^{3}Sen* abstracts hydrogen from LH and produces $L^{\cdot}$. The $L^{\cdot}$ can abstract hydrogen from neighbouring fatty acids to initiate the free-radical chain reaction. (ii) Excited triplet sensitizer (^{3}Sen*) transfers energy to ground state oxygen that becomes activated to singlet oxygen. Singlet oxygen reacts directly with double bonds in unsaturated fatty acids by 'ene' addition. Oxygen is thus added at either side of the *cis* double bond, which is shifted to yield lipid hydroperoxides [132]. Singlet oxygen oxidation yields conjugated and nonconjugated diene hydroperoxides [133]. Decomposition of the hydroperoxides yields free radicals which in turn initiate new chain reactions.

7.3 Antioxidants and lipid autoxidation

7.3.1 Antioxidants

An antioxidant is a substance that when present at lower concentrations than those of an oxidizable substrate (lipids, proteins, DNA, carbohydrates) considerably delays or avoids oxidation of that substrate [134, 135]. There is an increased interest on the use of natural phenolics as antioxidants in improving the shelf-life of lipid containing products since the widely used commercial antioxidants such as butylated hydroxyl toluene (BHT) are considered potential promoters of carcinogenesis [136]. Naturally occurring molecules with antioxidative properties are acid-phenols or flavonoids and their esters [137]. The antioxidant capacity of phenolic compounds is based on their ability to scavenge free radicals and, in the particular case of flavonoids, also by their binding of metal ions. In addition to antioxidant properties, natural phenolics such as rutin are associated with several health benefits such as antimicrobial, anti-inflammatory, anti-tumor and anti-angiogenic activities [138].

Phenolic acids consist of two groups: hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids have a carboxylic group (-COOH) and hydroxyl groups (one or more) in an aromatic ring. Hydroxycinnamic acids have CH=CH-COOH group instead of COOH as in hydroxybenzoic acid. Flavonoids are polyphenolic whose basic structure is a flavan nucleus that consists of 2 aromatic rings that are linked together by 3 carbon atoms that form an oxygenated heterocycle [139]. Hydroxyl groups linked with the phenolic compounds are often the most efficient free radical scavengers in foods since they can readily donate an electron or the hydrogen to intercept and convert free radicals to a more stable compound [140].

7.3.1.1 Lipophilization of phenolic acids and flavonoids

The use of natural phenolics in lipid based systems is limited due to their poor solubility which can result in a decrease in their ability to prevent oxidation of unsaturated fatty acids at oil-water or oil-air interfaces [141]. Acylation of natural phenols with lipophilic moieties with an aim of improving phenolics hydrophobicity has been used as one strategy to address this issue [142, 143]. Lipophilization of natural phenolics such as hydroxytyrosol [144], rutin [142, 145] ferrulic acid [146] and vanillyl alcohol [147], with fatty acids of varying chain lengths has been reported. Linolenic acid (C18:3) esterifed to rutin was shown to stabilise fish oil and emulsion systems against oxidation [142], implying that the fatty acid which is highly susceptible to oxidation due to presence of double bonds was protected

against oxidation. Similarly, esterification of long chain n-3 PUFA such as EPA and DHA with natural phenolics could help protect the highly oxidisable PUFA against oxidation while the PUFA may improve the hydrophobic properties of the phenolic compound.

As discussed in **Paper IV**, PUFA concentrate with 18 and 27% EPA and DHA, respectively was esterified with vanillyl alcohol or rutin using immoblzed *Candida antarctica* lipase (**Figure 11**). The different fatty acids in the PUFA concentrate were esterified to the phenolics (**Figure 12**).

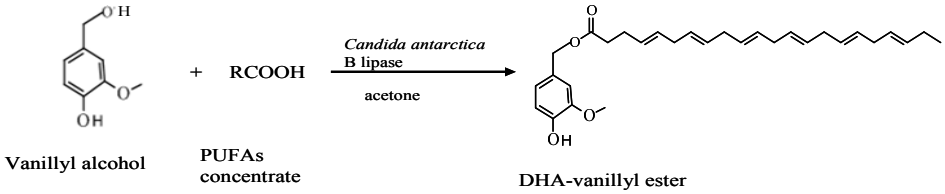


Figure 11. Reaction scheme of lipase catalysed esterification of vanillyl alcohol with fatty acids in PUFA concentrate e.g. DHA.

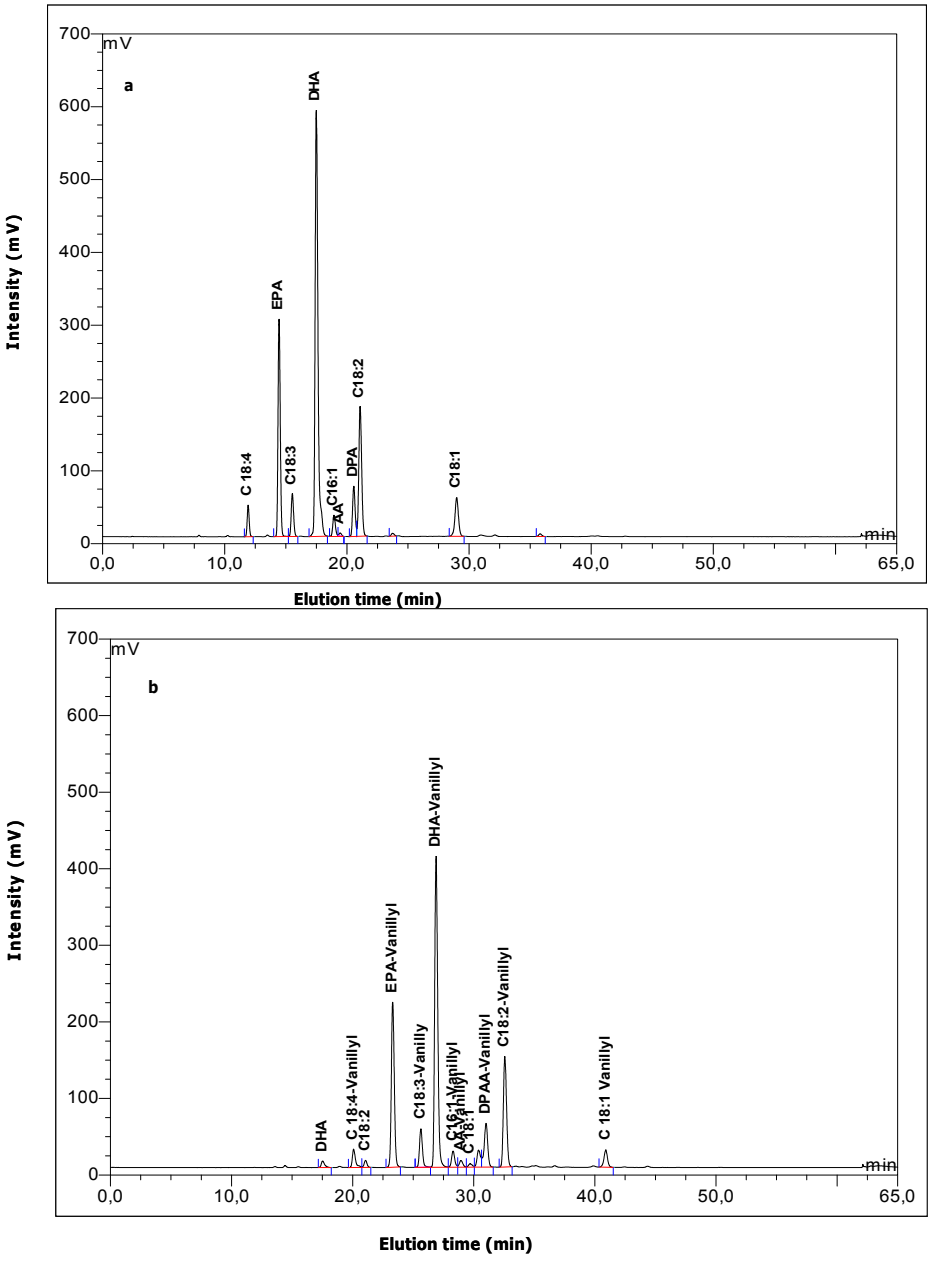


Figure 12. HPLC-ELSD chromatograms of PUFAs concentrate (a) and products of enzymatic esterification of PUFAs concentrate with vanillyl alcohol (b)

Esterification of vanillyl alcohol with PUFA greatly increased its hydrophobicity (**Figure 12**) and the esters stabilized both bulk and emulsion lipid systems against oxidation. In contrast, a slight increase in rutin hydrophobicity was observed with the rutin-pufa esters stabilising both bulk and emulsion lipid systems but to a lower degree in comparison with commercial antioxidants and the vanillyl-PUFA esters [**Paper IV**]**.**The difference observed in antioxidant activities of the two products could be attributed to the difference in the structure, solubility and the number of phenolic hydroxyls [148]. The results suggest that esterification of PUFA concentrate to natural phenolics improved their stability against oxidation and that is why the PUFA-phenolic derivative could stabilise the oil systems against oxidation. The advantage of such a derivative is that it carries the combined health benefits of PUFA and phenolics. Esterification of epigallocatechin (EGCG) with EPA or DHA resulted in esters which in addition to antioxidant activity showed antitumor activity that were absent in the parent phenolic compound EGCG, suggesting that the PUFA moieties contributed to the bioactivities of the ester derivatives [149].

8.0 Integration of omega 3-fatty acids and bio-fuel recovery from fish wastes

Enzymatic hydrolysis of fish waste to improve oil yields was employed as the first step towards recovery of omega-3 PUFAs. This resulted in four fractions: oil, emulsion, water soluble and insoluble fractions [**Paper I**]. To achieve better utilisation of fish waste, it is important to make use of the water non-soluble fraction as it forms a significant part of the hydrolysis products [50]. The fish sludge (insoluble fraction + emulsion) following enzymatic hydrolysis of salmon by-products presented 57% of the hydrolysis products. The potential of utilisation of such a fraction for biogas production through anaerobic digestion (AD) was evaluated in **Paper V.** AD involves the degradation of organic materials by microorganisms in absence of oxygen which leads to the formation of microbial biomass and biogas, a mixture of carbon dioxide and methane [150]. Following AD, specific methane yields of 828 and 742 m^3 CH_4/ton of organic material measured as volatile solids (VS) were attained from the fish waste (FW) before oil extraction and fish sludge (FS), respectively. The lower methane yield in the sludge was attributed to the reduced oil content. Lipids are known to have very high methane yields, approximately 1000 m^3 CH_4/ton VS. The methane yield from FS indicated that some lipids were present in the sludge since methane yield from proteins is about 490 m^3 CH_4 per ton of VS. Analysis of composition of different fractions obtained after hydrolysis of cod by-products showed the sludge contained a relatively high amount of lipids (up to 33.4

g/100 g of dry sludge) [49]. Also, as reported in **Paper I**, the oil yields obtained by enzymatic extraction method were lower than those obtained using solvents confirming this observation.

Omega-3 PUFAs in fish oil can be enriched as acylglycerol through hydrolysis of fish oil triglycerides as described in **Paper II**, enzymatic esterification of free fatty acids obtained by hydrolysis of fish oil or hydrolysis [**Paper III**]**,** enzymatic hydrolysis of fatty acid ethyl esters as described in **Paper III**, or urea crystallisation as described in **Paper IV**. In all these methods, saturated and monounsaturated fatty acids are the main by-products and can be converted to methyl/ethyl esters through an esterification reaction. Methyl/ethyl esters of fatty acids are regarded as biodiesel. Production of biodiesel from fish by-products has been reported [151].

8.1 Limitations of using fish residues as substrate for biogas production

Despite the high yields of methane observed for FW and FS, there are limitations of using fish waste for biogas production in a continuous anaerobic digester due to high content of lipids, proteins and light metals (sodium, potassium and calcium). These compounds are known to inhibit the methanogenesis [152, 153]. The intermediates of fat/lipid degradation are glycerol and long chain fatty acids (LCFAs). The inhibitory effect of lipids is commonly attributed to the free LCFAs [154]. This is due to their adsorption onto the cell wall/membrane interfering with the transport of soluble substrates to the biomass and consequently causing the conversion rate of substrates to decrease [150, 155]. Additionally, sorption of a light layer of LCFAs to biomass leads to the flotation of sludge and consequent sludge washout.

Ammonia is a product of urea hydrolysis or anaerobic degradation of protein-rich substrates such as fish sludge. Ammonia concentrations below 200 mg/L are beneficial to anaerobic process [150], but when present at high concentrations, it may inhibit methanogenesis. There are two forms of ammoniacal nitrogen in aqueous solution, ammonium (NH_4^+), and free ammonia (FA). FA is reported to be the main cause of inhibition since it is highly membrane-permeable and can therefore diffuse passively into the cell causing proton imbalance, and/or potassium deficiency [156]. As discussed in **Paper V**, both FW and FS had very high nitrogen concentrations (23 and 26 g/l, respectively), which would result in very high FA concentrations upon anaerobic digestion.

Metal ions are required for microbial growth when present in moderate concentrations [150]. For instance moderate levels of sodium (100-200 mg/l) are beneficiary to the anaerobes in formation of ATP and oxidation of NADH, while high levels may facilitate the passive influx of K^+ thereby neutralizing membrane potential. Fish sludge and FW were rich in light metals such as sodium, potassium and calcium with potassium concentrations as high as 8.7 g/l [**Paper V**]. Potassium is reported to be stimulatory at 200-400 mg/l and toxic above 3000 mg/l [157].

8.1.1 Co-digestion of fish waste for biogas production

The limitations of using FW and FS for biogas production in a continuous anaerobic digester can be overcome through co-digestion. This is defined as anaerobic treatment of a mixture of at least two different substrates with the aim of improving the efficiency of the AD process [158]. It helps in balancing the carbon: nitrogen (C:N) ratio in the co-substrate mixture, as well as macro and micronutrients, pH, dry matter and diluting inhibitors/toxic compounds [159]. Feasibility of co-digesting FS with residues from crop cultivation that are high in carbohydrate but low in fat, proteins and salts was evaluated and a full scale process outlined in **Paper V.** Co-digestion of 604 tonnes (t) of FS (residue that remained after removal of oil and soluble fish proteins from 1000 t of FW) with 2620 t of crop residues, at a ratio of 1:3 tonnes VS, would yield 538 t of biogas with 65% methane. The effluent, 4206 t was shown to be a good biofertilizer that can be used in agricultural land due to the low content of heavy metals while the ammonia-nitrogen, phosphorous and potassium were in line or higher than present in swine or cattle manure. Co-digestion of FS after enzymatic pre-treatment of FW for recovery of more valued PUFA and soluble fish proteins would thus ensure conversion of all parts of a smelly fish waste to useful products.

9.0 Conclusions and future perspectives

By-products from fish filleting present a potential resource instead of waste. Use of proteolytic enzymes to hydrolyse such biomass results in three major products, the oil fraction, soluble protein fraction and insoluble fraction that comprises of emulsion and sludge [**Paper I**]. The three fractions can be upgraded to products of more value such as omega-3 fatty acids [**Paper II, III** and **IV**], bioactive peptides and biogas [**Paper V**]. By use of proteases, the oil yields are improved when compared with the conventional cooking method. However, high water content in the samples results in reduced yields.

To add value to Nile perch oil, lipases that discriminate against EPA and DHA in fish oils were used to enrich these fatty acids. Based on results presented in this thesis, lipase catalysed enrichment of DHA/EPA as free fatty acids or ethyl esters [**Paper III**], resulted in better yields than enrichment as acylglycerols via hydrolysis [**Paper II**]. This is because during triglyceride hydrolysis, the full selectivity of lipases is not expressed as factors such as regio-specificity, non-homogenous distribution of DHA and EPA in the glyceride molecule as well as triglyceride structure comes into play. However, if DHA and EPA are required in aclyglycerol form, the DHA and EPA lost during the hydrolysis step could be enriched through esterification step. Enrichment of EPA/DHA in free form involves an extra step of splitting the fatty acids from the triglyceride molecule while enrichment in acylglycerol form involves hydrolysis of the native oil.

Saturated and monounsaturated fatty acids are the by-products of omega-3 recovery process. Such fatty acids can be used in production of biodiesel through esterification process while the sludge from the biomass hydrolysis step is used in biogas production [**Paper V**]. Integration of oil extraction for omega-3 recovery and biofuel production would minimise waste emanating from such a process. In fact, use of fish biomass as a feedstock would provide an excellent opportunity to initiate a fish waste biorefining industry. Several products as illustrated in scheme below (**Figure 13**) can be recovered using a variety of technologies.

Polyunsaturated fatty acids are highly prone to oxidation. This is usually minimised by addition of antioxidants such as α-tocopherol and BHT. Natural phenolic antioxidants are also used but the use is limited due to their hydrophilicity. Esterification of PUFA with natural phenolic compounds improves hydrophobicity of phenolic compounds [**Paper IV**]. PUFA esterified to phenolic compound is stabilised against oxidation and the lipophilic-phenolic derivative protects oil systems against oxidation. The PUFA-phenolic derivative also carries the health beneficial properties of PUFAs and phenolics. To synthesize such a kind of derivatives, prior separation of PUFA into individual fatty acids may not be necessary since using lipase from *Candida antarctica* which does not show fatty acid specificity, all fatty acids are incorporated into the phenolic derivative. Acylation of phenolic derivatives with a primary hydroxyl group such as vanillyl alcohol results in better yields.

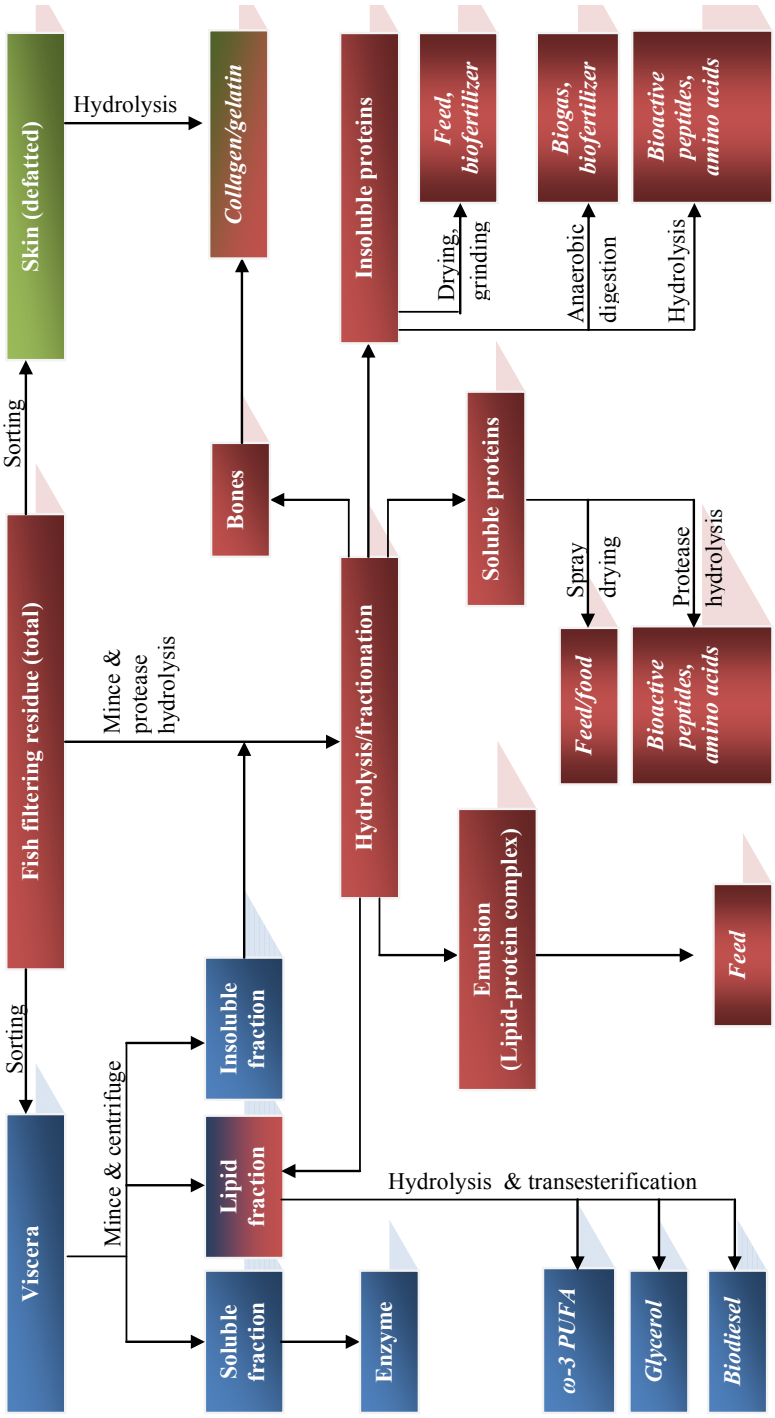


Figure 13. Schematic diagram of a fish waste biorefinery concept.

10.0 Acknowledgements

I would like to express my appreciation to all the people who in different capacities have greatly supported me during my PhD journey. There are a few who deserve special thanks.

My supervisors

Professor Bo Mattiasson, thank you for accepting me as a PhD student at the department of Biotechnology. I am also very grateful for the valuable discussions, suggestions and always taking time to answer my questions. Your encouragement and support gave a lot of motivation.

Professor Patrick Adlercreutz. Thanks for accepting me as your student when Dietlind left. Thanks for always being available for discussions, excellent advice and encouragement.

Professor Francis Mulaa, thanks for supervision and support while in Kenya.

Dr. Dietlind Adlercreutz, my co-supervisor in the first study (Paper I), thanks for introducing me to the world of lipidomics and being patient with my learning.

To co-authors of my papers Ally, Shiva, Ivo and Lo, thanks for good collaboration and excellent discussions.

To the departments' secretaries, Siv and Kersti, thanks for your kindness and help at all times since the first day I arrived in Lund. Frans-Peder, for your help with technical problems in the lab and office.

To all present and former environmental group members (Emma, Marika, Lo, Ivo, Valentine, Malik, Kassuwi, Maria, Maryam, Rosa, Danesh, Carla and Marisa): thank you for your friendship, and great moments during the dinners and fikas. Thanks too for bearing the fishy smell. Special thanks to my friends in the department Suhaila, Sindhu, Ally, Maryam, Laura, Tarek, Lesedi, Carla, Malik and Ivo for your help, participation and understanding during my difficult times in Lund. To my Kenyan Friends in Lund; thanks for making me feel home away from home. Suhaila, asante sana .

To the Bioorganic group members (Sindhu, Julia, Fabian, Carl, Gustav, Shiva and Pontus) thanks so much for your help with the analytical equipments (GC and HPLC).

To Marion, Maggie and Benny you are friends in deed. Thanks for standing in the gap and always being there for me.

I owe a lot to mum, dad, baba Jimmy and my siblings. Thanks for endless love, support, encouragement and prayers. Without you, I would not have come this far.

The Swedish International Development Agency (Sida/SAREC) sponsored BIO-EARN (East African Regional Programme and Research Network for Biotechnology, Biosafety and Biotechnology Policy development) program is gratefully acknowledged for the financial support of this work.

Finally, to the Almighty God, the pillar of my life in whom I trust.

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Paper I

Process Biochemistry 45 (2010) 815–819

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

Enzymatic oil extraction and positional analysis of ω-3 fatty acids in Nile perch and salmon heads

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ARTICLE INFO

Article history:
Received 24 August 2009
Received in revised form 24 January 2010
Accepted 9 February 2010

Keywords:
Salmon
Nile perch
Enzymatic extraction
Positional analysis
EPA
DHA

ABSTRACT

The use of commercial proteases, bromelain and Protex 30L for oil extraction/recovery of polyunsaturated fatty acids (PUFA) from Nile perch and salmon heads was evaluated. Four phases were obtained after hydrolysis, oily phase, emulsion, aqueous phase and sludge. An increase in water content during the hydrolysis resulted in a decrease in oil yield. Maximum oil yield was obtained when hydrolysis was performed with Protex 30L at 55 °C, without pH adjustment or water addition. An oil yield of 11.2% and 15.7% of wet weight was obtained from Nile perch and salmon heads, respectively, compared to 13.8% and 17.6%, respectively obtained using solvent extraction. Fatty acid distribution analysis showed 50% of palmitic acid was in *sn*-2 position in Nile perch triglycerides (TAG), while only 16% of this fatty acid was in *sn*-2 position in salmon oil TAG.

1. Introduction

Fish oils are an important source of omega-3 polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [1,2], which receive great interest in the scientific community because of their positive effects on human health. These fatty acids are believed to play a preventive role in cardiovascular diseases and alleviation of other health problems [3,4].

Fish oils from cold-water fish contain high proportions of ω-3 fatty acids [5]. Polyunsaturated fatty acids have also been found in tropic water fish [6]. *Lates niloticus* (Nile perch), a commercial fish in Lake Victoria and also found in central Africa's lakes and rivers, has been reported to contain considerable amounts of PUFAs [7]. Over 75% of the Nile perch catch in Lake Victoria is processed and exported as fillets. The by-products, which consist mainly of head, viscera, skeleton and skin are sold locally or wasted. One way to add value to Nile perch by-products is to recover omega-3 fatty acids. Oil from Nile perch heads has been extracted by crushing, heating and decanting, resulting in an oil yield of 15–18% of the dry weight, consisting of 16 mol% PUFA [7].

Fish oil can be produced by several methods which include hexane extraction [8], supercritical fluid extraction, which necessitates reduction of moisture content in sample prior to extraction, and heat treatment, which may affect quality of PUFAs [9,10]. Enzymatic oil extraction using commercial, low cost food grade proteases provides an attractive alternative as reactions can be carried out under mild conditions for short periods of time [11]. Commercial proteases have been used to release oil from marine by-products resulting in improved yields as compared to yields obtained after heat treatment [12]. In addition, the resulting hydrolysate provides a good source of soluble fish proteins. Hydrolysis conditions in these experiments involved pH adjustment and addition of water to the reaction medium. However, this is not industrially desirable since it adds to the process cost and bulkiness.

In the present work, use of proteases for extraction/recovery of oil from Nile perch and salmon heads without pH adjustment or water addition was evaluated.

2. Materials and methods

2.1. Materials

Salmon heads (*Salmo salar*) were collected from a local market in Lund, Sweden in month of September. Nile perch heads (*Lates niloticus*) were from Lake Victoria, Kenya (1°S, 33°E), collected in July. Protex 30L (≥2750 GSU/g) was a gift from Genencor, a division of Danisco A/S, Denmark. Bromelain (6 U/mg) and lipase from *Thermomyces lanuginosus* (≥100,000 U/g) (Amano lipase) were purchased from Sigma (St Louis, MO, USA). Qualmix fish S and methyl tridecanoate were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Silica gel 60 TLC plates were obtained from Merck (Darmstadt, Germany). All solvents and chemicals were of analytical grade.

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doi:10.1016/j.procbio.2010.02.010

2.2. Sample preparation and analysis

Three Kg each of salmon and Nile perch heads were ground and homogenized at 4 °C using Grindomix-GM 200 grinder to reduce particle size. The homogenates were preserved under nitrogen atmosphere at −20 °C until further analysis. This was done to minimize oxidation of long chain fatty acids and minimize endogenous lipolytic activity.

2.3. Enzymatic oil extraction from salmon and Nile perch heads

Ground and homogenized frozen salmon head fractions were thawed overnight at 4 °C. A 50 g sample was mixed with different amounts of water (0, 10, 25, 40 and 50 ml) in a 500 ml jacketed reactor heated from a re-circulating water bath. The mixtures were heated to a temperature of 55 °C (approximate 15 min) with stirring at 500 rpm after which enzymatic hydrolysis was initiated by addition of 0.5% bromelain (by wet weight of raw material). The contents were flushed with nitrogen and hydrolysis was allowed to proceed for 1 h.

Other parameters studied were enzyme concentration (bromelain 0.2–1% (w/w), Protex 30L 0.01–0.3% (v/w)), and hydrolysis time. The hydrolysates were centrifuged at 2000 × *g* at room temperature for 15 min. In order to recover the different fractions after centrifugation, the tubes were put upright at −20 °C for 2 h and the fractions separated by cutting the frozen content of the tubes and the wet weights determined. In all cases, control experiments were performed without enzyme addition.

Using the optimum hydrolysis conditions obtained with salmon (water content, hydrolysis time and enzyme concentration above), oil was extracted from 100 g of Nile perch heads homogenate using 0.16% (v/w) of Protex 30L. All experiments were performed in triplicates.

2.4. Chemical analysis

Dry matter content in salmon and Nile perch heads and fractions obtained after hydrolysis was determined gravimetrically after oven-drying the samples at 105 °C for at least 16 h [13]. Thereafter, ash content was quantified after heating the sample at 550 °C for 2 h. Total lipids were extracted from the by-products according to Folch method [14]. Crude protein content (N x 6.25) in the raw material and emulsion phase was determined using the Kjeldahl method [13] using FIAStar 5000 Analyzer (Foss-Tecator, AB Sweden).

2.5. Positional distribution of fatty acids

Positional distribution of fatty acids in the extracted oils was analyzed as described by Amate and Ramirez [15]. A 1,3-specific lipase from *Thermomyces lanuginosus* (1000 units) was used to hydrolyse 120 mg of oil and reaction stopped after 1 h. Hydrolysate was applied on thin layer chromatography (TLC) plates and eluted with cyclohexane/diethyl ether/acetic acid (50:50:1, v/v/v) to separate the monoglycerides (MAG), diglycerides (DAG), triglycerides (TAG) and free fatty acids [16]. Plates were viewed at 366 nm after spraying with 0.2% w/v 2,7-dichloroflourescene in ethanol. MAG fraction was scraped off and methylated for GC analysis. Fraction of fatty acid x at *sn*-2 position of TAG was calculated according to Eq. (1):

$$\text{Fraction}_{x\,\text{sn-2}} = \frac{MAG_x}{3 * TAG_x} \quad (1)$$

where X = mol% Palmitic acid, EPA or DHA.

2.6. Gas chromatography analysis of fatty acids

Extracted crude oils from Nile perch and salmon heads were transesterified into fatty acid methyl esters (FAME) using sodium methoxide [16,17]. FAME analysis was carried out using a Varian 3400 GC system equipped with a flame ionisation detector. Supelcowax 10 capillary column (60 m × 0.32 mm × 25 μm film thickness; Supelco, Bellefonte, PA, USA) was used to separate FAME. The carrier gas was helium at 1.79×10^{-1} MPa. Temperature programme for separation was: initial temperature of 50 °C was held for 5 min, increased to 220 °C at 25 °C/min and held for 21 min this was then increased to 240 °C at 15 °C/min and held for 10 min. Injector temperature was maintained at 60 °C for 0.1 min, and then increased to 250 °C at 60 °C/min and held for 2 min. Detector temperature was kept constant at 250 °C.

3. Results and discussion

3.1. Approximate chemical composition of salmon and Nile perch heads

Crude salmon heads contained (%): 38.2 ± 1.2 dry matter (DM); 3.3 ± 0.2 ash, 17.6 ± 1.5 lipid, and 11.9 ± 0.4 protein, while Nile perch heads contained (%): 37.3 ± 1.7 DM, 4.5 ± 0.4 ash, 13.8 ± 0.8 lipid. The low DM content in Nile perch and salmon heads indicated that above 60% of the Nile perch and salmon heads constituted water. Linder et al. [11] reported DM and ash content of 38.2 ± 1.3% and 2.6 ± 0.6%, respectively, from salmon heads.

Table 1
Yield of dry matter (g/100 g wet weight of raw material) obtained after enzymatic hydrolysis.

Water/substrate ratio	No water	1:1	1:2
Oil	12.2 ± 1.1	7.6 ± 0.3	8.8 ± 1.2
Fish protein hydrolysate	6.9 ± 0.8	10.8 ± 0.3	8.7 ± 1.2
Emulsion	5.8 ± 0.5	7.3 ± 0.1	5.4 ± 0.4
Emulsion protein content	0.6 ± 0.1	1.2 ± 0.1	0.7 ± 0.2
Control[a]	6.4 ± 0.7	7.0 ± 1.3	6.4 ± 0.8

Values are means ± standard deviation of triplicate determinations.
50 g salmon head homogenate hydrolysed for 1 h with 0.5% (w/w) Bromelain in the presence of different amounts of water and without pH adjustment.
[a] Control experiment is without enzyme

3.2. Enzymatic oil extraction from salmon and Nile perch by-products

3.2.1. Effect of water on oil yield

Enzymatic hydrolysis resulted in formation of four phases (an oily phase, emulsion phase, aqueous phase and sludge). A maximum oil yield of 12.2 ± 1.1% of wet weight was obtained when salmon heads were hydrolysed with 0.5% (w/w) bromelain with no water addition. The oil yield decreased with increasing water content with an oil yield of 7.6 ± 0.3% being attained when the sample was mixed with an equal amount of water (1:1, w/v) (Table 1). Decrease in oil yield with increasing water content during the hydrolysis could have been due to emulsion formation. An emulsion of 5.7 ± 0.5 and 7.3 ± 0.1 g dry weight per 100 g wet weight starting raw material, with protein content of 0.6 ± 0.1 and 1.2 ± 0.2 g was attained in the sample hydrolysed without water addition and at 1:1 (w/v) dilution, respectively. The emulsion has been reported to contain a high fat content compared to aqueous/fish protein hydrolysate (FPH) fraction, due to high contents of hydrophobic amino acids that retain more of the lipids than the more water-soluble FPH fraction [18]. During enzymatic hydrolysis of raw materials containing high amounts of lipids (10–30%), amount of added water has also been reported to have a significant influence on the amount of separated oil and emulsion fraction with the highest oil yield and lowest amount of emulsion being attained in the absence of water [19,20].

3.3. Effect of hydrolysis time on oil yield

By hydrolysing 50 g of salmon heads homogenate with 0.5% (w/w) bromelain without water addition, an oil yield of 11.8 ± 0.4% g lipids/100 g wet weight was achieved after 1 h of hydrolysis (Fig. 1). Prolonged hydrolysis (4–14 h) did not improve the oil yield further but resulted in a colour change of aqueous phase to brown. The formation of brown pigments could have resulted from reaction of carbonyls such as aldehydes produced from PUFA oxidation with amino acids and proteins [21]. Decrease in oil yield after 2 h could have been due to interaction of more lipids with the hydrolysed proteins as was the case observed when higher amount of water was added to the reaction. Linder et al. [11] reported that fat recoveries from salmon heads did not increase further with an increase in hydrolyzing time.

3.4. Effect of enzyme concentration on oil yield

The effect of varying amounts of enzyme on oil yield during hydrolysis was investigated using 50 g of salmon heads homogenate in condition where neither water was added nor pH

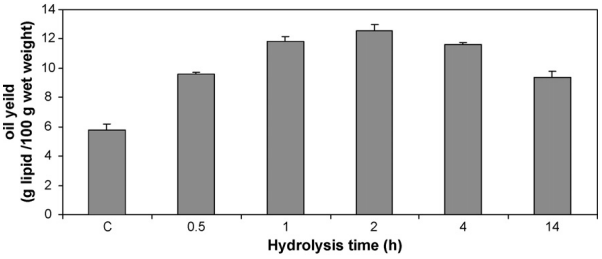

Fig. 1. Oil yield from 50 g of salmon heads homogenate incubated at 55 °C with 0.5% (w/w) Bromelain for 0.5, 1, 2, 4 and 14 h without water addition or pH adjustment. Column C shows oil yield from unhydrolysed sample.

adjusted. Maximum oil yield of 11.6 ± 0.2 g lipids/100 g wet weight was achieved when 0.5% (w/w) bromelain was used (Fig. 2a). A higher concentration did not result in further increase in oil yield. Using different concentrations of Protex 30L, an oil yield of 15.7 ± 0.4 g lipids/100 g wet weight was achieved with 0.05% (v/w) Protex 30L (Fig. 2b). Solvent extraction gave an oil yield of 13.8% in Nile perch and 17.6% in salmon. Compared with the control, hydrolysis with the two proteases resulted in an increased oil yield. However, the recoveries were lower than those obtained by solvent extraction. With 0.05% (v/w) Protex 30L, 88% of the total lipid was recovered while with 0.5% (w/w) bromelain, recovery was 65%. Hydrolysis of Nile perch heads homogenate with Protex 30L, yielded 11.2 ± 1.2 g lipids/100 g wet weight which was 81% of total lipids.

Increasing enzyme concentration would lead to an increased hydrolysis, but this does not necessarily lead to an increased oil yield. A study by Linder et al. [11] showed no significant difference in oil yield from salmon heads following enzymatic hydrolysis for 2 h with three enzymes (Neutrase®, 17.2%; Flavourzyme, 17.0%; Alcalase®, 17.4%). The degree of hydrolysis (DH) was in the range

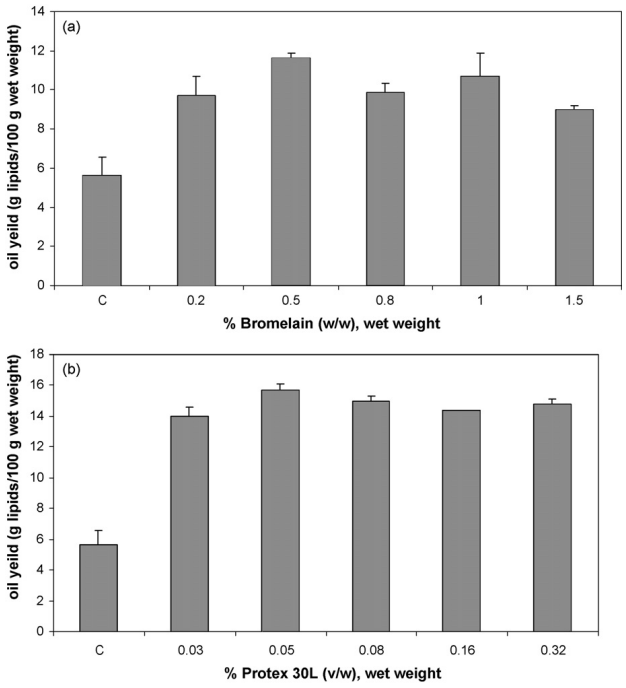

Fig. 2. Effect of enzyme concentration: (a) Bromelain, (b) Protex 30L on oil yield from 50 g of salmon heads homogenate incubated with different amounts of enzyme at 55 °C without water addition or pH adjustment. (C) is the oil yield from control sample incubated for 1 h without enzyme.

Table 2
Fatty acid composition (mol%) in crude oil recovered from Nile perch and salmon heads.

Fatty acid profile (mol%)			Fatty acid distribution (mol%) *sn 2* -position	
Fatty acid	Salmon oil	Nile perch oil	Salmon oil	Nile perch oil
C 14:0	3.4 ± 0.01	3.6 ± 0.03	2.1 ± 0.06	4.2 ± 0.53
C 15:0	0.3 ± 0.04	0.2 ± 0.47	0.3 ± 0.02	0.6 ± 0.18
C 16:0	12.3 ± 0.17	24.0 ± 0.20	5.9 ± 1.09	36.8 ± 1.45
C 16:1	4.6 ± 0.06	11.0 ± 2.29	5.2 ± 0.07	10.5 ± 1.05
C 18:0	3.0 ± 0.04	9.0 ± 0.25	0.6 ± 0.09	5.1 ± 1.11
C 18:1	34.9 ± 1.61	24.2 ± 0.43	39.6 ± 0.47	14.8 ± 0.45
C 18:2	10.2 ± 0.14	2.3 ± 0.50	11.5 ± 0.13	0.9 ± 0.19
C 18:3	4.2 ± 0.06	4.6 ± 1.13	6.2 ± 0.14	1.9 ± 0.5
C 18:4	1.1 ± 0.01	0.1 ± 0.88	1.7 ± 0.02	1.0 ± 0.07
C 20:1	1.0 ± 0.01	10.2 ± 0.43	2.7 ± 0.05	–
C 20:4	0.5 ± 0.01	2.7 ± 0.21	0.5 ± 0.01	0.7 ± 0.03
C 20:5	6.1 ± 0.09	3.4 ± 0.25	4.5 ± 0.28	3.2 ± 0.73
C 22:1	6.9 ± 0.1	0.1 ± 0.08	0.8 ± 0.05	1.3 ± 0.04
C22:4	0.1 ± 0.03	1.0 ± 0.47	–	–
C 22:5	3.0 ± 0.04	5.9 ± 0.27	3.8 ± 0.05	7.5 ± 0.5
C 22:6	8.4 ± 0.07	7.7 ± 0.29	14.8 ± 0.13	11.5 ± 0.01

of 6.9–7.2, 8.0–8.2, or 16.4–16.6 for Neutrase®, Flavourzyme or Alcalase®, respectively.

3.5. Fatty acid profile and positional distribution of fatty acids in Nile Perch and salmon oils

Fatty acid profile of unhydrolysed oil and monoglyceride fraction obtained after hydrolysis with 1,3-specific lipase is shown in Table 2. Nile perch oil showed a higher content of saturated fatty acid (36.8 mol%) than salmon oil (19 mol%) (Table 2). This could be a temperature effect since Nile perch is a warm water fish while salmon is cold water. Uysal et al. [22] observed that in the muscle of *Barbus plebejus escherichi* saturated fatty acid content was significantly higher in July (temperate conditions, 26 °C) than in January (cold conditions, 5 °C), while polyunsaturated fatty acid content was significantly higher in January than in July. A decrease of environmental temperature has been reported to lead to an increased proportion of unsaturated fatty acids that are important on maintaining membrane fluidity [23].

Of the total palmitic acid, EPA and DHA present in the crude oils, approximately 50, 13 and 48 mol% palmitic acid, EPA and DHA respectively, were in the *sn*-2 position in Nile perch TAG (Table 3). In salmon oil 17, 28 and 57 mol% palmitic acid, EPA and DHA respectively was in *sn*-2 position (Table 3). Lipase hydrolysis does not give the complete structure of the TAG molecule, but it offers reliable information about the *sn*-2 position. Turon et al. [7] reported a comparable distribution of 46, 25 and 57 mol% palmitic, EPA and DHA, respectively in *sn*-2 position of Nile perch TAG using chemical deacylation. Using high-resolution ^{13}C nuclear magnetic resonance spectroscopy, DHA has been reported to be concentrated in *sn*-2 position of TAG, whereas EPA is randomly distributed in all the three positions of Atlantic salmon TAG [24]. 1,3-specific lipases have been used to give an estimate composition of *sn*-2 position in squid, menhaden fish oils, tuna triglycerides, fungal triglycerides, egg phospholipids, and pig brain phospholipids [15,16].

Table 3
Positional analysis of palmitic acid, EPA and DHA found at the *sn*-2 and *sn*-1 and *sn*-3 positions of the TAGs in Salmon and Nile perch heads oils.

Oil	Position	Palmitic acid	EPA	DHA
Salmon	*sn*-2[a]	0.16 ± 0.03	0. 25 ± 0.04	0.59 ± 0.01
	sn-1 + 3[b]	0.84 ± 0.03	0.75 ± 0.04	0.41 ± 0.01
Nile perch	*sn*-2[a]	0.51 ± 0.01	0.13 ± 0.02	0.50 ± 0.01
	sn-1 + 3[b]	0.49 ± 0.01	0.87 ± 0.02	0.50 ± 0.01

[a] mol% fatty acid in monoglyceride (*sn* 2-MAG)/3*mol% fatty acid in initial triglyceride.
[b] *sn* -1 + 3 = 1 − *sn*-2.

4. Conclusion

This work shows that improved oil yields from fish by-products can be obtained by use of proteases with the added advantage of not requiring pH adjustment or addition of water. Since the pH of the by-products is close to neutral, enzymes with optimum pH within this range could be used. Simultaneous release of soluble proteins is another advantage. By-products of Nile perch processing are a potentially useful source of fish oil, which can be a source of economically useful omega-PUFAs. However, there is need to reduce the high concentration of saturated fatty acids, which constitute 36 mol% of total fatty acids.

Acknowledgements

We thank Dr. Suhaila Hashim for valuable input in manuscript preparation. The project is part of the Swedish International Development Cooperation Agency (SIDA/SAREC) sponsored BIO-EARN (East African Regional Programme and Research Network for Biotechnology, Biosafety and Biotechnology Policy development) program.

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Paper II

Research Article

Enzymatic enrichment of omega-3 polyunsaturated fatty acids in Nile perch (*Lates niloticus*) viscera oil

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Oil was extracted from fatty material obtained from Nile perch viscera using the protease Protex 30L. Enrichment of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the glyceride fraction was carried out by hydrolysis of extracted oils with lipases from *Candida rugosa*, *Thermomyces lanuginosus* and *Pseudomonas cepacia*. The unusual fatty acid distribution of the oil influenced the apparent lipase specificity to a large extent. In the unhydrolysed oil, only 16% of EPA was in *sn*-2 position while 51% of palmitic acid was located in this position of the triacylglycerol (TAG) molecules. Non-regioselective lipase from *C. rugosa* was the most effective in combined enrichment of both EPA and DHA. This was partly because it was able to hydrolyse off palmitic acid from the *sn*-2 position, which 1-, 3-specific lipases were unable to do. Hydrolysis with *C. rugosa* lipase enriched EPA from 3 to 6 mol% and DHA from 9 to 23 mol%, with recoveries of 42 and 55%, respectively. The 1-, 3-specific lipase from *T. lanuginosus* was ineffective in enriching EPA, but gave best DHA enrichment, 38 mol% with a recovery of 39%. DHA was rather equally distributed in *sn*-1, -2 and -3 positions of TAG. The results show that both the fatty acid specificity and regiospecificity of the lipase as well as the fatty acid distribution of the oil should be considered when choosing the strategy for fatty acid enrichment.

Keywords: DHA / EPA / Lipase-catalysed hydrolysis / Nile perch oil / Specificity constant

Received: January 12, 2010 / Revised: April 26, 2010 / Accepted: June 14, 2010

DOI: 10.1002/ejlt.201000009

1 Introduction

Nile perch (*Lates niloticus*) is a fatty fish from warm and fresh water and is the most dominant species in Lake Victoria, Eastern Africa. The bulk of by-products from Nile perch processing are wasted or sold at very low prices [1, 2]. Better utilization of the by-products could be achieved by recovering valuable products such as enzymes, vitamins, proteins and fish oil. Viscera and heads from Nile perch have the potential to be used as raw material for the production of fish oil since they contain a large amount of lipids rich in ω-3 polyunsaturated fatty acids (PUFA) [3, 4]. In addition, the oil has a high content of natural antioxidants (β-carotene and α-tocopherol) that are important in delaying PUFA oxidation [3].

Omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are associated with several health benefits [5–7] and consumption of appropriate amounts and proportions of these fatty acids are essential. When fish oil is suggested as a means of improving health, the content of saturated fatty acid (SFA) and PUFA composition should be considered and in some cases it is beneficial to concentrate EPA and DHA further. A number of techniques have thus been developed to prepare PUFA concentrates from fish oils, devoid of more SFA. They include low temperature crystallization, urea complexation, distillation, supercritical fluid extraction, chromatography and enzymatic methods [8].

Enzymatic enrichment of PUFA has shown potential in producing high quality product due to the mild conditions (*e.g.* neutral pH and low temperatures) of the process [9]. Concentrates of both EPA and DHA may be prepared by selective hydrolysis of fish oils using lipases which discriminate against ω-3 PUFA such as lipases from *Candida rugosa*, *Pseudomonas* sp., *Aspergillus niger* and *Thermomyces lanuginosus* [10–13] or by selective esterification [14, 15].

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Abbreviations: DAGs, diglycerides; **DHA,** docosahexaenoic acid; **DPA,** docosapentaenoic acid; **EPA,** eicosapentaenoic acid; **FAME,** fatty acid methyl esters; **MAGs,** monoglycerides; **MUFA,** Monounsaturated fatty acid; **PUFA,** polyunsaturated fatty acids; **SFA,** saturated fatty acid; **TAG,** triacylglycerol

Use of lipases to enrich ω-3 fatty acids in Nile perch oil has not been reported. In this study, preparation of ω-3 PUFA enriched acylglycerols from Nile perch viscera oil via enzymatic hydrolysis was evaluated using three commercial lipases. Effect of temperature on ω-3 PUFA enrichment and lipase specificity has also been evaluated.

2 Materials and methods

2.1 Materials

Protex 30L (≥2750 GSU/g) was a gift from Genencor, a division of Danisco A/S Denmark. Lipases from *T. lanuginosus* (≥100 000 U/g), *C. rugosa* (≥700 000 U/g) and *P. cepacia* (Amano lipase PS, ≥30 000 U/g) were from Sigma (St Louis, MO, USA). Qualmix fish S and methyl tridecanoate were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Silica gel 60 TLC plates were obtained from Merck (Darmstadt, Germany). All solvents and chemicals were of analytical grade.

2.2 Sampling and sample preparation

Fatty material obtained from Nile perch viscera was kindly donated by W. E. Tilly Ltd, Nile perch processing factory in Kenya and preserved at −20°C until use.

2.3 Enzyme assisted oil extraction and determination of fatty acid profile

Fatty material was defrosted at room temperature overnight. Hydrolysis with Protex 30L was performed at 55°C in a stirred jacketed reactor as described by Mbatia *et al.* [16]. The substrate was preheated to 55°C and enzymatic hydrolysis started by addition of Protex 30L, 0.05% v/w (wet weight of substrate). The contents were flushed with nitrogen and hydrolysis was allowed to proceed for 1 h with continuous stirring. This was followed by centrifugation at 10 000 × *g* for 10 min. The oil layer was decanted and preserved under nitrogen atmosphere at −20°C until further analysis.

In order to determine fatty acid profile of the extracted oil, a sample was transesterified into fatty acid methyl esters (FAME) using sodium methoxide [17, 18]. Methylation procedure was as described by Lyberg and Adlercreutz [13].

2.4 Lipase catalysed hydrolysis of Nile perch viscera oil

Oil extracted (Section 2.3) was hydrolysed with lipases from *C. rugosa*, *P. cepacia* and *T. lanuginosus* at 40°C (1000 U/g substrate). In order to determine the effect of temperature on enzymatic enrichment of EPA and DHA, a second set of hydrolysis was performed with lipases from *C. rugosa* and *T. lanuginosus* at 30, 40 and 50°C. In each reaction, 0.25 g of crude oil in 1.25 mL of 0.2 M sodium phosphate buffer (pH 7.2) was hydrolysed with continuous shaking at 500 rpm. A control experiment at 40 and 50°C was performed without enzyme addition. To minimize oxidation, the vials were bubbled with nitrogen prior to shaking.

Samples were withdrawn after 0, 1, 2, 4, 6, 12 and 24 h and diluted with 3 mL THF. Fatty acid composition of the hydrolysed lipids was determined by TLC and GC.

2.5 Thin layer chromatography

Triglycerides (TAGs), diglycerides (DAGs), monoglycerides (MAGs) and free fatty acids (FFAs) from lipase hydrolysed oil samples were separated on silica gel 60 TLC plates using the mobile phase cyclohexane/diethyl ether/acetic acid (50:50:1). In order to visualize the lipids and fatty acids in UV light (366 nm), the TLC plates were sprayed with 0.2% 2, 7-dichlorofluorescein in 99.5% ethanol. The bands containing TAGs (Rf = 0.74), DAGs (Rf = 0.41) and MAGs (Rf = 0.12) were scraped off and each fraction methylated as described by Lyberg and Adlercreutz [13] and analysed using GC.

2.6 Gas chromatography analysis

Fatty acid analysis was carried out using a Varian 3400 GC system equipped with a flame ionization detector (FID). Supelcowax 10 capillary column (60 m × 0.32 mm × 25 μm film thickness; Supelco, Bellefonte, PA, USA) was used to separate FAME. The carrier gas was helium at 1.79×10^{-1} MPa. The temperature programme for separation was as follows: initial temperature of 50°C was held for 5 min, increased to 220 at 25°C/min and held for 21 min. The temperature was then increased to 240°C at 15°C/min and held for 10 min. The injector temperature was maintained at 60°C for 0.1 min, and was then increased to 250°C at 60°C/min and held for 2 min. The detector temperature was kept constant at 250°C.

The instrument was calibrated with a one-point calibration method, using a standard mixture of fish oil FAME of known proportions (Qualmix fish S). The response factors of the different FAME were obtained from analysis of the standard mixture and were used to calculate the relative amounts of different fatty acids in a sample based on mol%. These data were compared with the internal standard (methyl tridecanoate) to determine the absolute amount (μmol) of the fatty acids in the sample. GC data provided are based on duplicate measurements.

2.7 The positional fatty acid composition

The positional fatty acid composition of the TAG was determined from fatty acid composition of the MAG fraction generated in the reaction catalysed by *T. lanuginosus* lipase at 30 and 40°C. MAG obtained after 1 h of hydrolysis at each temperature was used to calculate the fatty acid composition

of MAG (mol%). These data together with the fatty acid composition of the initial TAG in unhydrolysed oil were used to calculate the fraction of x (myristic acid, palmitic acid, EPA, DHA or docosapentaenoic acid (DPA)) at the *sn*-2 and *sn*-1 + 3 position of TAG according to Eqs. (1) and (2)

$$\text{Fraction}_{x\,sn-2} = \frac{\text{MAG}_x}{3^*\text{TAG}_x} \quad (1)$$

$$\text{Fraction}_{x\,sn-1+3} = 1 - \text{Fraction}_{x\,sn-2} \quad (2)$$

2.8 Lipase specificity at different temperatures

The specificity of lipases from *T. lanuginosus* and *C. rugosa* towards C16:1, C18:0, C18:1, EPA and DHA at the three temperatures was determined by calculating competitive factor, α defined by Eq. (3) [19, 20]. A log–log plot of Eq. (3) yields a linear curve with the slope equal to α value of the opposing substrate (B). From the α value, the specificity constant was calculated as $1/\alpha$ with palmitic acid as reference substrate and its specificity constant taken as 1.00

$$\alpha = \frac{\log(A_0/A)}{\log(B_0/B)} \quad (3)$$

where A_0 is the initial Palmitic acid concentration , A the Palmitic acid concentration at time t, B_0 the initial C16:1, C18:0, C18:1, EPA/DHA concentration and B is C16:1, C18:0, C18:1, EPA/DHA concentration at time t.

Two sets of competitive factors were calculated for *T. lanuginosus* lipase since it expresses regioselectivity. In the first case the competitive factors were calculated without taking into account the positional distribution of fatty acids and fatty acids in *sn*-1, -2 and -3 were considered as substrates. In the second case, only fatty acids in position *sn*-1 and *sn*-3 positions were considered as substrates. These were calculated as outlined below:

$$\text{Initial amount of fatty acid } (x) \text{ at } sn-1, -3\,\text{TAG} = \text{moles}\, x \times \text{Fraction}_{x\,sn-1+3} \quad (4)$$

$$\text{Initial amount of fatty acid } (x) \text{ at } sn-2 = \text{moles of } x \times \text{Fraction}_{x\,sn-2} \quad (5)$$

$$\text{Amount of fatty acid } (x) \text{ at } sn-1, -3 \text{ at time } t = \text{moles of } x_t - \text{initial moles of } x \text{ at } sn-2 \quad (6)$$

3 Results and discussion

3.1 Fatty acid profile in Nile perch viscera oil

Fatty acid composition of crude Nile perch viscera oil (mol%) is shown in Table 1. The SFA fraction was higher (36 mol%) than the PUFA fraction (27 mol%) with a ratio of PUFA to SFA of 0.73. Palmitic acid was the predominant fatty acid accounting for 23 mol%. Total ω-3 PUFAs (linolenic acid, EPA, DPA and DHA) accounted for 76% of the PUFA fraction. Monounsaturated fatty acid (MUFA) accounted for 37 mol% of total fatty acids. Ogwok *et al.* [3] reported a similar profile using oil extracted from belly flaps of Nile perch by cooking method. A PUFA/SFA ratio of 0.68–0.74 was reported in oil obtained from Nile perch of different size categories. Oil extracted from Nile perch head showed a similar profile with PUFA/SFA ratio of 0.55 [4].

Table 1. Fatty acid composition and distribution (*sn*-2 position) of oil from Nile perch viscera oil

Fatty acid	Fatty acid profile (mol%)	Fatty acid distribution (mol%) *sn*-2 position[a)]
C14:0	3.4 ± 0.3	5.5 ± 0.90
C15:0	0.5 ± 0.01	0.7 ± 0.03
C16:0	23.4 ± 0.1	35.3 ± 1.31
C16:1	12.7 ± 0.1	12.7 ± 1.23
C18:0	8.9 ± 0.01	5.3 ± 0.57
C18:1	23.7 ± 0.4	14.7 ± 1.87
C18:2	2.2 ± 0.1	2.2 ± 0.88
C18:3	2.3 ± 0.1	1.8 ± 0.17
C18:4	0.4 ± 0.03	0.0 ± 0.00
C20.1	0.6 ± 0.1	0.0 ± 0.00
C20:4	2.4 ± 0.1	1.9 ± 0.45
C20.5	3.0 ± 0.7	1.8 ± 0.14
C22:4	1.1 ± 0.4	1.2 ± 0.16
C22:5	6.2 ± 0.4	5.9 ± 0.75
C22:6	9.0 ± 0.5	10.8 ± 0.38

[a)] Fatty acid composition (mol%) in MAG fraction obtained after 1 h hydrolysis of TAG with *T. lanuginosus* at 30°C.

The SFA and MUFA content in Nile perch oils are higher than in menheden fish oil, while the ω-PUFA contents and PUFA/SFA ratio is lower. Menhaden oil has been proposed by USFDA [21] as PUFA supplement with ω-PUFA, MUFA and SFA fractions of 28.9, 19.4 and 20.2%, respectively, and a PUFA/SFA ratio of 2.87 [13, 22]. The high content of SFA, MUFA and low PUFA to SFA ratio in Nile perch oils indicates a need to concentrate PUFA prior to human consumption. Consumption of Nile perch PUFA concentrates may be more effective than the crude oil itself because the concentrates would contain less SFA thus allowing daily intake of total lipids to remain low.

The positional distribution of fatty acids in Nile perch viscera oil is shown in Tables 1 and 2. SFAs, palmitic acid (51%) and myristic acid (58%) were located in *sn*-2 position while EPA was mainly in *sn*-1 and *sn*-3 positions. DHA and DPA were equally distributed in the three positions. A similar distribution was found in oil extracted from Nile perch heads [4]. Myristic and palmitic acids were high in *sn*-2 position while unsaturated fatty acids were preferentially esterified in *sn*-1 and *sn*-3 positions [4].

Table 2. Distribution of Palmitic acid, myristic acid, EPA, DPA and DHA in different positions of TAG in Nile perch viscera oil

Position	C16:0	C14:0	EPA	DPA	DHA
sn-2[a)]	0.51 ± 0.014	0.58 ± 0.049	0.16 ± 0.057	0.37 ± 0.071	0.39 ± 0.021
sn-1 + 3	0.49 ± 0.014	0.42 ± 0.049	0.84 ± 0.057	0.63 ± 0.071	0.61 ± 0.021

Each value represents an average value of three independent experiments at 30 and 40°C and their SD.
[a)] Mol% fatty acid in monoglyceride (2-MAG)/3 × mol% fatty acid in initial TAG.

3.2 Fatty acid profiles after lipase hydrolysis

The three lipases used in this study caused changes in fatty acid composition, but in varying amounts. Ideally, enzymatic hydrolysis of fish oils followed by FFA removal will increase the concentration of PUFA while reducing SFAs and MUFAs. Figure 1a and b shows the changes in DHA, EPA, palmitic acid (C16:0) and oleic acid (C18:1) content in the glyceride fraction upon lipase hydrolysis. Among the lipases tested, 24 h hydrolysis with lipase from *C. rugosa* gave a better enrichment of combined DHA and EPA in the glyceride fraction than lipases from *P. cepacia* and *T. lanuginosus* (Fig. 1a). It also resulted in the highest decline of oleic acid after 24 h of hydrolysis (Fig. 1b). DHA and EPA contents were increased to 20 and 6 mol%, respectively, while oleic acid decreased to 14 mol%. Lipase from *T. lanuginosus* was the most efficient in enriching DHA (32 mol%). However, it was ineffective in enriching EPA (Fig. 1a). It also resulted in the least decline in palmitic acid (Fig. 1b). Lipase from *P. cepacia* increased DHA the least (14 mol%) while EPA was increased to 7 mol% (Fig. 1a). It also resulted in the highest decline of palmitic acid (Fig. 1b).

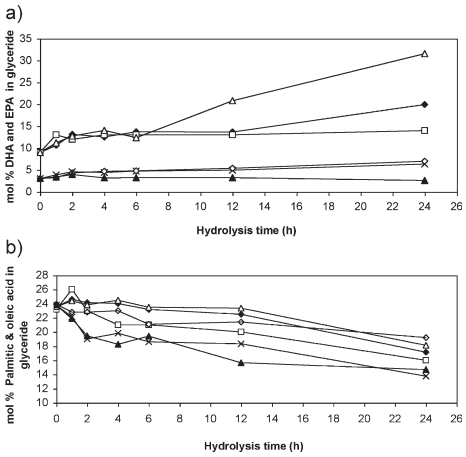

Figure 1. (a) Changes in DHA and EPA mol% in Nile perch viscera oil following hydrolysis at 40°C. ◆, □, △ mol% DHA and X, ◇, ▲ mol% EPA, after hydrolysis with *C. rugosa*, *P. cepacia* and *T. lanuginosus* lipases. (b) Changes in palmitic and oleic acid mol% in Nile perch viscera oil following hydrolysis at 40°C. ◆, □, △ mol% palmitic and X, ◇, ▲ mol% oleic acid, after hydrolysis with *C. rugosa*, *P. cepacia* and *T. lanuginosus* lipases.

The ability of *C. rugosa* lipase to enrich both DHA and EPA indicates ability of the lipase to discriminate SFAs and MUFAs from EPA and DHA in fish oils, most likely due to the reduced steric hinderance observed with SFAs and MUFAs [23]. In addition, *C. rugosa* lipase is non-regiospecific and catalyse hydrolysis of fatty acids at all positions in the TAGs [24, 25]. The lipase has been reported to be the most efficient in enriching combined ω-3 PUFA in marine oils [12, 26, 27]. Non-regiospecific lipase from *P. cepacia* gave a slightly better enrichment of EPA (7.0 mol%) compared to lipase from *C. rugosa* (6 mol%) although it increased DHA to 14 mol% compared to 20 mol% obtained with *C. rugosa* lipase. This was not surprising since lipases from *Pseudomonas* species (*P. cepacia* and *P. fluorescens*) have been reported to discriminate EPA more than DHA, resulting in an efficient increase in concentration of EPA in the acylglycerol form of fish oil [12, 13, 28]. *Chromobacterium viscosum* lipase is another lipase that has been reported to discriminate against EPA more than DHA [20].

Enrichment profile observed while using lipase from *T. lanuginosus* was probably influenced by its *sn*-1 and *sn*-3 position specificity. In the oil used in this study, EPA was mainly in *sn*-1 and *sn*-3 positions while 51% palmitic acid was in *sn*-2 position (Table 2). This was probably part of the reason why *T. lanuginosus* lipase was ineffective in enriching EPA and also hydrolysed palmitic acid least when compared to the other lipases. In the absence of lipases, there was minimal change in mol% EPA and DHA during the 24 h incubation (data not shown). This indicated that there was minimal oxidation of PUFA during the hydrolysis.

3.3 Competitive factors

Competitive factors for lipases from *C. rugosa* and *T. lanuginosus* with respect to C16:1, C18:0, C18:1, EPA and DHA were determined according to Eq. (3). Palmitic acid was chosen as a reference since it provided the highest concentration in the TAGs. Lipase specificity for a certain fatty acid was expressed as $1/\alpha$.

3.3.1 *C. rugosa* lipase specificity towards fatty acids in Nile perch oil at different temperatures

The specificity constants for *C. rugosa* lipase towards C16:1, C18:0, C18:1, EPA and DHA in crude Nile perch viscera oil at different temperatures are presented in Fig. 2. Ability of the non-regiospecific lipase from *C .rugosa* to enrich both DHA and EPA in the glyceride fraction was in agreement with specificity constants ($1/\alpha$ values) obtained. The lipase showed lower specificity to DHA and EPA compared to other fatty acids with DHA having the lowest specificity (Fig. 2). The lipase also showed a slightly higher preference for palmitoleic and oleic acid than palmitic acid. Haraldsson and coworkers [28], reported distinct discrimination of ω-3 PUFA for SFAs and MUFAs by *C. rugosa* lipase while considering hydrolysis of sardine oil ethyl esters, palmitic acid being the best substrate. The long chain PUFAs were also poorer substrates with the lipase displaying higher activity towards EPA than DHA [28].The lipase has been reported to have a strong discrimination against unsaturated fatty acids having the first double bond from the carboxyl end at an even number carbon such as (*cis*-4, such as DHA; and *cis*-6) than the others (*cis*-5, such as EPA; and *cis*-9) [20, 29].

Many factors such as regiospecificity, stereospecificity and structural specificity affect lipase specificity when considering hydrolysis of natural TAGs which have various fatty acids compositions and distribution within the glycerol backbone [25, 30, 31]. *C. rugosa* lipase preference for palmetoleic acid and oleic acid over palmitic acid observed in this study was not unusual. Lyberg and Adlercreutz [13] made a similar observation while hydrolysing oil from menhaden fish.

3.3.2 *T. lanuginosus* lipase specificity towards fatty acids in Nile perch oil at different temperatures

The specificity constants for *T. lanuginosus* lipase towards C16:1, C18:0, C18:1, EPA and DHA in crude Nile perch viscera oil at different temperatures are presented in Figs. 3 and 4. The $1/\alpha_{tot}$ values in Fig. 4 indicate that the lipase discriminates strongly against DHA in the oil, while discrimination against EPA seems quite low. The observed discrimination is partly caused by the fatty acid selectivity of the lipase. However, since this lipase is 1-, 3-specific, the distribution of fatty acids in the oil also influences the apparent discrimination. To obtain information on the true fatty acid specificity of the lipase, only fatty acids in the *sn*-1 and *sn*-3 positions of the TAG molecules should be considered as accessible substrate. When calculations are based on this, $1/\alpha_{1+3}$ values are obtained (Fig. 4). For EPA, the $1/\alpha_{1+3}$ value was about two times lower than $1/\alpha_{tot}$, because EPA was mainly present in the *sn*-1 and *sn*-3 positions while the reference, palmitic acid to a large extent was present in the *sn*-2 position (Table 2). This means that the enzyme discriminates against EPA, but this is compensated for by the uneven fatty acid distribution, which makes the apparent discrimination (shown as $1/\alpha_{tot}$) low. Similar effects were seen for C18:0 and C18:1. The $1/\alpha_{1+3}$ value for DHA were slightly higher than those of EPA at all the temperatures indicating higher discrimination of DHA. In addition to being regiospecific, lipase stereoselectivity has been reported [23, 30]. Lipase from *T. lanuginosus* has been reported to be more active at *sn*-1 position than at *sn*-3 position [23] which might have contributed to the results in the present study.

Previously, *T. lanuginosus* lipase has been reported to enrich EPA and DHA in menhaden fish oil [13]. The oil has a higher percentage of DHA in *sn*-2 position while EPA is equally distributed in *sn*-2 and *sn*-3 position and small amounts are in *sn*-1 position [13, 32]. A 1-, 3-specific *Rhizopus oryzae* lipase has been reported to enrich DHA in

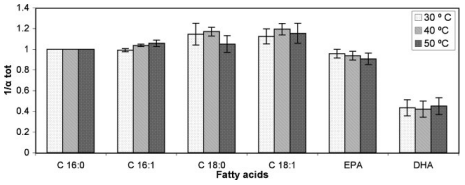

Figure 3. Specificity of *T. lanuginosus* lipase at different temperatures to fatty acids in *sn*-1, -2 and -3 positions of oil extracted from Nile perch viscera.

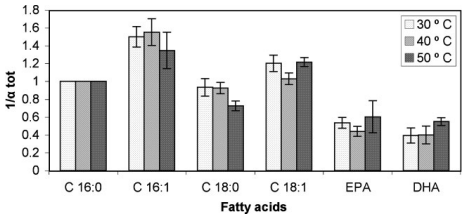

Figure 2. Specificity of *C. rugosa* lipase to fatty acids in Nile perch viscera oil at different temperatures.

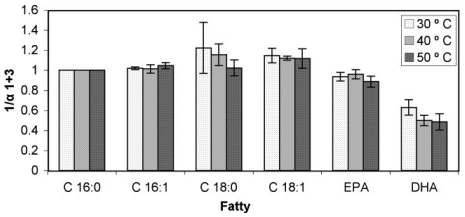

Figure 4. Specificity of *T. lanuginosus* lipase at different temperatures to fatty acids in *sn*-1 and *sn*-3 positions of oil extracted from Nile perch viscera.

seal oil but was ineffective in enriching EPA [32]. Seal oil has ω-3 PUFA located mainly in *sn*-1 and *sn*-3 positions of the TAGs with DHA mainly in *sn*-3 position and EPA random in *sn*-1 and *sn*-3 positions [32, 33]. These results indicate that positional distribution of PUFAs in TAGs influences there enrichment while using regiospecific lipases.

3.4 Effect of temperature on enrichment of EPA and DHA

Temperature can affect the reaction rate of lipases and thus enrichment levels. Figure 5a and b shows the effect of temperature on enrichment of EPA and DHA using *C. rugosa* lipase. Higher enrichment levels were associated with more EPA and DHA losses. At 30 and 40°C increment of DHA appeared similar for the first 6 h of hydrolysis. However, between 12 and 24 h of hydrolysis, a better DHA enrichment to 17 and 23 mol%, respectively, was attained at 30°C compared to 14 and 18 mol% at 40°C. After 24 h of hydrolysis at 30 and 40°C DHA recoveries were 55 and 75%, respectively. EPA levels increased to 6 mol% after 24 h hydrolysis at 30°C with a recovery of 42%. At 50°C only a slight increment of DHA to 13 mol% and EPA to 4 mol% was observed. A study by Sun *et al.* [27] showed that lipase from *C. rugosa* increased the EPA and DHA

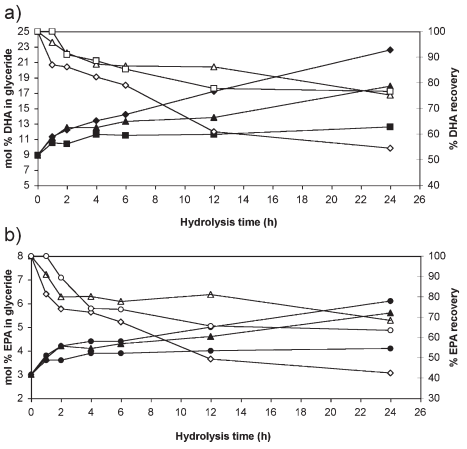

Figure 5. (a) Mol% DHA in the glyceride fraction (TAG + DAG + MAG) and recovery of DHA in this fraction as a function of time during hydrolysis of Nile perch viscera oil catalysed by lipase from C. *rugosa*. ◆ 30, ▲ 40 and ■ 50°C DHA mol%. ◇ 30, △ 40 and □ 50°C % DHA recovery. (b) Mol% EPA in the glyceride fraction (TAG + DAG + MAG) and recovery of EPA in this fraction as a function of time during hydrolysis of Nile perch Viscera oil catalysed by lipase from C. *rugosa* ◆ 30, ▲ 40 and ■ 50°C EPA mol%. ◇ 30, △ 40 and □ 50°C% EPA recovery.

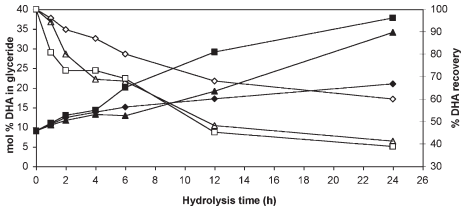

Figure 6. Mol% DHA in the glyceride fraction (TAG + DAG + MAG) and recovery of DHA in this fraction as a function of time during hydrolysis of Nile perch Viscera oil catalysed by lipase from *T. lanuginosus* ◆ 30, ▲ 40 and ■ 50°C DHA mol%. ◇ 30, △ 40 and □ 50°C % DHA recovery.

concentrations in salmon oil at 20, 35, and 50°C with 35°C being the optimum temperature. Inefficiency of *C. rugosa* lipase to enrich EPA and DHA at elevated temperatures could be due to the enzyme inactivation. *C. rugosa* lipase with an optimum activity at 40°C has been shown to maintain 20 and 10% of its original activity after 1 h incubation with 0.1 M sodium phosphate buffer, pH 7.5 at 40 and 50°C, respectively [34].

Hydrolysis with lipase from *T. lanuginosus* was effective in enriching DHA at the three temperatures (Fig. 6). However, EPA was not enriched at all temperatures (data not shown). During the first 4 h of hydrolysis, DHA increment appeared similar at the three temperatures. However after 12 and 24 h of hydrolysis, the enzyme appeared to perform better at 40 and 50°C with best DHA enrichment being achieved at 50°C. At 50°C DHA increased to 29 and 38 mol% at 12 and 24 h with recovery values of 45 and 39%, respectively, which shows that the lipase was still active at 50°C. A study by Zhu *et al.* [35] revealed that activity of wild type *T. lanuginosus* lipase and its mutant W89L increased with temperature up to 60°C then decreased upon further heating. Omar *et al.* [36] reported a similar finding with thermostable lipase from *T. lanuginosus* N0.3. The enzyme maintained 100% of its original activity for 24 h at 45°C, 20 h at 60°C and 1 h at 65°C.

Increase in temperature is associated with increased non-enzymatic acyl migration. This would not be preferred during enzymatic enrichment of fish oils with ω-3 PUFA located mainly in *sn*-2 position if a 1-, 3-specific lipase is used. However, in Nile perch oil acyl migration of palmitic acid from *sn*-2 to *sn*-1(3) position could have resulted in the rapid increase in enrichment levels from 6 to 24 h at 40 and 50°C. Palmitic acid was the predominant fatty acid in this oil with 51% located in *sn*-2 position (Tables 1 and 2). Omar *et al.* [36] detected 1, 3 DAG after prolonged incubation of triolein with *T. lanuginosus* lipase (45°C, 24 h), when in essence prolonged hydrolysis with 1-, 3-specific *T. lanuginosus* is expected to yield 2-MAG and 1(3), 2 DAG.

Change in temperature did not significantly affect substrate specificity for *C. rugosa* and *T. lanuginosus* lipases in this

study (Figs. 2–4). Lipase from *C. rugosa* showed low selectivity towards EPA and DHA as compared to other fatty acids with DHA having lowest selectivity at all temperatures. Similary, lipase from *T. lanuginosus* showed lowest selectivity towards DHA. Increase in temperature increases rate of reaction as long as the enzyme is stable. Higher temperature can also reduce the viscosity as well as improve the diffusion of substrate to the active site. Since competitive factors are a ratio of specificity constants (K_{cat}/K_m), they are often not affected by variations of individual reaction rates. A study of multi-competitive enzymatic reactions in organic media showed that changes in physiochemical conditions of the reaction, *e.g.* temperature, water content and substrate concentration did not significantly affect lipase specificity [19].

4 Conclusion

Lipase-catalysed hydrolysis of Nile perch viscera oil was demonstrated to be a feasible method for enrichment of Nile perch *n*-3 PUFA. This method offers a potential advantage for value addition of by-products from fish industries in Eastern Africa. Choice of lipase when performing hydrolysis of marine oils depends on the desired DHA and EPA enrichment and recovery levels. The ratio of DHA to EPA in the final product is also important. In the current study, *T. lanuginosus* lipase gave better DHA enrichment than *C. rugosa* lipase but showed low DHA recoveries and was ineffective in EPA enrichment. Lipase from *C. rugosa* could thus be better in catalysing hydrolysis of Nile perch viscera oil for enrichment of both DHA and EPA in acylglycerols. *C. rugosa* lipase has previously been recommended to be the enzyme of choice for preparation of ω-PUFA in the acylgycerol form in marine oils. On the other hand *T. lanuginosus* lipase would be the enzyme of choice if a high ratio of DHA to EPA is desired. For purposes of industrial application, lipases may be the most expensive part of the process and thus feasibility of using immobilized lipases needs to be investigated.

This project was part of the Swedish International Development Cooperation Agency (SIDA/SAREC) sponsored BIO-EARN (East African Regional Programme and Research Network for Biotechnology, Biosafety and Biotechnology Policy development) programme.

The authors have declared no conflict of interest.

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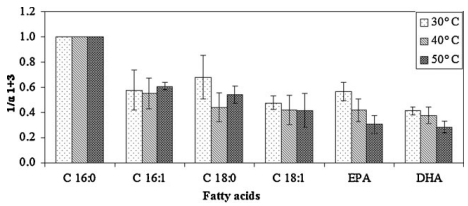
Correction

Eur. J. Lipid Sci. Technol. 2010, **112**, 977–984

Correction to: **Enzymatic enrichment of omega-3 polyunsaturated fatty acids in Nile perch (*Lates niloticus*) viscera oil**

Betty Mbatia, Patrick Adlercreutz, Francis Mulaa, Bo Mattiasson

Corrected Figure 4 provided by the Authors appears below:



Paper III

Strategies for the enzymatic enrichment of polyunsaturated fatty acids from fish oil

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Abstract.

Polyunsaturated fatty acids (PUFA) from oil extracted from Nile perch viscera were enriched by selective enzymatic esterification of the free fatty acids (FFA) or by hydrolysis of ethyl esters of the fatty acids from the oil (FA-EE). Quantitative analysis was performed using reverse phase high performance liquid chromatography coupled to an evaporative light scattering detector (RP-HPLC-ELSD). The lipase from *Thermomyces lanuginosus* discriminated DHA most, resulting in best DHA/DHA-EE enrichment while lipase from *Pseudomonas cepacia* discriminated EPA most, resulting in best EPA/EPA-EE enrichment. The lipases discriminated docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) more when present as ethyl esters (EE) than when in FFA form. Thus when DHA/EPA had been enriched to the same level during esterification and hydrolysis reactions, the DHA-EE/EPA-EE recoveries were better than those of DHA/EPA-FFA. In reactions catalysed by lipase from *T. lanuginosus*, at 26 mol% DHA/DHA-EE, DHA recovery was 76% while that of DHA-EE was 84%. In reactions catalysed by lipase from *P. cepacia*, at 11 mol% EPA/EPA-EE, EPA recovery was 79% while that of EPA-EE was 92%. Both esterification of FFA and hydrolysis of FA-EE were more effective at enriching PUFA than hydrolysis of the natural oil and are thus attractive process alternatives for the production of products highly enriched in DHA and/or EPA. When there is only one fatty acid residue in each substrate molecule, the full fatty acid selectivity of the lipase can be expressed, which is not the case with triglycerides as substrates.

Key words: EPA/DHA/lipase specificity/esterification/hydrolysis.

Introduction.

Marine oils are the major commercial source of the essential long chain polyunsaturated omega-3 fatty acids (n-3 PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [1, 2]. However, they also contain substantial amounts

of saturated and monounsaturated fatty acids [3, 4] which in some applications need to be removed in order to increase the overall content of DHA and EPA. Lipases have found applicability in such enrichment due to their fatty acid selectivity, which can be used in concentrating EPA and/or DHA by kinetic resolution [5]. There are numerous reports involving enrichment of EPA and/or DHA in the glycerol backbone through hydrolysis or transesterification of fish oil triglycerides with lipases [6-10]. The selectivity of lipases is however influenced by the distribution of fatty acids in the three positions of triglycerides and sometimes the glyceride structure [11, 12]. Therefore one lipase may concentrate EPA and DHA in one kind of fish oil but not be effective in another depending on the distribution in the triglyceride molecule. For example, lipase from *Aspergillus niger* increased DHA concentration in cod liver oil [13], but did not increase it in menhaden oil [14] or tuna oil [15]. Lipase from *Thermomyces lanuginosus* increased DHA and EPA contents in squid and menhaden oil [7, 9], but was ineffective in enriching EPA in Nile perch oil [12].

Eicosapentaenoic acid and DHA can also be enriched as free fatty acids by selective esterification of fish oil free fatty acids with an alcohol or glycerol [16-18] or by hydrolysis of fish oil fatty acid ethyl esters [19]. In this case, the fatty acid structure affects the lipase specificity in addition to the type of alcohol used during esterification reactions [18, 20]. Hydrolysis of sardine oil ethyl esters with several commercial enzymes revealed that all the lipases discriminated against n-3 PUFA and preferred the more saturated fatty acids as substrates [21]. The lipases also discriminated between EPA and DHA with most lipases such as lipases from *Geotrichum candidum*, *Candida rugosa* and *Thermomyces lanuginosus* showing preference for EPA over DHA while lipases from *Pseudomonas* species showed DHA preference over EPA.

The specificity of lipases (*Thermomyces lanuginosus* and *Pseudomonas cepacia*) towards PUFA in free fatty acids (FFA) and fatty acid ethyl esters (FA-EE) obtained from Nile perch oil was investigated in the current study and compared with previously obtained results concerning PUFA enrichment in the glyceride fraction by direct hydrolysis of the natural oil. The reaction mixtures were analyzed in a single run using RP-HPLC-ELSD.

2. Materials and methods

2.1 Materials

Protex 30L (≥2,750 GSU/g) was a gift from Genencor, a division of Danisco A/S Denmark. Lipases from *Thermomyces lanuginosus* (≥100,000U/g), *Candida rugosa* (≥700 000 U/g) and *Pseudomonas cepacia* (Amano lipase PS, ≥ 30 000U/g) were from Sigma (St Louis, MO, USA). Free fatty acids and fatty acid ethyl esters were purchased from Larodan Fine Chemicals AB (Malmö, Sweden) and Nu-check prep. Inc, USA, respectively. All solvents and chemicals were of analytical grade.

2.2 Sampling and sample preparation

Nile perch viscera was kindly donated by W. E Tilly LTD, Nile perch processing factory in Kenya and preserved at -20 °C until use. Oil extraction was performed as described by Mbatia et al [22] and the fatty acid composition determined. The DHA and EPA contents in the crude oil were 9 and 3 mol%, respectively.

2.3 Preparation of Nile perch viscera oil free fatty acids and fatty acid ethyl esters

Nile perch free fatty acids were prepared by saponifying 100 ml of Nile perch viscera oil with 250 ml of 90% ethanol containing 15 g of NaOH [23]. The reaction was carried out at 50 °C for 1 h. The saponified product was acidified to pH 2 using aqueous concentrated HCl and the free fatty acid fraction was separated from the aqueous phase using a separating funnel. The fraction was washed in excess water and analysed on silica plates using cyclohexane/diethyl ether/acetic acid (50/50/1) as eluent.

Fatty acid ethyl esters (FA-EE) were prepared as described by Ahmed et al [24]. 15 g Nile perch free fatty acids were refluxed overnight at 90 °C in 250 ml absolute ethanol containing 2% sulphuric acid. The refluxed sample was cooled to room temperature and the FA-EE was extracted in 50 ml hexane/water (1:1 v/v). The hexane fraction was washed with 2% bicarbonate solution and dried over anhydrous sodium sulphate. Esters were concentrated by vacuum distillation. Composition of generated FFA and FA-EE is shown in **Table 1.**

Table 1. Mol% fatty acids (FFA) and fatty acid ethyl esters (FFA-EE) produced after alkaline hydrolysis of Nile perch viscera oil or acid catalysed esterification of the free fatty acids.

Fatty acids	FFA- mol%	FFA-EE mol%
C 16:0	28.4 ± 1.5	33.6 ± 1.4
C 16:1	13.4 ± 0.8	18.2 ± 0.9
C 18:0	7.8 ±3.2	5.1 ± 0.1
C 18:1	21.8±0.8	20.1 ± 0.3
C 18:2	3.6 ± 0.5	1.2 ± 0.7
C 18:3	2.7 ± 0.2	1.8 ± 0.0
20:4	2.4 ± 0.1	2.2 ± 0.8
EPA	3.5 ± 0.1	3.5 ± 0.2
DPA	5.4 ± 0.1	5.1 ± 3.0
DHA	10.9 ± 0.8	9.2 ± 0.2

2.4 Immobilization of Lipases

The lipases were immobilised on Accurel MP1000 support by adsorption using a method similar to that described previously [25, 26]. Lipases from *T. lanuginosus* (2.5 mL), *C. rugosa* (1.21 g) or *P. cepacia* (2 g) were each dissolved in 20 mL of 20 mM sodium phosphate buffer (pH 7.0). The solution was centrifuged at 840 x g for 5 min and the supernatant was added to the MP1000 support, which had been pre-wetted with ethanol (3 ml/g support). The amount of MP1000 used was 1.0 g for the lipase from *T. lanuginosus*, 0.5 g for lipase from *C. rugosa* and 0.4 g for lipase from *P. cepacia*. The mixture containing enzyme and support was gently mixed overnight at room temperature. The enzyme preparation was then filtered and washed (3 x) with 20 ml sodium phosphate buffer (20 mM, pH 7.0). To the enzyme preparation, 1 ml sodium phosphate buffer (200 mM, pH 7.0)/g preparation was added and this preparation was dried under reduced pressure overnight. The protein content in the immobilized preparation was determined by the Bradford method [27] .

Activity assay was performed using a spectrophotometric assay based on hydrolysis of *p*-nitro phenyl butyrate (*p*NPB) to *p*-nitrophenol (*p*NP). 10 µl of 20 mM *p*NPB in ice cold dried methanol was added into a cuvette containing 1 ml phosphate buffer (200 mM, pH 7.0) with different amounts of enzyme samples (10, 20 and 30 µl) which were taken before immobilization and from the filtrate obtained after immobilization. The contents were mixed and absorbance recorded at 400 nm continuously for 2 min. Spontaneous hydrolysis of *p*NPB was determined by adding *p*NPB to phosphate buffer with no enzyme sample. Activities were 310, 510 and 728

U/g immobilized preparation for *T. lanuginosus*, *P. cepacia* and *C. rugosa* lipases, respectively. Activities for non-immobilizes enzymes were 980, 156 and 1340 U/g solid for *T. lanuginosus, P. cepacia* and *C. rugosa* lipases, respectively according to this assay.

2.5 Lipase catalyzed esterification of Nile perch free fatty acids

Esterification reactions were performed in 4 ml vials sealed with screw caps and Teflon lined septa. Nile perch free fatty acids (0.1 g) were esterified with ethanol, 1:3 molar ratios. The amounts of enzyme used were, 0.01 g *T. lanuginosus* lipase (3.1 U), 0.006 g *P. cepacia* lipase (3.1 U) or 0.004 g *C. rugosa* lipase (2.9 U). The reaction temperature was maintained at 30 °C in a thermoshaker (700 rpm). Vials containing 0.1 g samples were withdrawn after 0, 30 min, 1, 2, 4, 6, 9, 12 and 24 h and diluted with 3 ml acetonitrile. The lipid sample was further diluted to a final concentration of 1 mg/ml of which 20 µl was used for RP-HPLC-ELSD analysis. All experiments were performed in triplicates. The extent of esterification was determined as: $([A_i]-[A_t]/ [A_i])*100$. $[A_i]$ is initial concentration of FFA and $[A_t]$ is the concentration at time (t).

DHA/EPA recovery was determined as $([A_t]/[A_i])*100$, where $[A_t]$ is concentration of EPA or DHA in their original form (for example as free fatty acids in the case of the esterification approach) at time (t) and $[A_i]$ is the initial concentration.

2.6 Lipase catalyzed hydrolysis of Nile perch fatty acid ethyl esters

Hydrolysis of 0.1 g Nile perch fatty acid ethyl esters was performed in 0.5 ml of 0.2 M phosphate buffer containing either 0.06 or 0.28 units of *T. lanuginosus* and *P. cepacia* lipases, respectively in 10 ml glass vials. These enzyme units were based on the activity assay with *p*-nitrophenol as indicated in 2.4 above. The reaction temperature was maintained at 30 °C in a thermoshaker (700 rpm). Vials containing 0.1 g samples were withdrawn after 0, 30 min, 1, 2, 4, 6, 9, 12 and 24 h. Formation of a white precipitate was observed over hydrolysis time. To stop the reaction, samples were diluted with 8 ml acetonitrile. A further dilution to 1 mg/ml in acetonitrile was performed prior to RP-HPLC-ELSD analysis of which 20 µl samples were injected. All experiments were performed in triplicates. The extent of hydrolysis was determined as: $([A_i]-[A_t]/ [A_i])*100$. $[A_i]$ is initial concentration of FA-EE and $[A_t]$ is the concentration at time (t).

2.7 Calibration of evaporative light scattering detector (ELSD)

Different concentrations of free fatty acid standards were used. For DHA, EPA, C16:1, C18:0, C18:1, C18:2, C18:3, arachidonic acid (AA), docosapentaenoic acid (DPA) a concentration of 0.025-0.5 mM was used while for C16:0, a concentration of 0.05-0.8 mM was used. A plot of log peak areas versus log concentration was used to obtain the calibration curves of each fatty acid. For FA-EE, a concentration of 0.05-0.8 mM for C18:1, EPA, DPA was used while for C16:0 and C16:1 a concentration range of 0.02-1.4 mM was used. The standard curve for EPA-EE was used to estimate AA-EE concentrations while that of C18:1-EE was used to estimate the concentrations of C18:0, C18:2 and C18:3 ethyl esters.

2.8 Apparatus and RP-HPLC ELSD conditions

The HPLC system was Dionex ultimate 3000 connected with a Varian 385- LC evaporative light scattering detector (ELSD). A RP-C18 (3 x 250 mm, 5 µm) column was used for separations. The ELSD drift tube temperature was 25 °C, nebulisation temperature was 25 °C and gas flow 1.7 standard litres per minute (SLPM). The eluent programme used for free fatty acids and ethyl esters is shown in **Table 2**. For samples containing only free fatty acids, the programme shown in Table 2 was used except that the isocratic elution with acetonitrile was shortened to 5 min.

Table 2: Gradient elution programme for fatty acids and fatty acid ethyl esters in a RP-C_{18} column using a mobile phase of acetonitrile and aqueous acetic acid.

Time (min)	Acetonitrile (%)	0.05% aqueous acetic acid (%)	Flow rate (ml/min)
0	78	22	0.43
50	100	0	0.43
65	100	0	0.43
66	78	22	0.43
75	78	22	0.43

2.9 Determination of lipase specificity

The specificity of lipases from *T. lanuginosus* and *P. cepacia* towards fatty acids or fatty acid ethyl esters was determined by calculating the competitive factor (α). A log-log plot of equation (1) yields a linear curve with the slope equal to α value of

the substrate (B). From the α value, the specificity constant was calculated as 1/ α with palmitic acid as reference substrate (A); its specificity constant taken as 1.00.

$$\alpha = \frac{\log(A_0 / A)}{\log(B_0 / B)} \quad (1)$$

A_0 is the initial palmitic acid or palmitic acid ethyl ester concentration while A is the palmitic acid or palmitic acid ethyl ester concentration at time t. B_0 is the initial C16:1, C18:1, EPA and DHA fatty acid or the ethyl esters concentration while B is their concentration at time t.

Results and discussions

Conversion of FFA to FA-EE increased their retention times in the reversed phase HPLC method by approximately 15 min which made it possible to separate a mixture of FFA and FA-EE in a single run (**Figure 1**). Evaporative light scattering detector (ELSD) is a mass detector for non-volatile compounds that remain after solvent evaporation; therefore the samples were not derivatized. Gas chromatography (GC) is the most frequently used approach for fatty acid analysis due to its great efficiency. However, the fatty acids are derivatised to fatty acid methyl esters to increase their volatility and reduce polarity [28]. In addition, for analysis of different lipid classes for example FFA and FA-EE, a prior separation using thin layer chromatography is normally used before GC analysis [29] .

HPLC-ELSD was used for qualitative and quantitative analysis of fatty acids. The critical parameters of ELSD are the temperature of the drift tube, and the gas flow rate, which play a prominent role for the analyte's response [30]. The detector conditions in the current study were optimised using the ethyl esters since they were more volatile than the FFA. The use of a slow gradient assisted in avoiding co-elution of closely eluting pairs such as oleic/palmitic acids and arachidonic/palmitoleic acids (**Figure 1**). Unlike in GC where elution of fatty acids is influenced by chain length, in reverse-phase HPLC, unsaturated fatty acids are eluted substantially ahead of the saturated fatty acids of the same chain length [28]. In addition, the more the number of double bonds in unsaturated fatty acids of the same chain length, the shorter the elution time, therefore linolenic acid (C18:3) elutes ahead of linoleic acid (C18:2) and oleic acid (C18:1) [31]. The order of elution of some fatty acids also change with relative proportion of organic solvent in

the mobile phase with the retention time of the shorter fatty acids decreasing faster than that of the longer components with increasing solvent strength [28].

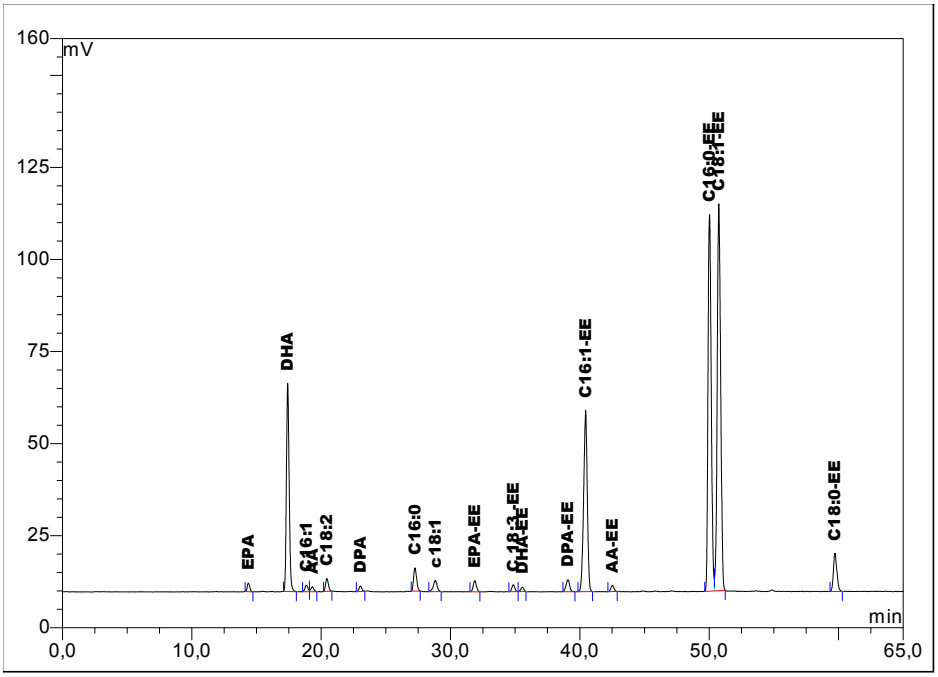

Figure 1. A RP- HPLC-ELSD chromatogram showing fatty acids and fatty acid ethyl esters after 6 h esterification of Nile perch free fatty acids with ethanol using immobilised lipase from *T. lanuginosus*.

Lipase specificity

The lipases were investigated in their ability to enrich EPA and DHA in either the acid (FFA) or ester (FA-EE) fraction. During esterification reaction, the lipases were expected to convert the bulk of saturated and monounsaturated fatty acids into ethyl esters leaving behind the more resistant DHA and EPA in the residual FFA. Similarly during the hydrolysis reaction, the lipases were expected to hydrolyse the bulk of saturated and monounsaturated fatty acids ethyl esters leaving behind the more resistant DHA-EE and EPA-EE in the residual FA-EE. The lipase from *C. rugosa* did not show any reaction at all for 24 h during the esterifiction reactions and thus was not used for further analysis. This observation agrees with previous reports that this lipase does not tolerate ethanolic conditions [9, 23]. The lipases from *T. lanuginosus* and *P. cepacia* were compared in their ability to discriminate between DHA and EPA in the form of free fatty acids or ethyl esters during the initial phase of

the reactions (**Figure 2**). The lipase from *T. lanuginosus* discriminated both forms of DHA more than the *P. cepacia* lipase while the latter was more effective in the discrimination of EPA and EPA-EE. Similar kind of discrimination for lipases from *T. lanuginosus* and *P. cepacia* is reported previously [7, 21]. The lipase from *Pseudomonas fluorescens* is another example of a lipase that has been reported to be more effective than other lipases in the enrichment of EPA in commercial fish oil (43.1% EPA) and from *Phaeodactylum tricornutum* oil (23% EPA), a single-cell oil, by selective enzymatic esterification [32]. The selectivity of the lipase for EPA depended on the content of EPA, with higher contents of EPA in the initial FFA mixture reducing the selectivity for EPA.

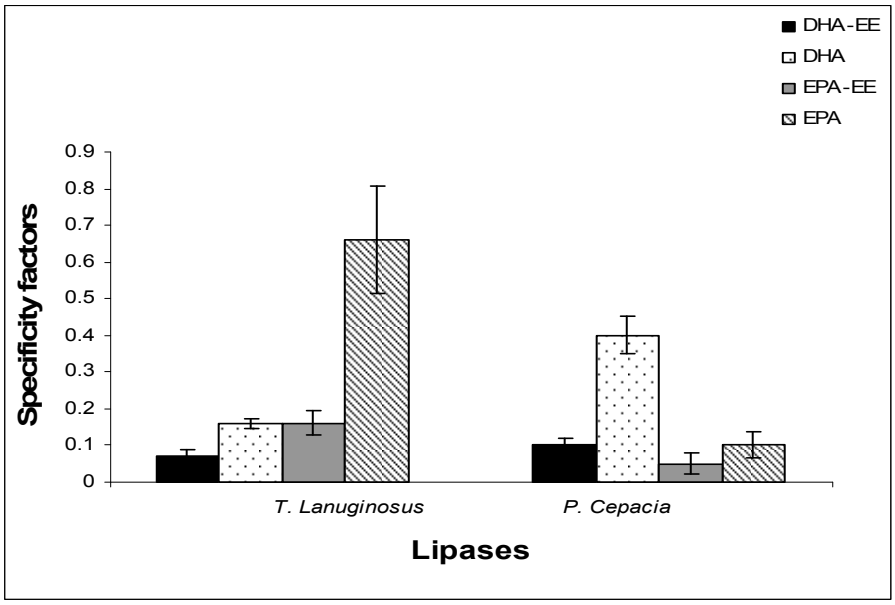

Figure 2. Specificity factors for lipases from *T. lanuginosus* and *P. cepacia* towards DHA, DHA-ethyl esters, EPA and EPA-ethyl esters. Palmitic acid/Palmitic acid ethyl ester was the reference substrate with a specificity factor of 1.00.

Both the *T. lanuginosus* and *P. cepacia* lipases showed a higher discrimination of DHA and EPA during the hydrolysis of FA-EE compared to the esterification reactions. The specificity factors obtained during esterification reaction were 0.16 and 0.66 for DHA and EPA using *T. lanuginosus* lipases and during hydrolysis of FA-EE they were 0.07 and 0.16 for DHA and EPA, respectively. When *P. cepacia* was used to catalyse esterification reaction, the specificity factors for DHA and EPA were

0.40 and 0.10, respectively while they were 0.10 and 0.05 during hydrolysis of FA-EE. The specificity factors obtained during hydrolysis of Nile perch oil triglycerides with *T. lanuginosus* lipase were 0.49 and 0.89 for DHA and EPA, respectively [12]. These were much higher than the specificity factors obtained in the current study. The lipase thus has a lower discrimination of DHA/EPA attached to glyceride structure than EPA/DHA in the form of ethyl esters or as free fatty acids. A similar observation was made when hydrolysing squid oil, fish oil and methyl esters of palmitic acid, EPA and DHA [7]. The specificity of *T. lanuginosus* lipase towards DHA/ EPA in the different reactions can thus be described in the order of TAG hydrolysis > FFA esterification > FA-EE hydrolysis. The lipase from *Rhizopus delemar* has also been reported to show similar specificity towards different forms of DHA obtained from tuna oil [33]. This can be explained by the fact that, when present in TAG, the regio, stereo triglyceride specificity of lipases affect lipase selectivity, whereas when present in free form (FFA or FA-EE), the fatty acid structure influence the selectivity.

When optimizing enzymatic enrichment reactions it is of crucial importance to monitor the enrichment of the fatty acids of interest as well as the recovery of those fatty acids in the desired fraction. When lipase from *T. lanuginosus* was used to catalyse esterification, the DHA content in the FFA fraction increased rapidly with increased degree of esterification up to 39 mol% beyond which it levelled off. DHA recovery however decreased with increased degree of esterification and at 39 mol% (82% esterification), only 68% of DHA was present in the FFA fraction (**Figure 3a**). At 80% of esterification, EPA content increased to 7 mol% with a recovery of 37% beyond which it declined (**Figure 3a**). When the same lipase was used to catalyze the hydrolysis of FA-EE, DHA–EE was enriched to 27 mol% with a recovery of 84% when a hydrolysis of 72% was achieved (**Figure 3b**) while EPA-EE increased to 9 mol% with a recovery of 71%.

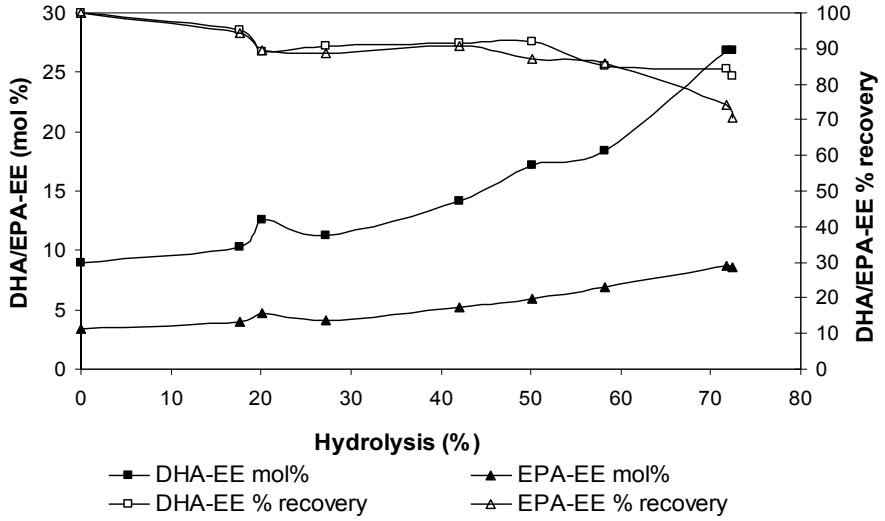

Figure 3a. Mol% DHA or EPA in the free fatty acid fraction and their recovery in this fraction against degree of esterification of Nile perch fatty acids with ethanol using immobilised *T. lanuginosus* lipase

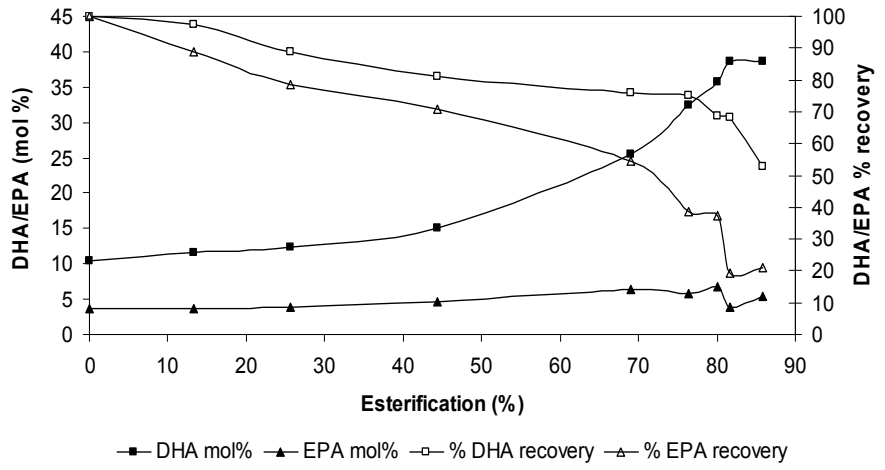

Figure 3b. Mol% DHA-EE or EPA-EE in the fatty acid ethyl ester fraction and their recovery in this fraction against degree of hydrolysis of Nile perch fatty acid ethyl esters catalysed by *T. lanuginosus* lipase.

When lipase from *P. cepacia* was used to catalyse the esterification reaction, EPA increased to 16 mol% with 70% recovery after 85% of esterification after which it started to decline (**Figure 4a**). The DHA content increased to 20 mol% with 40% recovery after 78% esterification beyond which it gradually declined. The marked decrease in EPA and DHA contents during the later part of the conversions shows that the *P. cepacia* lipase has an intermediate specificity for these fatty acids. In the beginning of the reaction mainly those fatty acids for which the lipase has high specificity are esterified, while EPA and/DHA are enriched in the FFA fraction. Later on EPA/DHA are esterified to a larger extent while fatty acids for which the lipase has especially low specificity are enriched in the FFA fraction. If the *P. cepacia* lipase catalyzed esterification reaction is to be used for enrichment of EPA/DHA it is thus of vital importance to stop the reaction at a suitable time. The maximum for DHA comes before that for EPA which is in agreement with the specificity results (**Figure 2**) where *P. cepacia* lipase showed a higher preference for DHA than EPA. In the hydrolysis reaction, the lipase increased EPA-EE to 11 mol% with a recovery of 92% while DHA-EE was increased to 21 mol% with 73% recovery when a hydrolysis of 68% was achieved (**Figure 4b**).

A comparison of the esterification and hydrolysis reactions at the same level of enrichment showed better recoveries in the hydrolysis reaction. For example when the lipase from *T. lanuginosus* was used, at 26 mol% DHA, the recovery was 84% in the hydrolysis reaction, while it was 76% in the esterification (**Figures 3a** and **b**). This was the same when both reactions catalyzed by *P. cepacia* lipase were compared. At 11 mol% EPA, the recoveries were 92 and 79% during hydrolysis and esterification reactions, respectively (**Figures 4a** and **b**). This result was in agreement with the specificity results since the lipases showed a lower specificity for DHA and EPA when they were present as ethyl esters (**Figure 2**).

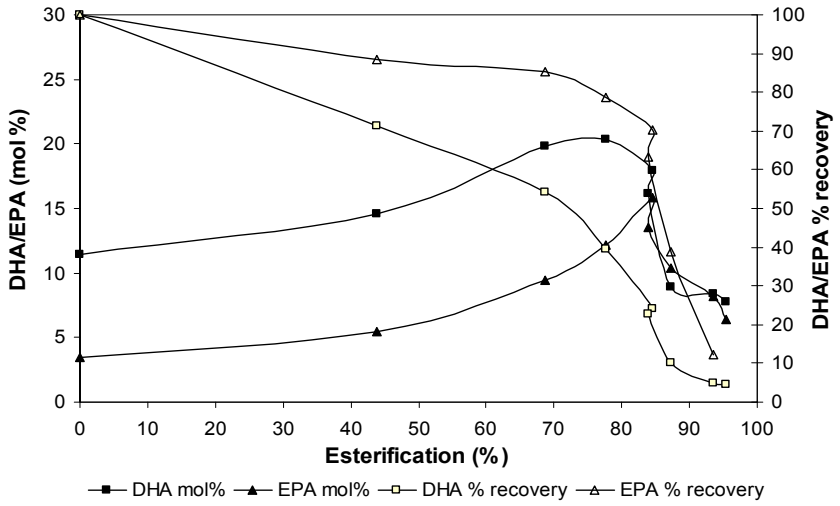

Figure 4a. Mol% DHA or EPA in the free fatty acid fraction and their recovery in this fraction against degree of esterification of Nile perch fatty acids with ethanol using immobilised *P. cepacia* lipase.

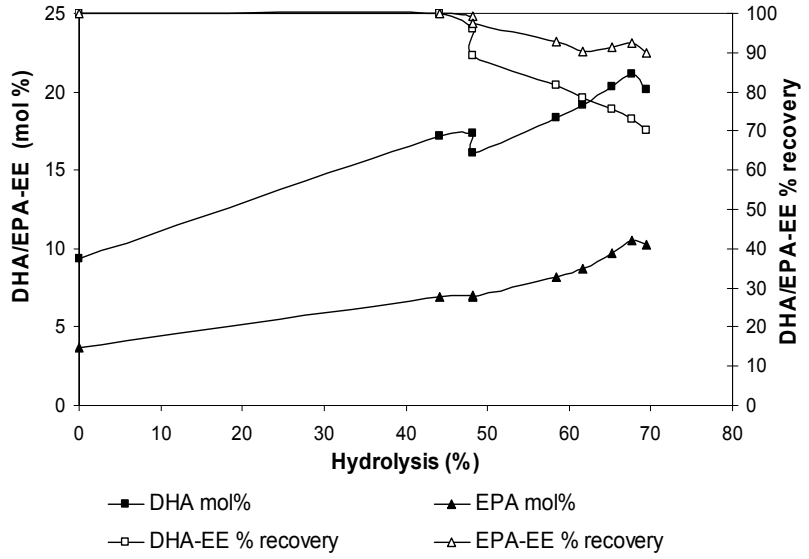

Figure 4b. Mol% DHA-EE or EPA-EE in the fatty acid ethyl ester fraction and their recovery in this fraction against degree of hydrolysis of Nile perch fatty acid ethyl esters catalysed by *P. cepacia* lipase.

Thermomyces lanuginosus lipase was able to enrich both EPA and DHA in the current study, although in a previous study, we observed that the same lipase was ineffective in enriching EPA but enriched DHA to 38 mol% during hydrolysis of triglycerides present in Nile perch oil [12]. This was attributed to the regioselectivity of the lipase and the fatty acid distribution in the triglyceride molecules [34, 35]. The content of EPA was high in the *sn* 1,3 positions of the triglyceride while DHA was equally distributed in the three positions [12]. Better yields (recoveries) of DHA and EPA obtained in the current study compared with those attained during enrichment of n-3 PUFA as acylglycerols [12] indicated that fatty acid selectivity was more pronounced when fatty acids were present as ethyl esters or in free form than when attached to glycerol. It should be pointed out that when the n-3 PUFAs to be enriched are mainly present in the *sn*-2 position, the 1,3-specificity of a lipase can help in the enrichment process. However, even in such cases enrichment by hydrolysis of esters of isolated fatty acids is often more efficient [7].

In conclusion, hydrolysis of simple esters and esterification of free fatty acids are more efficient process alternatives for the production of products highly enriched in DHA and/or EPA. When there is only one fatty acid residue in each substrate molecule, the full fatty acid selectivity of the lipase can be expressed, while the influence of combinations of different fatty acids in each substrate molecule reduces the observed selectivity in conversions of triglycerides. Nile perch oil is relatively inexpensive fish oil that is normally sold off as a by-product. The EPA and DHA content can be increased to favourable levels with excellent recoveries through esterification of FFA or hydrolysis of FA-EE, and thus these lipase-catalyzed conversions provide attractive routes to high value products. The previously studied direct lipase-catalysed enrichment in the glyceride fraction is another interesting option, mainly because it can be carried out directly on the natural oil. It should be pointed out that DHA and EPA lost during the enzymatic processes, could also be recovered from the side streams and re-cycled, thus ensuring maximum recovery of these essential fatty acids.

Acknowledgement

We thank Dr. Carl Grey for guidance in development of the HPLC-ELSD method. The project is part of the Swedish International Development Cooperation Agency (SIDA/SAREC) sponsored BIO-EARN (East African Regional Programme and Research Network for Biotechnology, Biosafety and Biotechnology Policy development) program.

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Paper IV

Enzymatic synthesis of lipophilic rutin and vanillyl esters from fish byproducts

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Abstract

Lipase-catalyzed synthesis of lipophilic phenolic antioxidants was carried out with a concentrate of n-3 polyunsaturated fatty acids (PUFAs), recovered from oil extracted from Salmon (*Salmon salar*) filleting by-products. Vanillyl alcohol and rutin were selected for the esterification reaction and obtained esters yields were 60 and 30%, respectively. The antioxidant activities of the esters were compared with those of commercial butylated hydroxytoluene (BHT) and α-tocopherol using DPPH radical scavenging assay and thiobarbituric acid assay. In the DPPH radical assay, rutin esters showed better activity than the vanillyl esters and on the contrary in lipophilic medium, the vanillyl esters were found to be superior to the rutin esters in the tested concentrations. In bulk oil system, the antioxidant activities of rutin and vanillyl derivatives were lower than that of BHT and α-tocopherol but in emulsion, they showed better activity than α-tocopherol. By attaching PUFAs to natural phenolics, the PUFAs are protected against oxidation while PUFA makes the phenolic antioxidant more hydrophobic which could enhance its function in lipid systems.

Key words: lipophilization, PUFA, phenolics, antioxidant

1. Introduction

The use of phenolic antioxidants to protect food based products from oxidation and also to improve the shelf life of lipid containing products has nutritional and pharmaceutical relevance (Figueroa-Espinoza et al, 2005). The use of natural phenolics as antioxidants has been increasing because the most widely used and commercially available antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) are not considered safe due to their suspected role as promoters of carcinogenesis (Iverson, 1995). The other alternative is the use of natural phenolics which is however limited due to their poor solubility in hydrophobic media. To address this, several reports on

the lipophilization of natural phenolics to prepare lipophilic antioxidants have been published (Guyot et al, 2000, Sabbally et al, 2005, & 2006, Kanjilal et al, 2008). Natural phenolics which are abundant in plant kingdom are of particular interest because of their potential biological properties, such as antioxidant, chelating, free radical scavenging, anti-inflammatory, anti-allergic, antimicrobial, antiviral and anticarcinogenic (Figueroa-Espinoza et al, 2005).

Fish oils are receiving increased interest due to their high content of omega-3 polyunsaturated fatty acids (PUFAs), which are reported to exert positive effects on human health (Uauy, 2000 & Calder, 2004). In addition, PUFAs serve a role as precursors of a wide variety of metabolites (prostagladins, leukotrienes and hydroxyl fatty acids) regulating critical biological functions. Since mammals have limited ability to synthesize these unsaturated fatty acids, they must be supplied in the diet. The practical use of omega-3 PUFAs for a preventive purpose is limited because of their high susceptibility to autoxidation, which is responsible for the unfavourable off-flavour in rancid oils. A solution would be to combine PUFA with natural antioxidants such as flavonoids, by forming an ester bond. Viskupicova et al, (2010) observed that long chain fatty acids conjugated to rutin provided improved stabilisation of sunflower oil against oxidation compared to conjugates with short chain fatty acids. Based on this observation, and the increasing demand of omega-3 PUFA, it was assumed that esterification of PUFAs concentrate with natural phenolic compounds can be helpful in protecting both the PUFAs and the product in which the PUFA-phenolic derivatives are present against oxidation. Also the products prepared would confer the beneficial effects of both the PUFA and the phenolic compounds. In addition to antioxidant activity, products of natural phenolics as esters of PUFA have been reported to show significantly improved anti-inflamatory activities as well antiviral and anticancer activities that were not present in the original phenolic molecule suggesting that PUFA moieties contribute to the bioactivities of the ester derivatives (Shahidi and Zhong, 2010).

Recently, we reported the utilization of by-products from fish industries as a source of omega-3 PUFAs (Mbatia et al, 2010 a & b). To the best of our knowledge, there are no reports on the utilization of PUFAs concentrate from fish oil to prepare lipophilic antioxidants and test them on stabilisation of fish oils. Two natural phenolics, rutin and vanillyl alcohol, were selected for this study. Enzymatic lipophilization of rutin with individual fatty acids (C4–C18) has been reported (Lue et al, 2010a, Viskupicova et al, 2010). Similarly, enzymatic esterification of vanillyl alcohol with individual fatty acids to prepare capsinoid derivatives have been

reported (Kobata et al, 2002, Kunduru et al, 2011). The objective of the present study was to prepare PUFA-phenolic esters exemplified by rutin and vanillyl alcohol through enzymatic esterification of PUFAs concentrate obtained from salmon by-products and to employ the products as lipophilic antioxidants in PUFA enriched oil and aqueous emulsion systems.

2. Materials and methods

2.1 Materials

Immobilized lipase B from *Candida antarctica* (Novozyme 435) was a kind donation from Novozymes A/S, Denmark. Protex 30L (≥2750 GSU/g) was a gift from Genencor, a division of Danisco A/S, Denmark. Rutin, vanillyl alcohol (4-hydroxy-3-methoxy benzyl alcohol), 2, 2- diphenyl-1-picrylhydrazyl radical (DPPH), butylated hydroxyl toluene (BHT), thiobarbuturic acid, α-tocopherol, molecular sieves 4Å were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). Pre-coated silica gel 60 F254 TLC plates and silica gel 60 for column chromatography were purchased from Merck. Salmon heads (*Salmo salar*) were a kind donation from Kalles Fisk, Göterborg, Sweden. All solvents and other reagents were of analytical grade or HPLC grade purchased from Merck (Darmstadt, Germany).

2.2 Preparation of n-3 PUFAs concentrate

The preparation of n-3 PUFA concentrate was achieved in 3 steps (a) Enzymatic oil extraction, (b) hydrolysis of the obtained oil and (c) urea complexation for PUFA enrichment. Oil was extracted from salmon heads (Salmon salar) using 0.15% v/w Protex 30L (Mbatia et al, 2010a). The recovered oil was hydrolysed to free fatty acids (FFA) using a method described by Haraldsson, (1998) with slight modifications. Crude oil (100 ml) was mixed with 250 ml of 90% ethanol containing 15 g of NaOH. The contents were refluxed for 1 h with stirring. The hydrolysis reaction was monitored by TLC and the reaction was complete within 1 h. The free fatty acids were recovered by lowering the pH to 2 using 12 N HCl. Recovered FFAs were washed with water to neutralise the acid and then dried over anhydrous sodium sulphate.

The obtained FFA mixture was enriched in PUFA content by the urea inclusion method as described by Hayes et al, (2000). Briefly, 50 g of FFA, 150 g urea and 550 ml 96% ethanol were heated at 65 °C, until a homogenous solution was obtained. The contents were rapidly cooled under running tap water for 10 minutes

to allow crystallisation. The crystallised and non-crystallised fractions were separated by filtration. The method for preparation of fatty acid methyl esters (FAME) and program for FAME analysis by GC was as described by Mbatia et al, (2010a).

2.3 Lipase catalyzed synthesis of lipophilic esters

2.3.1 Enzymatic synthesis of rutin fatty acid esters

This was performed following a method reported by Lue et al (2010a). Briefly, rutin (1 g) and PUFA concentrate (1.9 g) 1:4 rutin/PUFA molar ratio, were solubilised in dried acetone (300 mL). Immobilized lipase (6 g) and molecular sieves (15 g) were added and the reaction was agitated at 200 rpm, 50 $^{\circ}$C for 96 h. To terminate the reaction, enzyme and molecular sieves were filtered off and acetone was evaporated. The residue was transferred into 4 centrifuge tubes. Heptane/water 30 ml (4:1 v/v) was used to extract unreacted PUFA. The heptane phase was discarded. Rutin and rutin-PUFA esters were separated using 35 ml ethyl acetate/ hot water (60 $^{\circ}$C, 1: 6 v/v). Esters were extracted into the ethyl acetate phase. The ethyl acetate phases were pooled, dried over anhydrous sodium sulphate and evaporated using rota-vapour to recover the rutin esters of PUFA as a solid dark-yellow product (0.43 g, 30%).

2.3.2 Enzymatic synthesis of vanillyl fatty acid esters

This was performed as described by Kobata et al, (2002) with slight modifications. To a mixture of fish oil PUFA (6 g,) and vanillyl alcohol (4.5 g), (1:1.5 PUFA/vanillin molar ratio) in acetone (25 mL), lipase (2 g) and 2.5 g of molecular sieves were added and the mixture was agitated at 200 rpm for 48 h at 50 $^{\circ}$C. To stop the reaction, enzyme and molecular sieves were separated by filtration. The filtrate was washed with sodium bicarbonate in water. The organic phase was dried over anhydrous sodium sulphate and concentrated in vacuo. The crude product was purified by silica gel column chromatography using hexane/ ethyl acetate (96:4 v/v), to obtain the vanillyl esters of PUFA as oily colourless liquid (5.16 g, 60%).

2.4 Analytical methods

The reaction products of both rutin and vanillyl esters were monitored by TLC and HPLC and confirmed by LC-MS of purified samples. For rutin esters, mixtures of chloroform/methanol (80:20 v/v) and for vanillyl esters pure chloroform were used

as mobile phase for TLC analysis. TLC plates were visualised under UV light (254 nm).

HPLC analysis was carried out using DIONEX Ultimate HPLC system equipped with a Varian 385-LC evaporative light scattering detector (ELSD) and a Luna RP-C18 column (250 X 3.0 mm, 5 µm particle size). This system was also equipped with an autosampler, on-line degasser and a column heater. Injection volume was 20 µl. Acetonitrile and water containing 0.05% acetic acid were used as mobile phases A and B, respectively, in the following gradient elution: 78 to 100% A over 50 min, 100% A for 5 min, followed by 10 min re-equilibration time between samples. Column temperature was 25 °C, flow rate was 0.43 mL/min and detection was carried out in ELSD using evaporator and nebulizer temperatures of 25 and 40 °C, respectively, and a gas flow of 1.7 standard liters per minute (SLPM).

LC-MS analysis was carried out on a hybrid QSTAR Pulsar quadrupole time-of-flight mass spectrometer (PE Sciex Instruments, Toronto, Canada), equipped with electrospray ionization (ESI) source. The software used was Analyst QS 1.1, also from PE Sciex. LC-MS was used to characterise the purified vanillyl and rutin PUFA esters. The elution program was similar to that used during RP-HPLC analysis. The scan range was 200-1500 m/z. Negative ESI mode was used for PUFA and rutin-PUFA esters while positive ionisation mode was used for vanillyl-PUFA esters.

2.5 Determination of antioxidant activity

2.5.1. DPPH radical scavenging activity

The antioxidant activity was determined by the radical scavenging ability using the stable DPPH˙ radical as described by Akowuah et al, (2006). Briefly, 200 µL of methanolic solution of the synthesized phenolic lipids (1 mM or 2 mM) was added to 2 mL of methanolic solution of DPPH˙ radical (0.1 mM) and total volume was made up to 3 mL with methanol. After 60 min incubation at 30 °C in the dark, the absorbance of the mixture was measured at 517 nm against methanol as blank in an Ultrospec 1000 UV spectrophotometer.

BHT and α-tocopherol were used as positive controls and their concentrations were kept equal to that of synthesized phenolic lipids. The free radical scavenging activity (FRSA in %) of the tested samples were evaluated by comparison with a control (2 mL of DPPH radical solution and 1 mL of methanol). Each sample was measured in triplicates and an average value was calculated. Antioxidant activity was expressed

as a percentage of DPPH˙ radical scavenging activity compared to control. The FRSA was calculated using the formula: FRSA = $[(A_c-A_s) /A_c] \times 100$, where '$A_c$' is the absorbance of the control and 'A_s' is the absorbance of the tested sample after 60 min.

2.5.2 Stabilisation of fish oil against oxidation employing synthesised antioxidants.

The potential of the synthesised antioxidants to protect fish oil acylglycerol concentrate prepared in our laboratory was evaluated. The acylglycerol concentrate was spiked with vanillyl-PUFA, rutin-PUFA, α-tocopherol or BHT to a final concentration of 5 or 25 mM. A control sample with no antioxidant added was included. The samples were heated for 6 h in a water bath set at 70 °C with agitation at 170 rpm. The extent of oxidation was determined by the thiobarbituric acid assay (TBARS) as described in section 2.5.3.

2.5.2.1 Stabilisation of fish oil emulsion against oxidation using synthesised antioxidants.

The potential of synthesised antioxidants to protect an emulsion against oxidation was tested. An oil emulsion was prepared from the acylglycerol concentrate as described by Huber et al, (2009). Acylglycerol concentrate (10 mg/ml) was dissolved in buffer (pH 7.0) containing 50 mM Tris-HCl, 150 mM KCl and 1% Tween 20. The contents were sonicated for 20 seconds (ultrasonic cleaner BRANSON 200). The emulsion was maintained by agitating the tubes with the emulsion at 400 rpm. The emulsion sample (2 ml) was mixed with vanillyl-PUFA, rutin-PUFA, α-tocopherol or BHT to a final concentration of 5 or 25 mM. A control without antioxidant was included. The samples were heated at 70 °C for 6 h and extent of oxidation determined using TBARS assay as described in section 2.5.3. The experiment was performed in triplicate.

2.5.3 Thiobarbituric acid assay (TBARS)

This assay was performed based on the method described by Huber et al, (2009), with slight modifications. Briefly, 100 µl of lipid sample or 400 µl for emulsion sample was mixed with 2 ml of TBARS reagent (0.375% (w/v) TBA in 250 mM HCl) by agitating the contents at 200 rpm for 5 min. The contents were centrifuged at 2150 x g for 5 min. The lower phase (approximately 1 ml) was carefully transferred into Eppendorf tubes and heated at 80 °C for 20 minutes. The tubes were then allowed to cool to room temperature and again centrifuged at 2150 x g for 5

minutes. The absorbance of the lower phase was recorded at 535 nm. Distilled water was used as a blank. The degree of oxidation inhibition was calculated using the formula: % inhibition = $(1-(A_s/A_c))*100$, where 'A_s' is the absorbance of sample and 'A_c' is the absorbance of control.

3. Results and discussion

In the present study, by-products from fish processing were used as a source of polyunsaturated fatty acids (PUFAs). Annually, an estimated amount of 63.6 million metric tones (MMT) of fish waste is generated globally from an annual total fish production of 141.4 MMT (Rai et al, 2010). The oil content of by-products from fish ranges between 1.4 and 40.1% depending on the species and tissue (Zuta et al, 2003). Such waste represents a rich source of lipids that can be used for omega-3 PUFAs recovery. Since fish oils contain a mixture of saturated, monounsaturated and PUFAs, the PUFAs content was enriched by urea crystallisation. The fatty acid composition before and after urea enrichment is shown in **Table 1**. The concentrate yield was 19% of the starting free fatty acids. The content of omega-3 fatty acids, α-linolenic acid (ALA, C18:3 n-3), eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) accounted for 67.8% of all fatty acids in the concentrate and the mean molecular weight of the PUFAs concentrate was determined to be 312.

For clinical applications, concentrated forms of n-3 PUFAs devoid of saturated and monounsaturated fatty acids are preferred (Kapoor and Patel, 2011). However, 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 to the consumer due to their cytotoxic and genotoxic effects (Esterbauer et al, 1990, Fang et al, 1996). The high rate of oxidation of PUFA can be controlled by the addition of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butyl hydroquinone (TBHQ) and synthetic or natural α-tocopherol. Furthermore, lipophilic derivatives of natural polyphenolic compounds such as lipophilic rutin esters have been reported to inhibit oxidation of lipids (Viskupicova et al, 2010).

Table 1. Fatty acid composition of salmon oil and PUFAs concentrate obtained by urea complexation. The ratio of urea to fatty acids was 3:1 (w/w).

	Before enrichment	After enrichment
Fatty acid	mol%	mol%
C 14:0	5.4 ± 0.06	0.7± 0.06
C 15:0	0.4 ± 0.00	0.2± 0.01
C 16:0	15.8± 0.06	0.1± 0.02
C 16:1	5.6± 0.01	6.2± 0.39
C 18:0	3.5± 0.01	0.4± 0.41
C 18:1 n-9	28.0± 0.11	8.5± 1.46
C 18:1 n-7	3.6± 0.04	1.6± 0.25
C 18:2	7.9± 0.01	16.9± 0.01
C 18:3 (ALA)	2.8± 0.00	6.7± 0.06
C 18:4	1.2± 0.00	3.9± 0.11
C 20:0	5.1± 0.03	0.4± 0.09
C 20:4	0.5± 0.01	1.5± 0.05
C 20:5 (EPA)	5.5± 0.01	17.8± 0.58
C 22:1	4.3± 0.03	0.2± 0.05
C 22:4	0.2± 0.01	0.5± 0.02
C 22:5 (DPA)	2.5± 0.00	7.4± 0.32
C 22:6 (DHA)	7.6± 0.02	26.9± 1.26

3.1. Enzymatic synthesis of rutin and vanillyl esters of PUFA

The synthesis of lipophilic derivatives of rutin and vanillyl alcohol was performed employing reported methods with slight modifications (Lue et al, 2010a, Kobata et al, 2002). The yields obtained for isolated rutin and vanillyl PUFA esters after purification were 30% and 60%, respectively. Previously, lipophilization of phenolic derivatives is mainly reported with pure fatty acids in the range C4-C18 (Lue et al, 2010a, Viskupicova et al, 2010, Kanjilal et al, 2008 and Ardhoui et al, 2004). In this study, a PUFA concentrate with fatty acids mainly in the range C18-C22 was used. All the fatty acids were incorporated into the phenolic derivatives despite the differences in chain length and degree of unsaturation (**Figure 1b, c**). The shift in the retention times in reversed phase HPLC of the PUFA concentrate components after reaction with either vanillyl alcohol or rutin (**Figures 1a, b, c**), was an indication of ester formation. This was further ascertained by LC-MS. The observed and expected molecular weights of selected fatty acid esters are given in **Table 2**.

Table 2. Molecular weights of vanillyl-PUFA and rutin-PUFA esters observed in the LC-MS analysis (expected masses are shown in brackets).

Fatty acid	Vanillyl- PUFAa	Rutin- PUFAb
C 18:3	415.2869 (*415.2848*)	869.5341 (*869.3596*)
C18:4	413.2730 (*413.2692*)	867.5105 (*867.3439*)
C 20:4	441.2950 (*441.3005*)	895.5675 (*895.3752*)
EPA	439.2622 (*439.2848*)	893.5427 (*893.3596*)
DPA	467.2669 (*467.3161*)	921.6045 (*921.3909*)
DHA	465.2893 (*465.3005*)	919.5728 (*919.3752*)

[a] = M+H, [b] = M-H

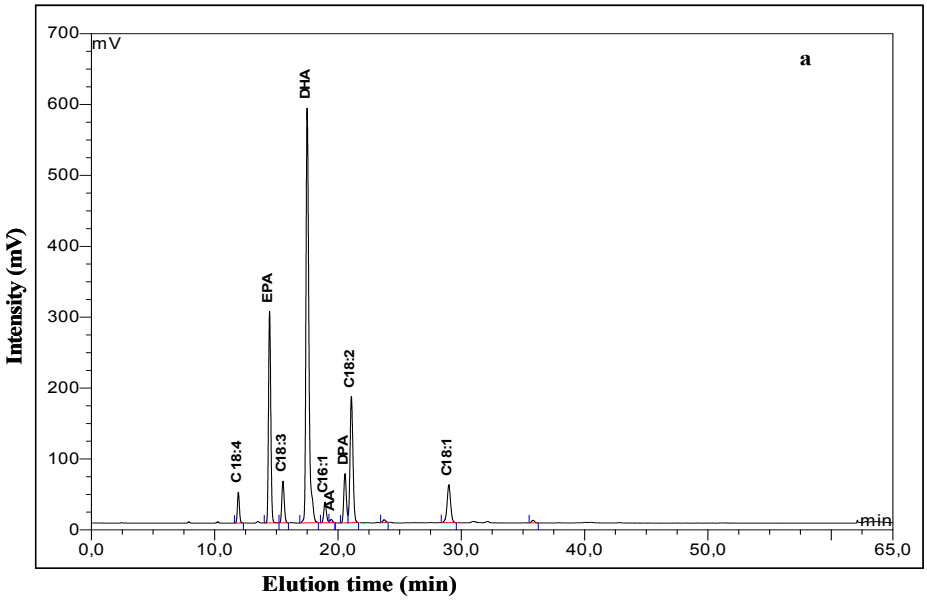

Figure 1a. HPLC–ELSD profile of salmon oil PUFA concentrate before esterifcation with vanillyl alcohol or rutin.

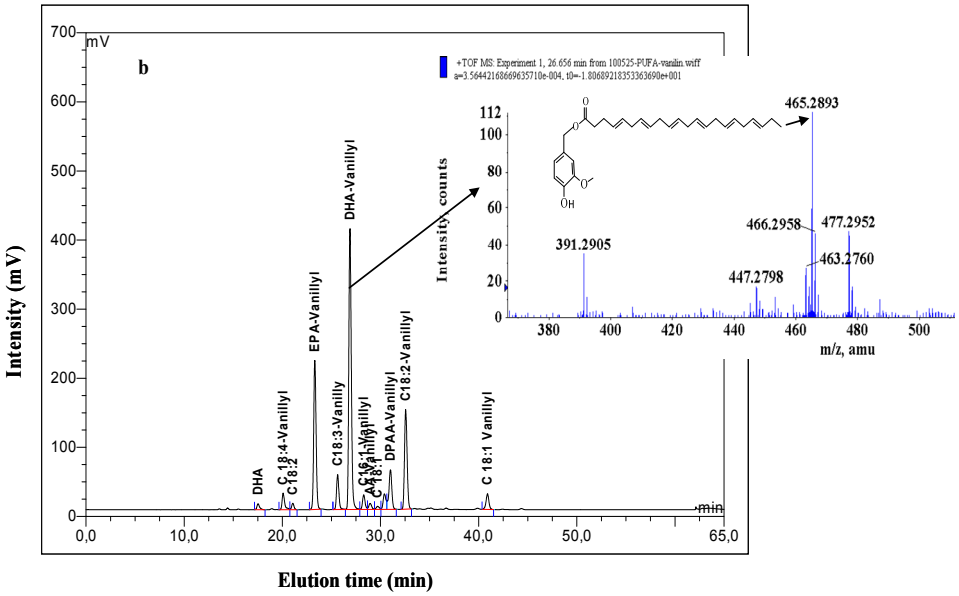

Figure 1b. HPLC–ELSD profile of products of enzymatic esterification of PUFA concentrate with vanillyl alcohol. LC-MS spectrums of DHA-vanillyl ester (one of the products) is shown along with the HPLC chromatogram.

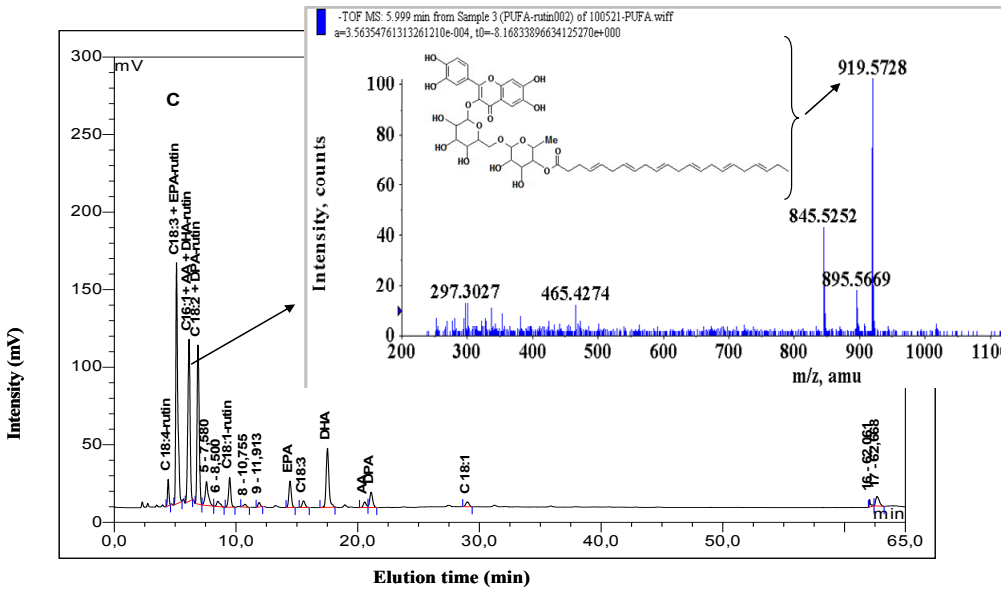

Figure 1c. HPLC–ELSD profile of products of enzymatic esterification of PUFA concentrate with rutin. LC-MS spectrums of DHA-rutin ester (one of the products) is also shown along with the HPLC chromatogram.

The better yields of vanillyl esters than those of rutin esters may be attributed to the lower reactivity of rutin with fatty acids compared to that of vanillyl alcohol. It has been reported that though Novozyme 435 is a non-specific lipase, it shows greater selectivity towards primary hydroxyl groups compared to secondary hydroxyls (Virto et al, 2000). Vanillyl alcohol has a primary hydroxyl group while incase of rutin, acylation occurs on the 4'''- hydroxyl group of the rhamnoside moiety (Viskupicova et al, 2010, Oliveira et al, 2009). Despite there being six secondary hydroxyl groups in rutin molecule, LC-MS data indicated that no rutin molecule was acylated with more than one fatty acid. A previous study by Lue et al, (2010a) in which rutin was esterified with lauric or palmitic acid reported rutin-laurate and rutin-palmitate esters yield of 82%. The lower rutin esters yields (30%) obtained in the current study could be due to presence of greater amounts of long chain PUFAs in the fatty acid mixture. It was earlier reported that as the chain length increases, the conversion yields of esters gradually decreases (Viskupicova et al, 2010, Ardhaoui et al, 2004). When using *Candida antarctica* lipase in 2-methylbutan-2-ol, esterification of rutin with fatty acids of chain lengths (C4-C12) gave a conversion yield greater than 50% while lower yields were obtained with longer fatty acids (C12-C18) (Viskupicova et al, 2010).

3.2 Antioxidant activity

Esterification of PUFA concentrate with vanillyl alcohol resulted in PUFA-vanillyl esters that were more hydrophobic than the PUFA concentrate while rutin-PUFA esters were less hydrophobic according to the retention times in reversed phase HPLC (**Figure 1b, c**). However, both products were more hydrophobic than either rutin or vanillyl alcohol with retention times of 2.26 and 2.65 min, respectively.

The well established DPPH radical scavenging activity was used to determine the antioxidant activity for the synthesized rutin and vanillyl PUFA esters in two different concentrations. The results were compared with those of the reference compounds BHT and α-tocopherol as well as the parent compounds rutin and vanillyl alcohol and are given in **Table 3**. Both rutin and vanillyl PUFA esters showed radical scavenging activity in the DPPH radical assay. The antioxidant activity of the synthesized rutin-PUFA esters was higher compared to that of vanillyl-PUFA esters in both the tested concentrations. The rutin-PUFA esters exhibited higher activity than the commercial antioxidant BHT, and an activity similar to that of α-tocopherol. The difference in antioxidant activities between the synthesised esters maybe attributed to the difference in the structure, solubility and the number of phenolic hydroxyls (Silva et

al, 2000). Unlike rutin which has four phenolic hydroxyl groups, vanillyl alcohol has only one phenolic hydroxyl group and a methoxy group. In addition, the solubility of the rutin derivatives in methanolic solution could be higher than that of vanillyl esters due to the presence of the carbohydrate moiety which may be the reason for the difference in the activity in DPPH radical assay. Lipophilization of rutin did not influence the radical scavenging activity as both rutin and rutin esters showed similar radical scavenging capacity. Similar patterns were also reported by Viskupicova et al, (2010) and Lue et al, (2010b). Lipophilization of vanillyl alcohol however lowered its $DPPH^{\cdot}$ scavenging activity. This could be due to the greatly increased hydrophobicity which may have resulted to decreased solubility in the tested medium.

Table 3. % antioxidant activity of commercial antioxidants, substrate phenolics and the synthesized rutin- and vanillyl-PUFA esters as determined by DPPH radical method.

Compound	1 mM	2 mM
α -tocopherol	92.3 ±0.69	92.6 ±0.71
BHT	65.8 ± 3.75	84.8 ± 0.21
Rutin	91.6 ± 0.57	95.6 ± 0.26
Vanillyl alcohol	87 ± 0.09	90.5 ± 1.48
Rutin-PUFA esters	91.1 ± 0.32	92.2 ± 0.31
Vanillyl-PUFA esters	52.3 ± 0.15	66.1 ±1.71

The antioxidant activity of the prepared lipophilic phenolic derivatives was also evaluated in two types of media rich in PUFA: an acylglycerol concentrate containing 53% PUFA obtained from enzymatic treatment of fish oil and an emulsion prepared using the same acylglycerol concentrate. Both the prepared lipophilic derivatives exhibited antioxidant activity as determined by the TBARS assay. BHT and α-tocopherol were used as reference antioxidants and the results are presented in **Table 4**.

Table 4. % oxidation inhibition after 6 h incubation of fish oil or emulsion at 70°C in the presence of commercial or synthesised antioxidants as determined by TBARS method.

Compound	Oil		Emulsion	
	5 mM	25mM	5mM	25 mM
BHT	43 ± 1.8	85 ± 2.7	87 ± 4.0	92 ± 2.8
α-Tocopherol	60 ± 6.1	80 ± 2.5	8 ± 1.0	38 ± 3.0
Rutin-pufa	42 ± 0.3	62 ± 4.9	22 ± 1.0	67 ± 4.7
Vanillyl-pufa	42 ± 3.7	77 ± 1.2	43 ± 5.1	63 ± 3.3

In the bulk oil system the antioxidant activities of rutin and vanillyl PUFA esters were similar at 5 mM concentration whereas at 25 mM the activity of vanillyl PUFA esters was higher probably because vanillyl-PUFA esters were more hydrophobic than the rutin esters. The activities for both the synthesized derivatives were however lower than those of reference compounds. In the emulsion system, vanillyl-PUFA esters exhibited antioxidant activity two times that of the rutin-PUFA esters, however, at 25 mM, the activities were comparable (**Table 4**). Both the products showed lower activity than one of the control, BHT but were superior than the lipophilic reference compound α-tocopherol, which exhibited the lowest antioxidant activity. BHT has previously been reported to provide a better stabilisation of PUFA in emulsion against oxidation than α-tocopherol (Rupasinghe and Yasmin, 2010).

The difference in activities in the tested methods is expected as the two media are different with respect to the solubility, distribution and location of antioxidants (Laguerre et al, 2010, Kunduru et al, 2011). Murata et al., 2004 studied the relationship between hydrophobic nature and antioxidant activity of flavonoids and found that hydrophobicity is an important determinant for antioxidant potency. Viskupicova et al, (2010) also observed that hydrophobicity may have an impact on antioxidant capacity of a compound in lipophilic food systems with long chain fatty acid rutin derivatives offering better protection against oxidation of sunflower oil and β-carotene–linoleate suspension than short chain derivatives. On the contrary, Laguerre et al, (2010) observed that an increase in hydrophobicity does not necessarily improve the antioxidant activity of phenolics. A maximum antioxidant efficiency to protect emulsions against oxidation was achieved with rosamarinic acid octyl esters with longer chain analogues showing a decreased activity. In the

present study, ranges of products with varying length of the fatty acid chain were synthesized, which can be a good strategy to assure that at least some molecules have ideal properties for use in each system.

4. Conclusion

In addition to their health beneficial effects, consumption of omega-3 PUFAs stimulate oxidation which necessitates their use in presence of an antioxidant. In this study, esterification of PUFA to natural phenolics resulted in lipophilic esters that were able to stabilise oil and emulsions against oxidation. Esterification of omega-3 PUFA to natural phenolics which have antioxidant properties thus protects the PUFAs from oxidation while the PUFA-phenolic derivative carry combined health beneficial properties of PUFA and the phenolic molecule.

Acknowledgements:

We thank Dr. Carl Grey for guidance in development of the HPLC-ELSD method and Martin Hedström for LC-MS analysis. The authors are also grateful to Kalles Fisk from Göterborg for supplying salmon heads.The project is part of the Swedish International Development Cooperation Agency (SIDA/SAREC) sponsored BIO-EARN (East African Regional Programme and Research Network for Biotechnology, Biosafety and Biotechnology Policy development) program.

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Paper V

Improved utilization of fish waste by anaerobic digestion following omega-3 fatty acids extraction

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ABSTRACT

Fish waste is a potentially valuable resource that can be used to harness high value products. Following an enzymatic pre-treatment to extract fish oil and fish protein hydrolysate, anaerobic digestion of the remaining fraction (fish sludge) and the original fish waste were evaluated for methane production. Results showed high biodegradability of both fish sludge and fish waste with specific methane yields of 742 and 828 m^3 CH_4/ton VS added respectively. However, chemical analyses showed high concentrations of light metals which together with high fat and protein content could be inhibitory to methanogenic bacteria. The feasibility of co-digesting the fish sludge with a carbohydrate-rich residue from crop production was demonstrated, and a full scale process was outlined, converting all parts of a smelly fish waste to useful products.

Keywords: Jerusalem artichoke, salmon waste, biogas

1. Introduction

The Food and Agricultural Organisation (FAO, 2005), puts the annual world fish harvest at 140 million tonnes, captured by commercial fishing in the wild fisheries and fish farms. Fish processing generates considerable quantity of waste in the form of edible and non-edible by-products. Considering 45% of the live weight to be the waste, it can be estimated that nearly 64 million tonnes of fish waste are generated annually (Rai et al., 2010). This waste is mainly composed of heads, viscera, bones and scales and is rich in lipids and proteins. Oxidation of the unsaturated fatty acids present in the lipids is the major factor responsible for the offensive odors associated with fish and fish waste (Rai et al., 2010). Fish waste is often underutilized (Berge, 2007) and mainly used in the production of low value animal feed products such as fish meal or fish silage (Crexi et al., 2009; Liaset and Espe, 2008). Arvanitoyannis and Kassaveti (2008) reported that also highly valuable compounds such as fish oils, biodiesel from fish oils, enzymes, omega-3 fatty acids and proteins can be obtained from fish waste. Much work has been done on the

extraction and purification of omega-3 polyunsaturated fatty acids (PUFA) from fish waste (Liaset and Espe, 2008; Linder et al., 2005; Mbatia et al., 2010a). PUFA are mainly present in marine oils and are associated with a several health benefits (Mozaffarian et al., 2010; Patel et al., 2010). The potential of using the soluble proteins or fish protein hydrolysate (FPH) for microbiological media has also been reported (Aspmo et al., 2005; Klompong et al., 2009). Extraction of PUFA and FPH from fish waste will however also leave a waste product which has to be properly handled.

Salmon (*Salmo salar)* heads have been used to represent oil rich fish waste (Mbatia et al., 2010a). Salmon constituted just over a million tonnes of the world fish harvest in 2006 (Gebauer and Eikebrokk, 2006). In a typical automated filleting line, the fillets count for approximately 59-63% of the total wet weight in a salmon with body weight of 5-6 kg (Liaset and Espe, 2008), hence about half a million tones of salmon waste is generated annually. Oils were extracted and PUFA enriched by enzymatic hydrolysis as described by (Mbatia et al., 2010a; Mbatia et al., 2010b). The extraction method applied also allowed straightforward removal of FPH, which was used for as an additive for *Lactobacillus sp* growth media. The residual product, called the fish sludge, was used for biogas production through anaerobic digestion (AD). As a comparison, the original fish waste was also investigated for biogas production.

AD is a technology that has been used for waste treatment and biogas recovery from many types of waste. Its numerous advantages such as the recovery of a renewable energy carrier, waste volume reduction and odour reduction are well documented (Parawira et al., 2008; Wu et al., 2009). Plant nutrients such as nitrogen and phosphorous are retained in the effluent (digestate) after AD, which could be used as a biofertilizer in agricultural production, provided it meets the required standards. The digestate heavy metals content is regulated by different certification schemes in different countries (e.g. SP, 2010). Also, legislation regarding handling of animal by-products may be applicable, which in the EU can involve heat treatment with a minimal particle size of 12 mm at 70 $^{\circ}$C for 1 hour (EC regulation No 178/2002 with amendment 208/2006).

Waste like fish waste and fish sludge, which are rich in lipids and proteins, have the advantage of giving high methane yields, and can be attractive as substrates in an AD process (Cirne et al., 2007). At the same time, these waste types also have properties that make them less suitable for anaerobic microbial degradation;

-free long chain fatty acids (LCFA) can inhibit methanogenesis (Cirne et al., 2007; Pereira et al., 2005).

-protein degradation will cause high concentrations of free ammonia in the process, which might inhibit aceticlastic methanogenesis (Schnurer and Nordberg, 2008).

-High concentrations of light metals such as calcium, sodium, potassium and magnesium are known to be inhibitory to methanogens (Chen et al., 2008).

Anaerobic digestion of protein-rich substrates such as meat and bone has been reported (Wu et al., 2009). A few studies have been done on biogas production from fish related waste, like sludge from saline fish farming and sludge from salmon farming (Arvanitoyannis and Kassaveti, 2008; Gebauer, 2004; Gebauer and Eikebrokk, 2006). Gumisiriza et al. (2009) and Mshandete et al. (2004) attributed poor results in AD of fish residues to the inhibitory effects of lipids and ammonia, and investigated co-digestion as an alternative. In this study, as a strategy to avoid inhibition by free ammonia (FA), LCFA and light metal ions, the possibility of co-digestion with biomass with a low content of the above compounds was investigated. The above ground part of Jerusalem artichoke was chosen to represent a residual biomass from crop cultivation, which is typically high in carbohydrates but low in fats, protein and salts.

In this study, as a step further from oils (PUFA) and FPH extraction, the potential of full utilization of the fish waste was evaluated. The study aims at evaluating the possibilities and limitations of using fish waste and fish sludge as substrates for biogas production. The evaluation was done through biochemical methane potential tests, chemical analyses and calculations based on the experimental data. The overall aim was to explore a biorefinery approach, in extraction of multiple products from waste, taking advantage of the differences in biomass components and intermediates, while limiting waste production (Lopez et al., 2010).

2. Materials and methods

2.1 Substrates and inoculum

2.1.1 Fish waste and fish sludge

The salmon heads were minced and homogenised with a grinder (GM 200, Retsch GmbH, Germany). This fraction, the fish waste, was investigated for biogas

production without further treatment. As described by (Mbatia et al., 2010a), heating, enzymatic hydrolysis and centrifugation was then used to extract the oil and separate the aqueous protein rich phase from the fish waste. The residue from this, the fish sludge, was also investigated for biogas production potential. The amounts of the products derived from 1 000 t (0.2% of the annual global salmon waste production) of salmon waste are illustrated in Figure 1 (Mbatia et al., 2010a).

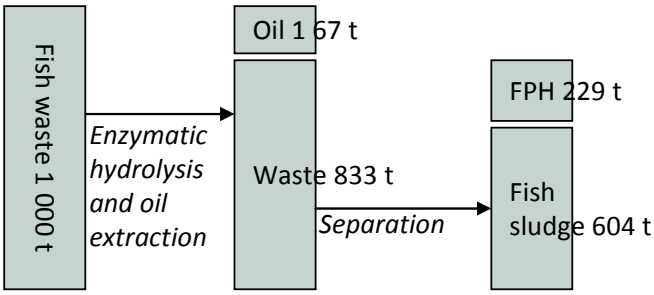

Figure 1. The products recovered and the waste (fish sludge) produced in processing of 1000 tonnes (t) of salmon waste.

2.1.2 Crop biomass

Jerusalem artichoke (*Helianthus tuberosus*) was chosen as representative of a residual crop biomass. The plant is native to North America, but grows well under many temperature and rainfall regimes. The tuber is used as a vegetable, while the above ground biomass is a residue from cultivation. The Jerusalem artichoke used in this study was cultivated in southern Sweden (55 40'N 13 6'E) and harvested in October. The leaves and stems were chopped with a garden shredder (AXT 2500 HT, Robert Bosch GmbH, Germany) into about 2 cm pieces. They were further minced in the laboratory with a grinder (GM 200, Retsch GmbH, Germany) to pass through a 6 mm mesh.

2.1.3 Inoculum

The inoculum was the effluent from a full-scale biogas plant (Söderåsens Bioenergi, Sweden). The biogas plant treats industrial waste from different sources and normally operates under high concentrations of ammonia-nitrogen. The inoculum was also rich in both macro and micronutrients. The buffering capacity (partial alkalinity) was 5.9 g/l, NH_4-N was 4.0 g/l and the pH was 8.

2.2 Biochemical methane potential assay

The biochemical methane potential (BMP) was determined as previously described by (Kreuger, 2011). Exceptions were that 37 °C was used as the digestion temperature, 300 mL of inoculum was used per assay, the control was crystalline cellulose (Avicel PH-101, Sigma-Aldrich, St. Louis, MO, USA) and no nutrients were added to the assays. During the experiment, gas composition and total gas volume were monitored every other day. Ammoniacal-nitrogen and pH were determined at the end of the experiment. The experiments were terminated after 33 days of incubation.

In laboratory triplicates, fish waste, fish sludge and Jerusalem artichoke were digested separately, and the fish sludge was co-digested with Jerusalem artichoke in ratios 1:1 and 1:3 based on the content of organic material measured as volatile solids (VS). All gas volumes are given as dry gas normalised to standard temperature and pressure (0 °C, 1 atm) and the methane yield is reported as normalized volume of methane per added amount of VS of each substrate or substrate mixes.

2.3 Analytical methods

Total solid (TS), VS and pH were determined according to standard methods (APHA, 1995). Total nitrogen, macrominerals and light metals (S, P, K, Na, Ca, Mg), and heavy metals (Zn, Ni, Cr, Pb, Cd, Cu, Hg) were analysed by Kjelldahl, ICP-OES (inductively coupled plasma–optical emission spectrometer) and ICP-MS (inductively coupled plasma–mass spectrometer) respectively by LMI AB (Helsingborg, Sweden). The ammonia concentrations were measured with the Dr Lange test kit LCK 303 (Dr. Bruno Lange GmbH, Dusseldorf, Germany) after diluting a 0.45 μm filtered sample to fall within the detection range. Biogas composition was determined by gas chromatography as described elsewhere (Parawira et al., 2008). Total gas volume was measured using a graduated 100-ml gas-tight glass syringe with a sample lock (Fortuna, Germany).

2.4 Calculations

The non-ionised fraction of the ammoniacal nitrogen (free ammonia) was calculated as described elsewhere (Angelidaki and Ahring, 1993).

Based on the experimentally determined methane yields and the chemical analyses, calculations were made to illustrate the potential full scale conditions in an anaerobic co-digestion process. The mass loss and VS-reduction during digestion was calculated by subtraction of the total mass of CH_4 and CO_2 formed, these latter quantified by using the experimentally determined methane yields. The potential loss from other compounds, e.g H_2O, H_2S, NH_3, through the raw gas was assumed to be negligible. These gases would hence influence the results very little and were not included in the calculations. This means that all minerals in the ingoing substrate remained in the effluent, the biofertilizer. The only change was that organically bound nitrogen was partly mineralized. The degree of mineralization of organically bound nitrogen was assumed to be equal to the degree of VS degradation. The lower heating value (9.97 kWh/m^3) was used to convert normalized methane volumes to energy units.

2.5 Statistical analyses

A significance test (one-way ANOVA P = 0.05) was performed to verify if co-digestion led to any significant difference in methane yield in the batch experiments. Grubb´s test (P = 0.05) was used to make certain there were no outliers in the batch test replicates and a t-Test (P = 0.05) was performed to compare the means in the batch experiments.

3. Results and discussions

3.1 Material flow and chemical analyses

The amount of residual fish sludge extracted from fish waste is outlined in **Figure 1**. The fish sludge made up about 60% of the total amount after removal of fish oil and FPH. The analyzed parameters for the investigated materials and the inoculum are shown in **Table 1**. The fish sludge retained 52 and 49% of the TS and VS of the original fish waste respectively, hence the amount of remaining waste after extraction of oil and FPH is significant. Table 1 shows the very high nitrogen concentration of both fish sludge and fish waste as compared to that of a crop residue (Jerusalem artichoke) and the concentration in the effluent from a large scale biogas process (inoculum). Already this parameter shows that it is not possible to digest either of these substrates (fish waste and fish sludge) alone in a biogas process due to the very high ammonia-nitrogen concentrations that will occur. The concentrations of light metals are also high, while heavy metals with the exception

of zinc, which could jeopardize the biofertilizer quality, are not higher than in the investigated crop and biogas effluent samples.

Table 1 Characteristic of substrates and inoculum used in the anaerobic degradation trials

Variables	*Inoculum*	*Fish waste*	*Fish sludge*	*J. artichoke*
TS (%)	3.7	41.2	37.7	24.7
VS (%)	2.0	35.5	31.4	21.7
Concentrations (mg/kg wet weight)				
N	8010	23800	26100	5340
P	440	7700	8700	380
S	360	1800	1900	280
K	1200	1600	1500	1430
Na	1500	1800	1800	64
Ca	760	11000	13000	6170
Mg	66	380	410	1480
Cd	0.02	0.01	0.03	0.09
Pb	0.3	0.3	0.2	0.5
Zn	8.6	88	71	4.8
Ni	0.50	0.06	0.17	0.23
Cr	0.49	0.29	0.25	0.15
Cu	4.00	0.90	0.91	1.24
Hg	<0.01	0.02	0.03	0.01

3.2 Biochemical methane potential

The characteristic pungent smell that can emanate from fish was completely absent in the BMP assays after digestion. The pungent smell from fish waste has been blamed on the oxidation of unsaturated fatty acids (Rai et al., 2010), and oil extraction (Mbatia et al., 2010a) followed by a further biodegradation step could be expected to greatly reduce the smell.

3.2.1 Digestion of Jerusalem artichoke, fish waste and fish sludge

The extent of conversion of various substrates in terms of methane yield is shown in **Figure 2**. Jerusalem artichoke showed the lowest methane yield amongst the investigated feedstocks (283 ± 14 m^3 CH_4/ton VS added, corresponding to 61 ± 3 m^3 CH_4/ton WW added). Gunnarson et al. (1985) achieved 315 m^3 CH_4/ton VS added in the anaerobic digestion of the above the ground part of Jerusalem artichoke. The

cellulose control used in the BMP assay rapidly reached the theoretical methane yield, showing that the inoculum had cellulolytic activity for crystalline cellulose (results not shown). The low methane yield for Jersusalem artichoke leaves in this study can be attributed to recalcitrant compound such as the lignin embedded cellulose in plant fibres (Amon et al., 2007).

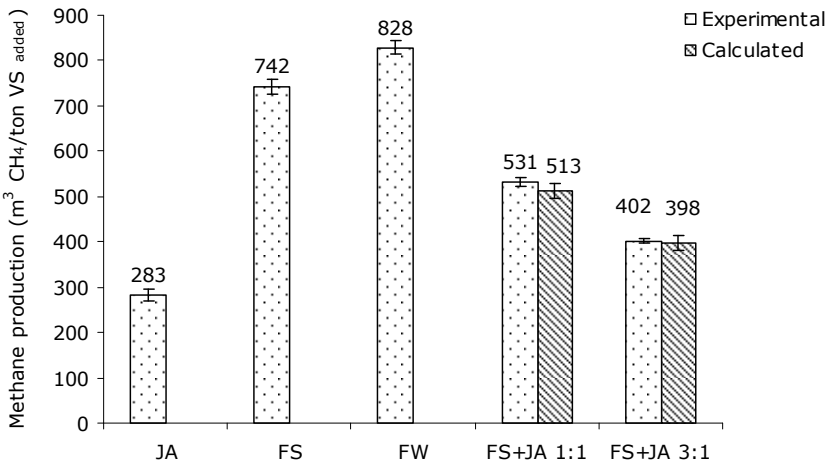

Figure 2. Methane yields from the separate digestion of Jerusalem artichoke residues (JA), fish waste (FW), fish sludge (FS,) and co-digestion of mixtures of FS and JA at VS ratios of 1:1 and 1:3.

Fish waste and fish sludge can be expected to have very high methane yields due to their lipid and proteineous nature. The theoretical yield for lipids is about 1000 m^3 CH_4/ton VS while the theoretical yield for protein is about 490 m^3 CH_4/ ton VS (Moller et al., 2004). The enzymatic oil extraction method applied is known to give lower oil yields as compared to solvent extraction (Mbatia et al., 2010a), its is therefore plausible to think that some residual oil remained in the fish sludge after enzymatic oil extraction. Analysis of variance (ANOVA) for fish waste and fish sludge showed that there was a significant difference in methane yield ($p = 0.05$). Fish waste digestion reached a methane yield of 828 ± 15 m^3 CH_4/ ton VS added corresponding to 294 ± 6 m^3 CH_4/ton WW added while fish sludge reached 742 ± 17 m^3 CH_4/ton VS added corresponding 234 ± 5 m^3 CH_4/ton WW added. The difference can be explained by the removal of oils and proteins from the fish waste, but the high yield of fish sludge also shows that lipids were remaining in this sample.

In other studies on the AD of fish residues, inhibiting conditions, and resulting low methane yields have been reported. Gebauer and Eikebrokk (2006) reported methane yields in the range of 260-280 m^3 CH_4/ton VS added in the mesophilic treatment of salmon sludge (generated from salmon farming). Low yields were attributed to high concentrations of VFAs and ammonia, a condition reported elsewhere as inhibited steady-state (Angelidaki and Ahring, 1993). Also, poor methane yields have been reported by Gebauer (2004) as due to high concentrations of sodium and ammonia. The methane yield reported by Mshandete et al. (2004) for fish waste was 390 m^3 CH_4/VS, lower than the theoretical yield of proteinous substrate (Moller et al., 2004), the authors commented on the inoculum to substrate ratio as reason for the low yield. The high methane yield in the present study indicates that the BMP assay was probably performed under non-inhibited conditions.

3.2.2 Co-digestion of fish sludge and Jerusalem artichoke

The methane yields from the co-digestions of fish sludge and Jerusalem artichoke residues are shown in **Figure 2**. The figure also shows the expected methane yield calculated from the individual digestion of fish sludge and Jerusalem artichoke. Co-digestion might lead to either synergism or antagonism. Synergism is occurs when an additional substrate e.g. contributes essential nutrients needed for bacterial growth, or dilutes the toxic effect of already present compounds. In antagonism, the toxic effect of a compound is further exacerbated by the addition another compound. In this study, co-digestion did not readily lead to either synergism or antagonism. Analysis of variance (ANOVA) for methane yield for the 1:1 and 1:3 VS-ratios of fish sludge/Jerusalem artichoke combinations experimented and calculated from the individual yields showed that there were no significant differences ($p = 0.05$). The experimented yield for the 1:1 mixture was 531 ± 10 m^3 CH_4/ton VS added as compared to 402 ± 3 m^3 CH_4/ton VS added for the 1:3 mix of fish sludge/Jerusalem artichoke combination. If co-digestion trials would have shown higher methane yields that those calculated from digestion of the individual substrates, inhibition in the individual digestion of fish sludge could have been suspected, but this observation further supports the fact that no inhibition occurred in the BMP assays.

The average CH_4 content of the biogas produced from the 1:1 and 1:3 co-digestion trials were 70% and 67% respectively. These values are higher than those normally obtained from conventional anaerobic digestion of organic waste conducted in single

stage-slurry digesters (Samani, 2001). This is due to the high methane content in the biogas from the fish residues, 75-80%, which improves the methane concentration typical for a carbohydrate dominated waste like Jerusalem artichoke (50-60%).

3.3 Limitations of using fish residues as substrate for biogas production

The potential problems of using fish waste/fish sludge as substrate in biogas production can not be fully demonstrated in a BPM assay, where the substrate is mixed with a high amount of inoculum. The chemical analysis of the waste in combination with interpretations of results from the BMP assay will however allow for an analysis of the problems that might occur.

3.3.1 Ammonia inhibition

Ammonia is produced through biological degradation of protein-rich material. Ammonium, NH_4^+, and free ammonia (FA), NH_3, are the principal forms of inorganic ammoniacal-nitrogen in aqueous solution. FA has been suggested to be the main cause of inhibition since it is freely membrane permeable (Chen et al., 2008). The hydrophobic molecule may diffuse passively into the cell, causing proton imbalance and or potassium deficiency (Chen et al., 2008).

The inoculum had an ammoniacal-N concentration of 4.0 g/l at a pH of 8. A rapid methane production from the control substrate in the BMP assay (the crystalline cellulose) indicated that the inoculum was well adapted for degradation under high concentration of FA (results not shown). The fish sludge had a total nitrogen concentration of 26.1 g/kg (**Table 1**), but the measured concentration of ammoniacal nitrogen after the BMP assay was only 4.2 g/kg since the inoculum was added in large excess to the FS. The calculated FA at completion of the BMP assay was 532 mg/l (Angelidaki and Ahring, 1993). This is a relatively high value and can be inhibitory, especially in non acclimated systems. In non acclimated systems, a free ammonia level of 150 mg/l can cause growth inhibition but acclimated system can withstand up to 1100 mg/l free ammonia levels, an adaptation attributed to a shift in the microbial degradation pathway (Hansen et al., 1998; Schnürer and Nordberg, 2008). In designing and operating an anaerobic digestion process, a substrate contributing with so much nitrogen must be added with care not to cause free ammonia inhibition.

3.3.2 Light metals

The fish sludge abounds in light metals such as sodium, potassium and calcium with calcium concentrations as high as 13 g/l (**Table 1**). Generally, high salt concentrations may dehydrate bacterial cells due to osmotic pressure though microbes in saline environment have been reported to accumulate or synthesize substances (osmolytes) which might aid in water retention in the cells. Though moderate levels of sodium (100-200 mg/l) are beneficiary to the anaerobes as in formation of ATP and oxidation of NADH, high levels can be detrimental. High levels of potassium for example may facilitate the passive influx of K^+ thereby neutralizing membrane potential (Chen et al., 2008). This may negatively impact on the membrane integrity thereby jeopardizing transport, protective and nutritional functionalities. The high concentration of calcium in fish sludge means that carbonates and phosphate may be precipitated leading to loss in buffering capacity, scaling of reactors pipes, scaling of biomass hence reducing specific activity.

3.3.3 Long chain fatty acid (LCFA) inhibition

Fish waste abounds in oils or lipids (**Figure 1**). The lipid concentration in the fish sludge was not analyzed, but the presence of good amounts of lipids is evident by the high methane yield (**Figure 2**). Lipid degradation products, LCFA, have been reported to severely inhibit methanogenesis (Cirne et al., 2007; Luostarinen et al., 2009). This inhibition though reversible is mainly due to adsorption of LCFA to bacterial biomass, causing floatation and precipitation. This creates a physical barrier and hinders the transfer of substrates and products, leading to a delay in the initial methane production rate (Pereira et al., 2005). Due to this, substrate with high amounts of lipids are attractive due to the high methane yields, but must be blended with other types of substrates.

3.4 Features of a biogas process using fish sludge

To illustrate that a feasible biogas process can be designed to utilize fish sludge as feedstock, calculations were made based on the laboratory scale results. The calculation was based on the annual processing of 1 000 t fish waste, creating 604 t fish sludge after extraction of the oils and FPH (**Figure 1**). This fish sludge was mixed with Jerusalem artichoke in the experimentally investigated ratio of 1:3 based on the VS content. This means that 2 620 t of the leaves and stems of Jerusalem artichoke should be added per year (**Figure 3**). In **Table 2**, the experimentally achieved input data are summarized, and the calculated outputs for a process are

outlined. The selected process for calculations was a one stage continuous stirred tank reactor, which ideally mixed gives concentrations in the reactor equal to concentrations in the outflow. The organic loading rate was set to 3 kg VS/m^3, d, which is normal for a co-digestion process, and the TS in the reactor was set to a maximum of 8%. At reactor TS of 9% and above, increases in viscosity has been observed, making proper mixing of the reactor content difficult (FNR, 2010).

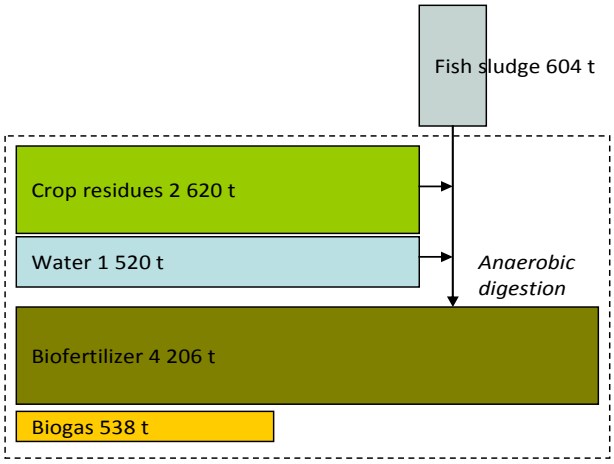

Figure 3. A feasible anaerobic digestion process for the fish sludge remaining from extraction of high-value products from 1 000 t fish waste as shown in Figure 1.

The calculated outputs called scenario A in **Table 2** are with addition of the above mentioned substrates only. The effluent concentration in scenario A, which also represents the concentrations in the reactor, of both TS and ammoniacal nitrogen will then be too high, 12.5% and 8.6 g/l respectively. In scenario B, water is added to reach the preconditioned limit of 8% TS in the reactor. This causes a dilution to 5.5 g/l ammoniacal nitrogen. This is within the range where the microbial consortia will be influenced by FA, but the process is likely to be feasible (Schnürer and Nordberg, 2008). Adding water but keeping the reactor volume constant will decrease the hydraulic retention time from scenario A to B, but the resulting 53 days is satisfactory and likely to give a stable and high methane production also in combination with a high FA concentration (Schnürer and Nordberg, 2008). **Figure 3** shows the total input and output in a co-digestion process that enables biodegradation of the fish sludge under non-inhibited conditions. The produced 538 ton, or 466 000 m^3, of biogas has a calculated methane content of 65%, and an energy content of 3 GWh, or close to 300 m^3 diesel per year.

Table 2. Input and output data for a calculated process based on experimental data.

Input data		
	Fish sludge	*Jerusalem artichoke*
Amount (t/year)	604	2 620
Amount (t VS/year)	190	570
Methane yield (Nm^3/t VS)	742	283
Methane content in biogas (%)	79	55
Outputs		
Scenario	**A**	**B**
Methane production (MWh/year)	3 007	
Methane content in biogas (%)	65	
Active reactor volume (m^3)	690	
Hydraulic retention time (d)	78	53
Effluent (biofertilizer)		
Amount (t/year)	2 690	4 210
TS (%)	12.5	8.0
Ammoniacal nitrogen (g/l)	8.6	5.5
Process water addition (t/year)	-	1 520

In **Table 3**, the calculated composition of the effluent from the biogas process based on scenario B is summarized. The heavy metal content meets in all cases the given guidelines, allowing certification of the effluent as an approved biofertilizer according to Swedish regulations (SP, 2010). The content of ammonia-nitrogen, P and K are in line with or higher than that of e.g. swine or cattle manure, which should make this effluent an attractive biofertilizer. However, waste of animal origin must also meet the demands on hygienic quality, which can be achieved by treating the waste at 70 °C for 1 hour. Alternative treatment with proven similar effects on pathogen reduction can also be approved, and 55 °C for 10 hours is one suggested alternative. During oil extraction (Mbatia et al., 2010a) the fish waste was pretreated at 55 °C for 1 hour, and prolonging the holding time did not affect the oil yield. The holding time could thus be extended to 10 hours in this extraction step, to ensure that the hygienic quality standard is met, and no separate hygienization would be needed. All together, the biofertilizer produced in the suggested process is deemed to be an attractive high quality product.

Table 3. Characteristics of the effluent (bio-fertilizer) for a calculated full scale AD process. Values in brackets: maximum concentrations for certification of biogas plant effluent as biofertilizer (SP, 2010).

Parameters	**Biofertilizer scenario B**
TS (%)	8.0
VS (%)	5.2
Concentrations (mg/kg wet weight)	
Ntot	7100
NH4-N	5500
P	1500
S	450
K	1100
Na	290
Ca	5710
Mg	980
Cd	0.06 (0.08)
Pb	0.35 (8.0)
Zn	13.2 (64)
Ni	0.17 (4.0)
Cr	0.13 (8.0)
Cu	0.90 (48)
Hg	0.01 (0.08)

4. Conclusion

The study demonstrates how all the different components of fish waste can be converted to useful products. The fish sludge is through anaerobic digestion converted from a smelly residue to a renewable energy carrier and a high quality biofertilizer. The fish sludge could, however, not be digested alone due to the demonstrated high content of potentially inhibitory compounds. Co-digestion, in this study exemplified with a residue from crop production, could mitigate the inhibitory effect of FA, light metals and LCFAs as these inhibitors are degraded or diluted to acceptable levels.

Acknowledgements

The funding from Swedish International Development Cooperation Agency (SIDA/SAREC) and the faculty of engineering at Lund University are gratefully

acknowledged. Kalles Fisk Göterborg, Sweden is also acknowledged for supplying fish heads.

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