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# Repeated hydrolysis process is effective for enrichment of omega 3 polyunsaturated fatty acids in salmon oil by *Candida rugosa* lipase

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ABSTRACT

Enrichment of omega 3 polyunsaturated fatty acids (PUFA) in the glyceride fraction of salmon oil was performed by *Candida rugosa* lipase (CRL)-catalysed hydrolysis. Total omega 3 PUFA content in the product was 38.71% (mol.%), more than double of the initial level. The hydrolysis reaction was scaled up to 1 l in a stirred tank reactor without any decrease in the selectivity of the reaction. Moreover, non-steroselectivity and acyl chain specificity of CRL-catalysed hydrolysis, as well as CRL's recognition of the whole triacylglycerol molecule, were proved. The product was purified by short path distillation, which was also shown to contribute to the recovery of omega 3 PUFA in residue. After the removal of free fatty acids, the final product was subjected to a second round of hydrolysis to concentrate omega 3 PUFA further to 50.58%. Recoveries of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) decreased slightly after the second round of hydrolysis, while oleic acid (OA) content was not affected.



## 1. Introduction

Omega 3 polyunsaturated fatty acids (PUFA) are essential fatty acids (FAs) that cannot be synthesized in the human body. Humans lack the necessary enzymes to convert omega 6 FAs, which are found in high amounts in the typical Western diet, into their omega 3 counterparts, so the latter must be obtained from dietary sources. The main source of omega 3 PUFA is marine products. Omega 3 PUFA are synthesized by phytoplankton and algae, transferred through the food web and incorporated into the lipids of fish and marine mammals (Shahidi, 2008).

Since the 1970's, when the low incidence of coronary heart disease amongst Greenland Eskimos had been linked to their high level of oily fish intake (Bang, Dyerberg, & Hjorne, 1976), there have been a tremendous focus on health effects of omega 3 PUFA, especially on eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6), the two most important omega 3 PUFA with well-documented effects. According to the current knowledge, omega 3 PUFA play an important role in the prevention and treatment of cardiovascular diseases, hypertension, diabetes, arthritis and other inflammatory and autoimmune disorders, as well as cancer, and are essential for normal growth and development, especially for the brain and retina (Riediger, Othman, Suh, & Moghadasian, 2009; Yashodhara et al., 2009). Omega 3 PUFA mainly function by altering membrane lipid composition, cellular metabolism, signal transduction, and regulation of gene expression. They regulate the expression of genes in various tissues, including the liver, heart, adipose tissue, and brain (Sampath & Ntambi, 2004).

Marine oils, such as salmon oil, are rich sources of omega 3 PUFA. In order to benefit from omega 3 PUFA, fish oil supplements are suggested instead of fish meal consumption. Ward and Singh (2005) stated that 60–135 g of salmon per day is needed to maintain a daily intake of 1 g of EPA and DHA, which raises the interest in fish oil supplements. The concentrated form provides higher omega 3 PUFA content while reducing the intake of saturated and monounsaturated fatty acids (SFA and MUFA, respectively), as well as the total fat intake. Moreover, commercial fish oil capsules were shown to have mercury levels from non-detectable to negligible (Foran, Flood, & Lewandrowski, 2003), which is a major concern related to the adverse effects of fish consumption (Domingo, 2007).

The main methods for concentration of omega 3 PUFA are chromatographic separation, fractional or molecular distillation, low temperature crystallisation, supercritical fluid extraction and urea complexation, and have been recently reviewed elsewhere (Rubio-Rodríguez et al., 2010). Lipase-catalysed hydrolysis, on the other hand, has been shown to be a straightforward and selective process for production of omega 3 PUFA concentrates over 40 years ago (Bottino, Vandenbu, & Reiser, 1967). The unique feature of the process is the discrimination of the lipases against omega 3 PUFA. The molecular conformation of *cis* carbon–carbon double bonds in omega 3 PUFA, particularly EPA and DHA, causes

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steric hindrance and results in bending of the FA chains, which brings the terminal methyl groups very close to the ester bonds. Due to this steric hindrance effect, enzymatic active sites cannot reach the ester-linkages of these FAs with their glycerol backbones, which protects EPA and DHA from lipase-catalysed hydrolysis (Okada & Morrissey, 2007). Many different lipases from both fungi and yeast have been employed for the purpose.

Scaling up enzymatic reactions requires the consideration of downstream processing steps. In the case of hydrolysis, separation of aqueous phase, together with the water-soluble lipase, from the oil phase is needed, which will then be followed by purification of the oil. Free fatty acids (FFA) produced can be removed by conventional means of lipid fractionation such as column chromatography, solvent extraction, and short path distillation. The latter would be advantageous since it is possible to process large amounts of product continuously and efficiently.

The level of omega 3 PUFA concentration is highly affected by their initial content in the substrate, which is relatively low when by-products are utilised. The aim of the present study was to employ lipase-catalysed hydrolysis to enrich omega 3 PUFA in salmon oil obtained from salmon processing waste. The reaction was scaled up to 1 l in a stirred tank reactor, and the effect of scaling up on the selectivity of the reaction was investigated. A one-step process was not efficient enough to obtain a high value-added product. To concentrate the omega 3 PUFA further, a novel approach, repeated hydrolysis of oil after removal of FFA by short path distillation, was investigated. The omega 3 PUFA content of waste oil was increased by more than 300% after repeated hydrolysis. The proposed method is feasible since the substrates are waste oil and water; moreover, it is suitable for large scale production since the product from the first reaction can be circulated to the same reactor after purification without an additional set-up.

## 2. Materials and methods

### 2.1. Materials

Salmon oil from by-products of farmed salmon was provided by Marine Bioproducts A/S (Bergen, Norway) according to the patented enzymatic process (Sorensen, Sandnes, Hagen, & Pedersen, 2004) involving hydrolysis of by-products by a protease enzyme. The FA profile of the salmon oil is shown in Table 1. Pelagic oil was supplied by Marine Bioproducts, while EPAX 1050 and EPAX 3000 were obtained from EPAX A/S (Aalesund, Norway). *Candida rugosa* lipase (CRL; 64,000 U/g) was donated by Meito Sangyo Co., Ltd. (Tokyo, Japan). All other reagents and solvents used were from Sigma–Aldrich Co. (St. Louis, MO, USA) and were of chromatographic grade.

### 2.2. Lipase-catalysed hydrolysis

Reaction conditions were chosen based on the previous optimisation study (Kahveci & Xu, 2011). 3 grams of oil and 9.5 ml of distilled water (at 3.16 water-to-oil ratio, w/w) were placed in a sealed jacketed glass reactor of 25 ml volume and heated to 45 °C under 300 rpm stirring. After the substrates reached the reaction temperature, lipase (3 wt.% based on the oil amount) was added to initiate the reaction. Samples were taken periodically for analysis.

In the case of repeated hydrolysis, 3 g of residue obtained from short path distillation, as described below, was used as the substrate under the same reaction conditions.

All the reactions were conducted in duplicates. The means of duplicated determinations were used for result evaluation. All analysis and measurements were done in duplicates as well. The adopted values in figures and tables are the average of duplicates at the 95% confidence limit.

**Table 1**
Fatty acid composition of salmon oil.[a]

| Fatty acid | Content (mol.%) |
|---|---|
| 14:0 | 3.09 |
| 16:0 | 11.46 |
| 16:1 | 4.41 |
| 18:0 | 2.85 |
| 18:1 n-9 | 31.40 |
| 18:1 n-7 | 3.13 |
| 18:2 | 10.29 |
| 18:3 | 3.58 |
| 18:4 | 0.99 |
| 20:0 | 0.34 |
| 20:1 | 4.17 |
| 20:2 | 0.76 |
| 20:3 n-6 | 0.21 |
| 20:4 n-6 | 0.52 |
| 20:3 n-3 | 0.33 |
| 20:4 n-3 | 1.21 |
| 20:5 EPA | 5.46 |
| 22:1 | 4.38 |
| 22:5 DPA | 2.79 |
| 22:6 DHA | 8.11 |
| 24:1 | 0.52 |
| ΣSFA | 17.74 |
| ΣMUFA | 48.00 |
| ΣPUFA | 34.25 |
| ΣOmega 3 PUFA[b] | 16.36 |

[a] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b] EPA + DPA + DHA.

### 2.3. Scaling up lipase-catalysed hydrolysis

Two hundred grams of salmon oil and 632 ml of distilled water were placed in a stirred tank glass reactor of 1 l volume and heated to 45 °C under 300 rpm stirring. After the substrates reached the reaction temperature, 6 g of lipase was added to initiate the reaction. The hydrolysis reaction took place for 4 h. Substrates and enzyme mixture was centrifuged twice at 10,000 rpm for 15 min to separate oil from both water and lipase. Due to the losses during the process, centrifugation ended up with 169 g of product.

### 2.4. Purification of product by short path distillation

100 g of product was fed to the short path distillation (SPD) unit (KDL 5, UIC GmbH, Germany) at a rate of 2 ml/min under $10^{-3}$ mbar of vacuum. The feeding tank, condenser and evaporator temperatures were set at 35, 45 and 145 °C, respectively. Both residue and distillate fractions were collected for lipid class and FA composition analyses.

### 2.5. Fractionation of lipid classes by TLC

25 μl of samples from residue and distillate obtained from SPD were dissolved in 100 μl of chloroform/methanol (2:1, v/v) and spotted on silica gel 60 plates (Sigma–Aldrich Co., St. Louis, MO, USA). The developing solvent was diethyl ether/*n*-hexane/formic acid (60:40:1). Separated lipid classes (triacylglycerols, TAG; both isomers of diacylglycerols, 1,3-DAG and 1,2(2,3)-DAG; monoacylglycerols, MAG; free fatty acids, FFA) were visualised by placing the developed and dried plate into an iodine chamber. Each band was scraped off separately and extracted twice by 2 ml of chloroform/methanol (2:1, v/v). Solvent was evaporated under nitrogen