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Enrichment of Omega-3 from Anchovy (*Stolephorus sp.*) Fish Oil by Enzymatic Hydrolysis

Wawan Kosasih[1,3*], Raden Tina Rosmalina[1], Mohamad Robi Muhdani[2], Dede Zainal Arief[2], Endang Saepudin[3], and Sri Priatni[1]

1) Research Unit for Clean Technology, Indonesian Institute of Sciences, Jl Sangkuriang Bandung 40135, Indonesia
2) Pasundan University, Jl Setiabudhi 193, Bandung 40153, Indonesia
3) Department of Chemistry, Faculty of Mathematics and Natural Sciences University of Indonesia, Building G, UI Depok Campus, Depok 16424, Indonesia

**Corresponding author* : wawan.kosasih7@gmail.com

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Abstract

Anchovy (*Stolephorus sp.*) is an economically important fish in Indonesia. Anchovy contains Omega-3 that is important to maintain the health of the heart and brain. This study aimed to enrich the omega-3 content of anchovy oil from the North Sea of West Java. The extraction of anchovy oil was carried out by the soxhlet method. Enrichment of omega-3 from anchovy fish oil is carried out by hydrolysis with a commercial lipase enzyme at a concentration of 500, 1000, 1500, and 2000 unit/0.6 g fish oil for 5, 10, 15 and 20 hours. Before enzymatic hydrolysis by lipase, fish oil was added with water and organic solvent (ethanol, toluene, n-hexane). The Omega-3 content of fish oil products were analyzed by using Gas Chromatography (GC) with FID detector with retention time 14.068 min and 15.506 min for α-Linolenat (ALA) and eicosapentaenoic (EPA), respectively. The results showed that the highest omega-3 content of ALA and EPA were 0.54 and 1.103%, respectively that produced by the addition of n-hexane was 0.5 mL and lipase activity of 1000 units for 15 hours of hydrolysis.

1. INTRODUCTION

Indonesia was awarded a vast ocean with various of fish resources. Indonesia is the largest archipelago country in the world because it has a large sea area and a large number of islands. The length of Indonesia's coast reaches 95.181 Km with sea area of 3.25 million Km^2, mainland 2.01

million Km^2 and 2.55 million Km^2 exclusive economic zone total territorial area of 7,81 million Km^2[1].

Indonesia as a country endowed with large marine resources, including the greatest richness of marine biodiversity. Anchovy is widely available in Indramayu's market. In this research, enrichment of omega-3 content is carried out by enzymatic reaction using commercial lipase and anchovy oil obtained from fishermen of Indramayu beach.

Omega fatty acids (omega-3 and omega-6) are types of long-chain unsaturated fatty acids, Poly Unsaturated Fatty Acids (PUFA), which are essential or cannot be produced by a human body. Therefore, the intake of omega-3 and omega-6 nutrients derived from the food is needed. Adequate omega-3 and omega-6 intakes can optimize the growth of brain cells related to children's intelligence. The lack of omega-3 in the human body can cause fatigue, weak memory, depression, Alzheimer's, and schizophrenia [2,3]. In addition to omega-3, the intake of omega-6 is also important, although the amount needed is less than the amount of omega-3 [4]. The lack of omega-6 fatty acids can cause a decrease in growth rates, hair fall, infertility, fat infiltration in the liver, and scaly skin [5].

The most important omega-3 PUFAs are eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) [6,7] and α-linolenic acid (ALA; C18:3n-3) is important for the precursor of EPA and DHA [8]. On the other hand, high intakes of long-chain omega-6 polyunsaturated fatty acids (Omega-6 PUFAs) have opposite properties than those of omega-3 PUFAs [9,10]. However, excessive consumption of omega-6 without the consumption of omega-3 can reduce LDL (Low-Density Lipoprotein) cholesterol and also decreases HDL (High-Density Lipoprotein) cholesterol. If the level of LDL and HDL is not balanced, it will cause blood clots that trigger coronary heart disease [11].

Oleic acid (OA) is the major monounsaturated fatty acid (MUFA) present in salmon oil and belongs to the family of omega-9 fatty acids, which correlates with the high MUFA content of fish oil with cardioprotective effects [12].

On the contrary, Palmitic acid (PA; C16:0) and OA affect cholesterol and fatty acid metabolism oppositely than oleic acid [13]. Saturated fatty acids (SFAs) affect involved factors in cholesterol metabolism[14]. For the aforementioned reasons, an in-depth knowledge of the presence and concentration of lipids in fish oil is mandatory.

Omega-3 fatty acids DHA (docosahexaenoic acid, C22:6 n-3), EPA (eicosapentaenoic acid C20:5 n-3) and Linolenic acid (C18:3 n-3) derived from fish oil are widely marketed across the world as valued dietary supplements offering numerous health benefits for children and adults alike [15].

DHA and EPA are important to prevent some diseases, including coronary heart disease, inflammation, hypotriglyceridemic effect, and diabetes [16,17].

Omega-3 PUFAs can be obtained from fish oils and marine microalgae and they have been purified using several procedures: urea complexation, chromatography, distillation, low-temperature crystallization, supercritical fluid extraction and enzymatic methods [18,19]. The main advantage of the enzymatic method is that low temperatures and non-aggressive reagents are used, which allows to maintain the PUFA structure unchanged. However, stable lipases, which are not deactivated in the reaction conditions, are essential for these processes to be economically viable [20].

During the last decade, several studies have shown positive effects of fish oils on cognitive development and vision enhancement in newborns, as well as in young children [21]. The aim of this study was to enrich the Omega- 3 (EPA and ALA) content of anchovy oil by the enzymatic hydrolysis method.

2. EXPERIMENTAL

2.1. Materials

Anchovy (*Stoleophorus sp*.) was obtained from Indramayu, lipase enzyme (200,000 units / g) obtained from Xi'an Lyphar Biotech Company, Boron trifluoride-Methanol (BF3, 14%) (E.Merck), n-Hexan (E. Merck), Methanol (E. Merck), DHA and α-Linolenat (Sigma Chemical Co. St. Louis, USA), phosphate buffer pH 5.7 and other reagent were analytical grade.

2.2. Methods

Drying anchovy and extraction of fish oil was done one time, while the measurement of enzymatic reaction results was duplicated using GC.

2.2.1 Anchovy flour Preparation [22]

Anchovy sample was dried at 50°C, for 48 hours. The dried sample was then crushed with a chopper to become anchovy flour. Fish oil was extracted from anchovy flour by using the soxhlet method.

2.2.2 Anchovy Oil Extraction

The extraction of fish oil was carried out by the soxhlet method. 80 grams of fish flour was wrapped in filter paper, then the wrapped flour was placed in the Soxhlet, and 300 mL of n-hexane was added. This process was carried out for 8 hours, and n-hexane was evaporated.

2.2.3 Characterization of Anchovy Oil

2.2.3.1 Determination of Iodine Numbers [23]

0.3 grams of fish oil was added with 10 ml of carbon tetrachloride and 20 ml of iodine-bromide. The solution was placed in a dark room for 30 minutes and shaked for each 10 minutes. 10 mL of 15% KI and 100 mL of distilled water free of CO_2 were added to the solution and then titrated with 0.1 N $Na_2S_2O_3$ until the solution was pale yellow. Furthermore, into these solution was added 1 mL of starch solution and titrated until the blue color disappears. Iodine numbers can be calculated using the following equation:

$$Iodine\ number = \frac{(b-a)XS.Thiosulphate\ X\ 126.9\ X\ 100}{g\ X\ 1000}$$

Where:

b = Number of mL $Na_2S_2O_3$ blank titration

a = Amount of mL $Na_2S_2O_3$ titration of test substance

g = Sample Weight

1 mL of $Na_2S_2O_3$ = 0.01269 g Iodine

2.2.3.2 Numbers of Free Fatty Acid (FFA) [24]

Ten grams of oil was added 50 ml of 95% ethanol and boiled for10 minutes. The solution was added with 2 drops of 2% phenolphthalein and titrated with 0.1 N KOH to form a pink color. The number of free fatty acids was calculated by equation.

$$\%FFA = \frac{V\ KOH\ x\ N\ KOH\ x\ MW fatty\ acid}{Sample\ Weight\ x\ 10}$$

Where :

V = KOH volume of the titration result

N = Normality KOH used

MW = Molecular weight of dominant fatty Acids

2.2.3. Enzymatic hydrolysis of fish oil

0.6 gram of fish oil was added with 0.5 mL of an organic solvent (toluene, n-hexane, ethanol, water), lipase 0.5 mL (1000 unit, as written on the packaging 20.000 unit/g) in phosphate buffer and 0.1 M of phosphate buffer pH 5.7 until 3 ml of total volume. The solution was then incubated at room temperature and shaked at 150 rpm and 20 hours. The reaction was stopped by adding 2 mL of methanol. The same way was done for effect of n-hexane volume variations (0.5, 0.75, 1 and 1.25 mL), effect of incubation

time (0, 5, 10, 15 and 20 hours) and enzyme concentration (0, 1000, 1500 and 2000 unit).

2.2.4 GC and GC-MS Sample Preparation (Sample Esterification)

Firstly 0.125 g of fish oil was put in a test tube. Then, 0.5 ml of boron trifluoride (BF_3) in MeOH (14%) was added to the test tube. Next, the test tube containing the fish oil and boron trifluoride (BF_3) in MeOH (14%) was incubated in an incubator shaker at 55ºC for 1.5 hour. Then, 0.5 ml of saturated sodium hydrogen carbonate ($NaCHO_3$) and 1.0 mL of n-hexane was then added to the test tube. The mixture was mixed and shaken well using a vortex for about 30 second. Then, the mixture was stored at freezer for 10 minutes, so that it will form two layers. Lastly, 0.5 ml of upper layer contain hexane was carefully transferred into a vial for GC and GCMS analysis.

2.2.5. Gas Chromatography (GC) analysis

Fatty acids composition of fish oil samples were analyzed using gas chromatography (GC) Agilent Technologies 7890B equipped with split injector, detector flame ionization detection (FID) system to separate and quantify each FAMEs components. FAMEs were separated using HP-5 column (30 m x 0.32 mm i.d, 0.25). Oven temperature was held at 100ºC, 2 min, then increased to 240ºC at 10ºC/min, held for 1 min. Temperatures for injector and detector were set at 250ºC and 300ºC. 1 µl of sample was injected with ratio 100:1 at column temperature 100ºC. Carrier gases that were used for the system are helium gas 3.0 mL/min controlled at 15.726 psi, hydrogen and air used for FID was held at 30 and 400 mL/min.

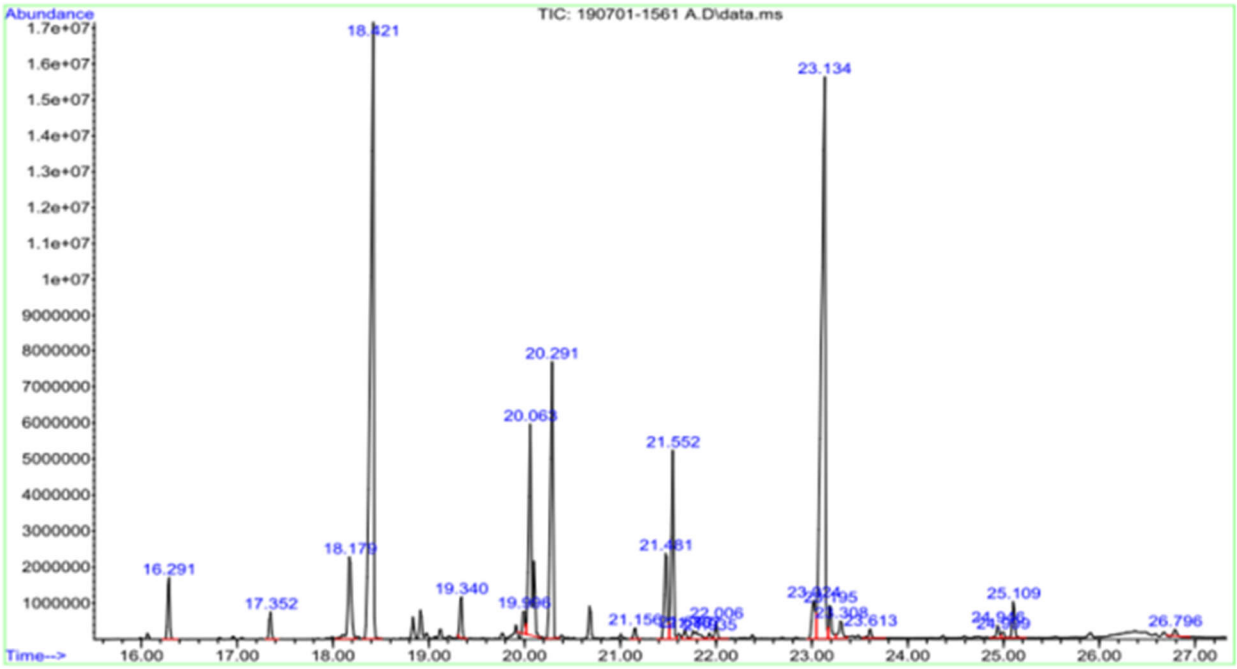

Fig. 1. Analysis of Anchovy Oil Used GCMS

3. RESULT AND DISCUSSION

Enrichment of omega-3 content in Anchovy oil has been carried out by enzymatic hydrolysis method using commercial lipase. Anchovy oil has been extracted from anchovy flour with the yield 5.09%. The iodine number of Anchovy oil was 95.35g I_2 / 100 g According to SNI proper oil consumed > 45-46 mg / g. The higher the iodine number of oil, the more double bonds it has, the better its quality for consumption [24]. The fatty acid content of anchovies oil has been analyzed using GC-MS and the chromatogram was presented in Figure 1.

Table 1. Fatty Acid profile in Anchovy Oil

RT (Retention Time)	Library/ID	% Normal
16.291	Methyl tetradecanoate	1.916
17.350	Pentadecanoic acid, methyl ester	0.795
18.181	9-Hexadecenoic acid, methyl ester, (Z)-	3.815
18.421	Hexadecanoic acid, methyl ester	25.954
19.341	Heptadecanoic acid, methyl ester	1.262
19.996	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	0.635
20.059	11-Octadecenoic acid, methyl ester	9.435
20.286	Methyl stearate	9.883
21.156	Nonadecanoic acid, methyl ester	0.358
21.483	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	3.404
21.546	5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)-	5.946
21.685	.omega.-3 Arachidonic Acid methyl ester	0.328
21.761	cis-11,14-Eicosadienoic acid, methyl ester	0.703
21.937	11-(3,4-Dimethyl-5-propyl-2-furyl)-undecanoic acid, methyl ester	0.215
22.000	Eicosanoic acid, methyl ester	0.520
23.021	Methyl 4,7,10,13,16-docosapentaenoate	1.665
23.134	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	28.513
23.197	Docosapentaenoic Acid methyl ester	1.262
23.311	11-(3,4-Dimethyl-5-pentyl-2-furyl)-dodecanoic acid, methyl ester	0.884
23.613	Docosanoic acid, methyl ester	0.397
24.949	15-Tetracosenoic acid, methyl ester, (Z)-	0.450
24.100	15-Tetracosenoic acid, methyl ester, (Z)-	0.217
25.113	Tetracosanoic acid, methyl ester	1.261
26.802	Hexacosanoic acid, methyl ester	0.183

Based on the data from GSMS chromatogram, The types of fatty acids profile derived from anchovy oil was presented in Table 1.

Based on the data from GCMS database, Anchovy oli contain 24 fatty acids in esters form with the highest content of unsaturated fatty acid was successively 4,7,10,13,16,19-Docosahexaenoic acid or DHA (28.513%), 11-Octadecenoic acid (9.43%) and 5,8,11,14,17-Eicosapentaenoic acid or EPA (5.95%). On our previous study, fish oil from Boso fish using soxhlet extraction and hydrolyzed by lipase produced 10.76% of omega-6 and 13.84% of omega-9, higher than without lipase (6.31% of omega-6 and 12.81% of omega-9) [22]. Anchovy has better potential than Boso fish for omega-3 production. Boso fish oil before the enzymatic reaction or after the enzymatic reaction does not appear to contain omega-3s.

In this study, the effect of the organic solvent in enzymatic process was evaluated by using some solvent such as water, toluene, ethanol and hexane. The fatty acids content linolenic acid (ALA) and eicosapentaenoic acid (EPA) of the hydrolysis products was then analyzed by using GC-FID. The effect of the organic solvent in enzymatic process on ALA and EPA content was presented in Table 2.

Table 2. The effect of organic solvent on enrichment of Omega-3 (ALA and EPA) content in lemuru fish oil

Type of Solvent	Content (%)	
	EPA	ALA
Control	0.14	0.32
Water	0.12	0.32
Toluena	0,09	0.25
Ethanol	0.15	0.35
n-Hexane	0.16	0.44

The data on Table 2 showed that the highest EPA (0.16%) and ALA (0.44%), was obtained when n-hexane is used in enzymatic reaction. Enzymatic hydrolysis with the addition of hexane, heptane and toluene solvents can increase the catalytic activity of enzymes [25]. The study was continued to find the optimum concentration of n-hexane in hydrolysis of

Omega-3 in Anchovy fish oil and the results is presented in Figure 2.

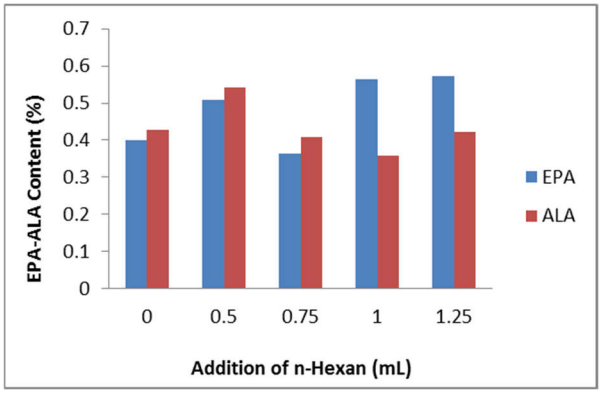

Fig. 2. The effect of n-hexane concentration on enrichment of Omega-3 (ALA and EPA) content in Anchovy fish oil

The data on Figure 2 showed that the optimum addition of n-hexane was 1.25 mL for EPA and 0.5 mL for ALA (total volume of reaction 3 mL). The addition of n-hexane volume affected the EPA and ALA content. The addition of organic solvent can increase the thermostability of lipase. Hydrophobic properties of organic solvent will depend on some parameters such as dielectric constant, dipole moment and log P value. Hexane is known has a log P value (3.5) higher than toluene (2.5). [26]

To optimize the enzymatic hydrolysis of anchovy oil by lipase, in this study was evaluated the effect of incubation time and enzyme concentration to Omega-3 content. The results was presented in Figure 3.

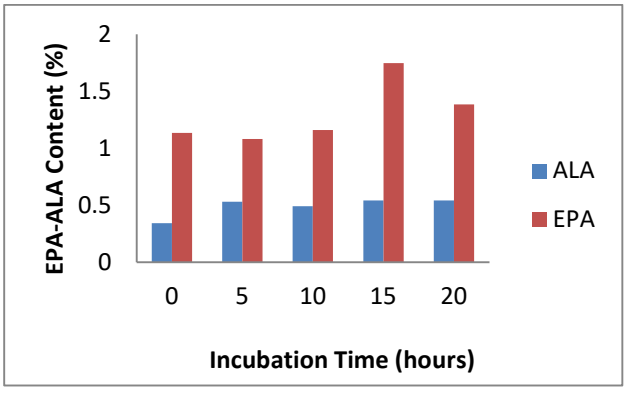

Fig. 3. The effect of incubation time on enrichment of Omega-3 (ALA and EPA) content in Anchovy fish oil

Reaction time is one of parameters for an enzyme to catalyze and generate the product. Long reaction time is not effective and likely to suffer from substrate reformation due to reversible property of an enzyme. This phenomenon can be observed in Figure 4 as Lipase is used to hydrolyze triglycerides:

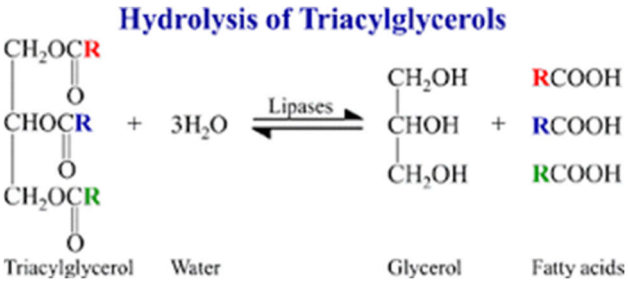

Fig. 4. The mechanism of the triacylglycerol hydrolysis reaction by the lipase enzyme [28].

The data on Figure 3 showed that the highest of EPA (1.75%) and ALA (0.54%) content was reached after 15 hours incubation time.

The study was continued to evaluate the optimum of enzyme concentration in enzymatic hydrolysis process.

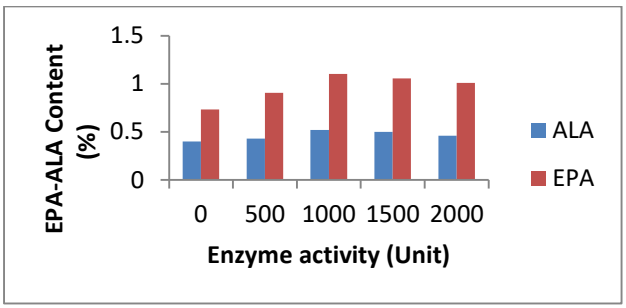

Fig. 5. The effect of enzyme activity on enrichment of Omega-3 (ALA and EPA) content in Anchovy fish oil

On Figure 5 showed that the optimum enzyme concentration was 1000 units per 0.6 g of fish oil with the concentration of EPA and ALA are respectively 1.103% and 0.520%. Lipase was concentrated the polyunsaturated fatty acids such as EPA and DHA. The ester bond between glycerol and monounsaturated fatty acid and saturated fatty acid was cleaved by lipase [27]. Based on the data on Figure 5, the increasing of

EPA and ALA concentration are between 30-50%, after hydrolysis with lipase (1000 unit) for 15 hours. A probable explanation for the low lipase activity is the structure of enzyme, which do not accommodate the esters of PUFA from Anchovy oil in their active sites.

4. CONCLUSION

Enrichment of Omega-3 anchovy oil by hydrolysis using the commercial lipase enzyme was successfully carried out. The addition of 0.5 mL n-hexane solvent, lipase activity of 1000 units / 0.6 gram fish oil and 15 hours incubation time were the most optimum conditions to produce EPA and ALA with concentration of 1.103 and 0.520%, respectively.

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