Improvement of Omega-3 in Lemuru (Sardinella sp) Fish Oil by Using Enzymatic Reaction

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Abstract. This study aims to scale-up the production of the omega-3 enrichment generated from Lemuru fish oil using a 1 L reactor without experimental design through enzymatic reactions that might have increased the economic value of lemuru fish oil. The enrichment of omega 3 fish oil was carried using a 1 L reactor through hydrolysis with commercial lipase enzymes' help. Enzymatic reactions were carried out with variations in temperature (40, 45, 50 and 55°C), time of reaction (0, 6, 12, 18, 24, 30, and 36 hours), the enzyme concentration of 1000 units, and agitation (50, 100, and 150 rpm). The fatty acid content of fish oil before and after the enzymatic reaction was analyzed using GC-MS. At the same time, the omega-3 fatty acid content of fish oil that had been hydrolyzed with lipase was determined using Gas Chromatography (GC). The results show that the optimum conditions for enzymatic reactions were 24 hours reaction time, 50°C temperature, and 150 rpm agitation. Enzymatic reactions using commercial lipases can increase the omega-3 levels of Lemuru fish oil consisting of the content of ALA, EPA, and DHA, 10, 18, and 35-fold, respectively.

1. Introduction
As the largest archipelago country in the world, Indonesia has 17,499 islands from Sabang to Merauke. Indonesia’s total area is 7.81 million square kilometers, consisting of 2.01 million km² of land, 3.25 million square kilometers of ocean, and 2.55 million square kilometers of the Exclusive Economic Zone (EEZ). Indonesia is well known as a maritime country due to the greater water area [1]. Therefore, Indonesia has enormous amounts of marine biodiversity as a resource along with non-marine resources.

Lemuru fish (Sardinella sp.) is a group of fish that owned high protein and fish oil content, which generally can be found in East Java waters, particularly in Banyuwangi Muncar Beach Fishing Port. This fishing port located in Banyuwangi district is one of the largest fish producers in East Java, and the second greatest in Indonesia. Lemuru (Sardinella sp), as a raw material for canned fish such as sardines, is the primary commodity of the fishery industry in Banyuwangi. Based on data from the Technical Service Unit for Quality Testing and Development of Marine and Fisheries Products in Muncar, Banyuwangi, lemuru production during 2008-2009 has reached 27,833 tons as an average. Nevertheless, the number dropped significantly to 1,651 tonnes in 2011. However, the number increased in 2015 by 10,267, then considerably decreased to 54 tonnes in 2017[2].
In 2010 the European Food Safety authorities recommended a good 250 mg/day for EPA plus DHA [3]. The World Health Organization since 2008 has recommended a daily intake of EPA plus DHA of 250 mg as primary prevention of coronary heart disease and two gas secondary prevention [4]. The American Heart Association recommends a higher daily dose (500 mg/day) for healthy adults. In contrast, the Linus Pauling Institute recommends that healthy adults, increasing their omega-3 fats intake by consuming fish twice a week or substituting regular consumption of fish oil supplements at least two times a week [5].

Long-chain omega-3 fatty acids in recent years have become increasingly popular as nutritional and nutraceutical supplements, as proven by the increasing number of new dietary supplements and functional foods. Omega-3 fats, eicosapentaenoic acid (EPA; C20: 5n-3), and docosahexaenoic acid (DHA, C22: 6n-3) are widely claimed to be very beneficial health foods. During the last decade, the number of products contains unsaturated fatty acid tends to be increased. Omega-3 fatty acids can be obtained from several types of fish (see Table 1), and anchovy is the greatest source of omega-3 fatty acids among them [6].

| Fish               | % EPA+DHA                      | Tons  |
|--------------------|-------------------------------|-------|
| Refined anchovy oils| 30% (18/12TG, for high-level EPA concentrates) | 41,600|
| Concentrates       |                               | 13,600|
| Cod liver oil      | 30%                           | 11,200|
| Menhaden oil       |                               | 8500  |
| Salmon             | ≤ 15%                         | 6100  |
| Tuna               | 20–24% (0.5/25TG) for high level EPA concentrates | 2600  |

[Data from GOED, cited in Starling (2015)]

Hydrolysis of fish oil (triglycerides) using lipase enzyme can increase free fatty acids (omega 3) fish oil. The hydrolyzing process of fish oil facilitates free fatty acids (omega 3) releasing from glycerol, thus forming free fatty acids. Enrichment of free fatty acids (omega-3) by hydrolysis with lipase enzyme has been reported by [6][8][9]. The study aimed to produce the omega-3 enrichment generated from Lemuru fish oil using a 1 L reactor without an experimental design.

2. Instruments and Materials

2.1. Instruments
The instruments used in this study was 1 L reactor stirred (homemade), water bath (Memert), Centrifuge (Tommy MX-301), Gas chromatography (Agilent 7860B GC System), Gas Chromatography-Mass Spectrometry (Agilent Technologies with Agilent HP-5MS column), glassware (pyrex), and other laboratory types of equipment.

2.2. Materials
The materials used in this study was fish oil from Muncar Banyuwangi, Lipase enzyme (Xi'an Lyphar Biotech Company), n-Hexane (Merck), Boron trifluoride-methanol solution (Merck), standard omega-3 (all-cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester (DHA), Methyl cis-5,8,11,14,17-Eicosapentaenoic acid (EPA), Methyl cis, cis, cis-9,12,15-octadecatrienoate (ALA) products of Sigma-Aldrich) and other materials with analysis grade.

3. Methodology

3.1. Survey and Sampling of Fish Oil
The survey was conducted in several factories that produce Lemuru fish oil located in Banyuwangi City, East Java. Fish oil was obtained as a by-product of the fish processing industry.
3.2 GC and GC-MS Sample Preparation[10]

Prior to incubation, a fish oil sample (0.125 g) was put in the test tube, and 0.5 ml of boron trifluoride (BF$_3$) in MeOH (14%) was added. The test tube containing the fish oil and boron trifluoride (BF$_3$) in MeOH (14%) was incubated in an incubator shaker at 55$^\circ$C for 1.5 hours. Then, 0.5 ml of saturated sodium hydrogen carbonate (NaCHO$_3$) and 1.0 mL of n-hexane were then added to the test tube. The mixture was mixed and shaken well using a vortex for about 30 seconds. Then, the mixture was stored in the freezer for 10 minutes, and 0.5 ml of supernatant was transferred into a vial for GC and GCMS analysis.

3.3 Analysis of Lemuru Fish Oil Content Using GC-MS (Gas Chromatography-Mass Spectroscopy)

The fatty acid composition of Lemuru fish oil was analyzed using Agilent Technologies Gas chromatography-mass spectrometry (GCMS) with an Agilent HP-5 MS column (30 m x 250 μm x 0.25 μm). The oven temperature was held at 100$^\circ$C, 2 minutes, then increased to 240$^\circ$C at 10$^\circ$C / min, held for 1 minute. The temperature for the injectors and detectors was set at 250$^\circ$C and 300$^\circ$C. Sample (1 μl) was injected with a split ratio of 100:1 at 100$^\circ$C column temperature. The carrier gas used for this system was helium gas 3.0 mL/min controlled at a pressure of 7.0699 psi.

3.4 Fish Oil Enzymatic Reaction Using 1 L Reactor[12]

A total of 160 g of Lemuru fish oil was put into the 1 L reactor, then 133 mL of n-hexane (p.a.) along with 80 mL (2000 U / mL) enzyme and 427 mL 0.1 M phosphate buffer pH: 5.7 was added, thus the total volume was 800 mL. The reactor was closed, then N$_2$ gas flows through one of the reactor holes for 1 minute with low pressure. Enzymatic reactions were carried out to determine the effect of time, temperature, and agitation. As much as 10 ml was sampled for each condition, the enzymatic reaction was stopped by adding 2 mL of methanol. The sample was then esterified, and the omega-3 levels (EPA, DHA, and ALA) were measured using GC.

3.5 Analysis of Omega-3 Content Using GC (Gas Chromatography) of The Enzymatic Reaction Result

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

4. Results and Discussion

4.1 Fish Oil Sample used in this study

The survey was conducted at three factories producing lemuru fish oil in Muncar, Banyuwangi, which produce raw materials for animal feed in fish meal and fish oil. There is only one factor that can provide sufficient samples to be used as raw materials.

4.2 Analysis of Fatty Acid Content Before and After an Enzymatic Reaction Using GC-MS

An analysis was carried out using a GC-MS (Gas Chromatographic Mass Spectra) instrument both before and after the enzymatic reaction. The analysis results of fish oil content before the enzymatic reaction can be seen in Figure 1 and Table 2, while the enzymatic reaction is presented in Figure 2 and Table 3.
Figure 1. GC-MS chromatogram before enzymatic reactions.

Table 2. Results of Lemuru fish oil analysis before the enzymatic reaction

| Pk# | Area% | RT     | Library/ID                                      |
|-----|-------|--------|-------------------------------------------------|
| 1   | 5.300 | 16.240 | Methyl tetradecanoate                            |
| 2   | 0.870 | 17.321 | Pentadecanoic acid, methyl ester                |
| 3   | 6.180 | 18.131 | 7-Hexadecenoic acid, methyl ester, (z)-         |
| 4   | 14.990| 18.320 | Pentadecanoic acid, 14-methyl-, methyl ester    |
| 5   | 3.520 | 19.870 | 1,4,8-Dodecatriene, (E,E,E)-                    |
| 6   | 35.590| 20.009 | 9-Octadecenoic acid (Z)-, methyl ester          |
| 7   | 3.590 | 20.223 | Methyl stearate                                  |
| 8   | 2.200 | 21.420 | 5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-|
| 9   | 6.790 | 21.483 | 5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)-|
| 10  | 0.800 | 21.635 | omega-3 Arachidonic Acid methyl ester           |
| 11  | 5.810 | 21.748 | 11-Eicosenoic acid, methyl ester                |
| 12  | 10.590| 23.021 | 4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-|
| 13  | 1.360 | 23.134 | Docosapentaenoic Acid methyl ester              |
| 14  | 2.400 | 23.349 | 13-Docosenoic acid, methyl ester, (Z)           |
Figure 2. GC-MS chromatogram after the enzymatic reaction.

Figure 2 shows that the enzymatic reaction has taken place at 50°C for 48 hours. The 48 hours was the longest time chosen to ensure that the reaction was completed, while the temperature at 50°C, while the temperature at 50°C was chosen as the median between the maximum and minimum temperature.
Table 3. Results of analysis of Lemuru fish oil as a result of enzymatic reactions.

| Pk# | Area% | RT   | Library/ID                                                                 |
|-----|-------|------|-----------------------------------------------------------------------------|
| 1   | 7.320 | 16.266 | Methyl tetradecanoate                                                       |
| 2   | 0.230 | 16.921 | Methyl 13-methyltetradecanoate                                               |
| 3   | 0.880 | 17.321 | Pentadecanoic acid, methyl ester                                             |
| 4   | 7.070 | 18.156 | cis-9-Hexadecenoic acid methyl ester                                         |
| 5   | 0.160 | 18.232 | 7-Hexadecenoic acid, methyl ester, (Z)-                                     |
| 6   | 18.150| 18.383 | Hexadecanoic acid, methyl ester                                              |
| 7   | 0.860 | 18.875 | 6-Hexadecenoic acid, 7-methyl, methyl ester (Z)                              |
| 8   | 0.400 | 18.938 | Hexadecanoic acid, 15-methyl-, methyl ester                                  |
| 9   | 0.570 | 19.076 | Methyl 8-heptadecenoate                                                     |
| 10  | 0.980 | 19.291 | Heptadecanoic acid, methyl ester                                             |
| 11  | 2.250 | 19.883 | 1,4,8-Dodecatrione, (E,E,E)                                                 |
| 12  | 26.200| 20.059 | 11-Octadecenoic acid, methyl ester 1                                        |
| 13  | 0.280 | 20.160 | 11-Octadecenoic acid, methyl ester, (Z)-                                    |
| 14  | 5.880 | 20.248 | Methyl stearate                                                             |
| 15  | 0.240 | 21.105 | Nonadecanoic acid, methyl ester                                              |
| 16  | 0.980 | 21.433 | 5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-                      |
| 17  | 7.400 | 21.509 | cis-5,8,11,14,17-Eicosapentaenoic acid                                       |
| 18  | 0.150 | 21.572 | Methyl 6,9,12-hexadecatrienoate omega-3 Arachidonic Acid methyl ester        |
| 19  | 0.770 | 21.635 |                                                                                |
| 20  | 10.040| 21.786 | cis-Methyl 11-eicosenoate                                                   |
| 21  | 0.390 | 21.962 | Eicosanoic acid, methyl ester                                                |
| 22  | 7.170 | 23.046 | 4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-                 |
| 23  | 1.630 | 23.147 | cis-5,8,11,14,17-Eicosapentaenoic acid                                      |

The comparison between the fatty acid content before and after treatment showed that the types of fatty acid after enzymatic reaction varied, consisting of 14-23 types. The fatty acid released from glycerol due to the enzymatic reaction is why this phenomenon took place. As much as 35.59% of Lemuru fish oil dominantly consists of 9-Octadecenoic acid (Z) -, methyl ester, omega-9. Meanwhile, the omega-3, cis-5,8,11,14,17-Eicosapentaenoic acid (EPA) and 4,7,10,13,16,19-Docosahexaenoic acid methyl ester (DHA) was found 6.790 and 10.590%, respectively. Maulana et al. 2014 reported that the omega-3 content of Lemuru EPA and DHA fish oil were 8.97 and 6.56%, respectively, while
Suci Istiqlaal et al. (2018) reported that tuna fish bones contained 20.50% DHA. The content of EPA and DHA after enzymatic reaction were 9.03% and 7.17%, respectively. For EPA, the number was increased due to the release of free fatty acids from glycerol. Meanwhile, decreasing DHA content might be due to the oxidation of the long C chain of free fatty acid and double bonds during enzymatic reactions. However, this could be minimized by adding antioxidants during the reaction.

### 4.3 Effect of Temperature on Omega-3 Recovery

Enzymatic reactions were carried out at 40, 45, 50, and 55°C and agitated at 100 rpm. Furthermore, the reaction time was carried out at purposive random times, at 0, 4, 20, and 24 hours. In this study, the types of Omega-3 analyzed were EPA and ALA to determine the impact of temperature on Omega-3 recovery. The results of the enzymatic reaction at various temperatures are presented in Figure 3 and Figure 4.

![Figure 3](image1.png)

**Figure 3.** Enzymatic reaction curve the effect of temperature to increase ALA

![Figure 4](image2.png)

**Figure 4.** Enzymatic reaction curve for the effect of temperature to increase EPA

Figure 3 and 4 shows that that 50°C is the optimum temperature for the enzymatic reaction, especially at 24 hours both for the Linolenic acid (ALA) and EPA. Sapta Raharja et al. (2011) reported that the optimum temperature of fish oil hydrolysis reaction is 45°C. The recovery of ALA and EPA, both in temperatures 45°C and 55°C, were below the yield resulted from temperatures 40°C and 50°C. Moreover, 55°C was the temperature where the ALA and EPA recovery were lowest. This might be happened due to enzyme damage caused by high temperature. This result was similar to the study...
performs by Sapata Raharja et al. (2011). Therefore, the most optimum temperature in this study is 50°C.

4.4 Effect of Agitation on Omega-3 Recovery
Enzymatic reactions were carried out at 50, 100, and 150 rpm agitation at a temperature of 50°C. Similar to the previous analysis, the reaction times and Omega 3 ALA and EPA were also used to find out the impact of agitation on Omega-3 recovery. The time and omega-3 analyzed were the same as the effect of temperature. The enzymatic reaction results and the impact of agitation on Omega-3 recovery are presented in Figure 5 and Figure 6.

![Variasi agitasi](image1)

**Figure 5.** Enzymatic reaction curve for the effect of agitation to increase ALA.

![Variasi Agitasi](image2)

**Figure 6.** Enzymatic reaction curve for the effect of agitation to increase EPA
The graphic shows that the optimum agitation at 24 hours was 150 rpm for ALA and EPA fatty acid recoveries. According to Sapta Raharja et al. (2011), which performed enzymatic reactions using a glass container (4 cm in diameter and 7 cm in height) followed by agitation using a shaker at a speed of 200 rpm. The agitation at 100 and 150 rpm increased the recovery of Omega-3 for about 20-24 hours, but not with the agitation of 50 rpm. The agitation at 50 rpm, the curve looks flat, tends to fall at the 20th and 25th hour. This phenomenon shows that due to the different polarity characteristic between the fish oil and the enzyme, the high agitation was needed to provide perfect condition for contact, thus the Omega-3 recovery will be increased.

4.5 Effect of Reaction Time on Omega-3 Recovery
The effect of reaction time was carried out at an optimum temperature and optimum agitation conditions (50ºC and 150 rpm). The result of the enzymatic reaction is presented in Figure 7.

Figure 7. Enzymatic reaction curve for the effect of time to increase ALA, EPA and DHA

Figure 7 shows that the enzymatic reaction time of 24 hours produces the optimum ALA, EPA and DHA. In this condition, all substrate which contains triglycerides were all hydrolyzed. The study performed by Warna Sundara et al. (1998) in which hydrolysis of Menhaden fish oil using hydrolysis reaction time of 72 hours has obtained EPA and DHA for 14 and 10% respectively, while EPA and DHA generated from the seal buber fish oil was 7 and 10% respectively. Meanwhile, Sapta Raharja et al. (2011) study shows that the enzymatic hydrolysis reaction time of 48 hours has generated EPA and DHA as much as 17.75 and 1.21% respectively. The hydrolysis reaction’s optimum time is required when producing fish oil rich in omega-3 by hydrolysis using commercial lipase enzymes. Measurement results of enzyme reactions performed every two times, the standard deviation of the measurement results with GC so small that it does not appear in the curve.

5. Conclusion
The enrichment of omega-3 Lemuru fish oil has been successfully carried out using a 1 L reactor (160 grams of fish oil). The 50ºC, 150 rpm, and 24 hours were the optimum conditions for temperature, agitation, and enzymatic reaction time, respectively.
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