Isolation and characterization of Boso fish (*Oxyeleotris marmorata*) oil and their recovery using enzymatic reaction

W Kosasih1,*, S Priatni2, E Saepudin2, R T Rosmalina1, S Nurasiah1 and E S Endah1

1 Research Unit for Clean Technology, Indonesian Institute of Sciences (LIPI), Komplek LIPI Bandung, Jalan Sangkuriang, Gedung 50, Bandung 40135, Indonesia
2 Department of Chemistry, Faculty of Mathematics and Natural Sciences Universitas Indonesia, Building G, UI Depok Campus, Depok 16424, Indonesia

* E-mail: wawankosasih@yahoo.com

**Abstract.** *Oxyeleotris marmorata* (Boso) fish is abundant and widespread in West Java Sea area, primarily in Indramayu. This study was aimed to improve the extraction method of essential fatty acids from Boso fish oil, so it would increase the economic value and the benefits of Boso fish. The extraction of fish oil was carried out by n-hexane for 2, 4, 6, 8, 10 and 12 hours. Fish oil was hydrolyzed by a commercial lipase enzyme at various concentration for 48 hours. The fish oil products were analyzed for acid level, acid value and saponification value. Fatty acid contents of fish oil with and without hydrolysis with lipase were determined by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrophotometry (GC-MS) methods. GC-MS results show that fish oil without lipase hydrolysis contained 6.31% omega-6 and 12.31% omega-9, while fish oil hydrolyzed with lipase contained 10.76% omega-6 and 13.84% omega-9. The optimum methyl linolenic acid concentration of 8276.6 ppm was obtained with 9 units lipase concentration, whereas the optimum EPA (eicosapentaenoic acid) concentration of 3937.6 ppm was obtained with 6 units lipase concentration. Enzymatic reactions using lipase can increase the level of omega-3, omega-6 and omega-9 from Boso fish oil.

1. Introduction

Omega fatty acids (omega-3 and omega-6) are types of long-chain unsaturated fatty acids, polyunsaturated fatty acids (PUFA), which are essential or cannot be produced by the 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, and schizophrenia [1,2]. 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 [3]. The lack of omega-6 fatty acid can cause a decrease in growth rates, hair fall, infertility, fat infiltration in the liver, and scaly skin [4]. 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 [5].

Omega-3 fatty acids DHA (docosahexaenoic acid, C22:6 n-3) and EPA (eicosapentaenoic acid, C20:5 n-3) derived from fish oil are widely marketed across the world as valued dietary supplements.
offering numerous health benefits to children and adults alike [6]. DHA and EPA have been shown to be of major importance in the prevention of a number of diseases, including coronary heart disease, inflammation, hypotriglyceridemic effect and diabetes [7–11]. 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 [12].

Omega-3 is often found in marine fishes such as salmon, tuna, mackerel, and herring. The content of omega-3 (DHA) in these fishes is very high, but those fish are expensive [13], so we need to find alternative sources that are cheap and available around us. One of the alternatives that can be offered is producing fish oil from fishes that are currently not consumed (rejected), therefore have low economic value. Isolating fish oil from rejected fishes can increase the economic value of these fishes. Therefore, this study aimed to isolate fish oil from Boso fish (Oxyeleotris marmorata) by the Soxhlet method. Afterwards, the fish oil was hydrolyzed by lipase. Both hydrolyzed and un-hydrolyzed fish oil were characterized using Thin Layer Chromatography (TLC), Gas Chromatography (GC), and Gas Chromatography-Mass Spectrophotometry (GC-MS) to determine the types of fatty acid present.

2. Experimental

2.1. Materials

The materials used were Boso fish, commercial lipase (stated activity 200,000 unit/g), n-hexane, EPA and linolenic acid standards, bentonite, 0.1 N and 0.5 N KOH, methanol, phenolphthalein, 0.5 N HCl, ethanol, 0.01 N Na₂S₂O₃ solution, 15% KI solution, Hanus solution, saturated amylum, CCl₄, aquadest, phosphate buffer pH 7, 0.2 M p-nitrophenyl butyrate, acetic acid glacial, Na₂HPO₄, 14% BF₃, iso-octane, saturated NaCl, Na₂SO₄, and petroleum benzene 40/60.

2.2. Equipment

The equipment used were standard chemical glassware, a set of Thin Layer Chromatography (TLC) (Merck silica gel 60 F₂₅₄), Gas Chromatography (GC) Agilent 7860B GC System, Gas Chromatography-Mass Spectrophotometry (GC-MS) Shimadzu GP2010 Ultra, Spectrophotometer Hitachi U-2800 and Soxhlet extractor.

2.3 Methods

2.3.1. Survey and sampling. The survey and sampling of the rejected fish were done at a fish auction market in Indramayu area, a fish-producing area in West Java, Indonesia.

2.3.2. Making the fish flour. The Boso fish was washed and fish scales were removed. After Boso fish was clean, the fish was weighed and dried using an oven at temperature 60°C for 48 hours to remove the water content. After drying, the fish were crushed using a blender to increase the surface area of contact with the solvent during the extraction. The dry weight of the fish flour was determined.

2.3.3. Extraction of fish oil. The fish oil was extracted from the tissue of Boso fish by the Soxhlet method. Thirty grams of fish meal was wrapped using filter paper, then the fish meal was put into extractor into which 150 mL of n-hexane was added. Soxhlet method was selected due to its low cost, however, the method has two main disadvantages namely the large volumes of organic solvent and several hours to days can be required to achieve an exhaustive extraction [14]. To determine the optimum extraction time, time variation of 2, 4, 6, 8, 10, and 12 hours were tested. The extracted fat was evaporated to remove the solvent. The residue was weighed.

2.3.4. Purification of fish oil by bentonite. Fish oil (0.5 mL) was added with 3% bentonite and mixed until homogeneous. The mixture was allowed to stand for 10 minutes, then it was centrifuged for 10
minutes at room temperature at a speed of 5000 rpm. The supernatant was poured to a new tube, then 3% bentonite was added. The next step was the same as the step above, repeated two times.

2.3.5. Lipase activity test. The enzyme solution (0.45 mL) was put into a screw cap. Then the solution was added with 0.54 mL of 0.1 M phosphate buffer (pH 7) solution and shaken by vortex until the solution was mixed well. After that 0.01 mL of 0.2 M p-nitrophenyl butyrate solution was added and shaken again by vortex until the solution was mixed well. The sample was incubated at 37°C for 30 minutes. The absorbance was measured by a spectrophotometer at 410 nm. The blank was made using the same procedure for the sample without the addition of enzyme solution. The enzyme activity was calculated using the following formula [15]:

$$\text{Unit/mL enzyme} = \frac{(A-B) \times Vt \times fp}{t \times Ve \times K}$$

where:
A = Absorbance of sample
B = Absorbance of blank
t = Time (minutes)
Vt = Total volume sample (mL)
fp = Dilution factor
K = Standard conversion value of p-nitrophenyl butyrate (0.0148 μmol)
Ve = Volume of enzyme solution (mL)

2.3.6. Enzymatic reaction (hydrolysis). Fish oil (0.6 grams) was added with n-hexane, phosphate buffer and enzyme in pH 5 buffer solution at volumes presented in Table 1. The mixture was incubated at 45°C for 48 hours and stirred occasionally. The enzymatic reaction was stopped by adding 2 mL of methanol. Afterwards, the hydrolyzed oil was separated by centrifuging at 5000 rpm for 10 minutes.

### Table 1. The composition of the enzymatic reaction.

| Test code | Fish oil (gram) | Hexane (µL) | 0.1 M phosphate buffer pH 5 (µL) | Enzyme concentration (Unit) |
|-----------|----------------|-------------|--------------------------------|-----------------------------|
| Control   | 0.6000         | 30          | 1,970                          | 0                           |
| 100       | 0.6000         | 30          | 1,870                          | 100                         |
| 200       | 0.6000         | 30          | 1,770                          | 200                         |
| 300       | 0.6000         | 30          | 1,670                          | 300                         |
| 400       | 0.6000         | 30          | 1,570                          | 400                         |
| 1000      | 0.6000         | 30          | 1,000                          | 1000                        |
| 1500      | 0.6000         | 30          | 500                            | 1500                        |
| 2000      | 0.6000         | 30          | 0                              | 2000                        |

*a Test codes refer to the initially planned enzyme concentration*

2.3.7. Determination of fatty acid contents. The oil layer was esterified and tested with TLC, GC and GC-MS to determine the content of fatty acids. Standards for GC analysis were omega-3 EPA and linolenic acid. Analysis using GC-MS was carried out on un-hydrolyzed fish oil and fish oil hydrolyzed with 400 units of lipase.

2.3.8. Determination of acid level and value of fish oil. Fish oil (0.2032 g) was dissolved into 50 mL of methanol and heated at 60°C until it dissolved completely. Three drops of phenolphthalein were added then the solution was titrated with 0.1 N KOH. The same steps were done for the blank. Acid level and value were calculated using the following formulas:

$$\text{Acid levels} = \frac{(m \text{ KOH})}{(m \text{ fish oil})} \times 100\%$$
Acid value = \( \frac{V \text{ KOH} \times N \text{ KOH} \times M_r \text{ KOH}}{m \text{ fish oil}} \)

where:

- \( m_{\text{KOH}} \) = The amount of KOH added (gram) to neutralize the sample
- \( m_{\text{fish oil}} \) = The amount of sample
- \( V \text{ KOH} \) = Volume of KOH added to neutralize the sample
- \( N \text{ KOH} \) = Concentration of KOH
- \( M_r \text{ KOH} \) = Molecular weight of KOH

2.3.9. Determination of saponification value. The saponification value analysis aims to show the relative size of the fatty acid molecules contained in the oil. Oil composed by short carbon chain fatty acids, consequently having a relatively small molecular weight, will have a high saponification rate and conversely, oil composed by long chain fatty acids will have a relatively low saponification rate. Fish oil (0.5 g), 25 mL KOH (0.5 N in ethanol) and boiling stone were placed in a 500 mL flask that has been connected to a condenser. The mixture was refluxed for 60 minutes and let cool. Phenolphthalein indicator (5 drops) was added and the mixture was titrated with 0.5 N HCl until the purple indicator disappeared. In the same way, it was also done for the blank (without fish oil). Saponification value was calculated using the following formula:

\[
\text{Saponification value} = \frac{m_{\text{KOH}} \times V_{\text{titrant}}}{m_{\text{sample}}}
\]

2.3.10. Sample preparation for GC. In a boiling flask, 0.6 grams of fish oil, KOH in 5 mL methanol and a few boiling stones were refluxed for 5-10 minutes until the free fatty acids formed. Subsequently, 5 mL of 14% BF3 in methanol was added followed by boiling for 2 minutes, then 3 mL of iso-octane was added followed by boiling for 1 minute. The mixture was then chilled to room temperature. Saturated NaCl (15 mL) was added and stirred while warmed. The top solution was transferred into a separating funnel, extracted two times with 25 mL petroleum benzene 40/60. Petroleum benzene was then washed with distilled water several times until the distilled water was neutral. Anhydrous Na2SO4 was added to the solution in an evaporator flask followed by evaporation of the solvent. The solution containing free fatty acids was finally dissolved in iso-octane at a final concentration of 5-10%.

3. Results and discussion

3.1. Production of fish oil
Fish oil was produced from Boso fish flour. From 321.9 grams of wet Boso fish, 66.3 grams of fish flour with 9.65% water content could be produced (20.6% yield). Data from the extraction of fish flour into fish oil with variations of extraction time can be seen in Table 2.

| Time (hour) | Boso fish flour (gram) | Extracted fish oil (gram) |
|------------|------------------------|---------------------------|
| 2          | 30                     | 1.1195                    |
| 4          | 30                     | 1.1425                    |
| 6          | 30                     | 1.1578                    |
| 8          | 30                     | 1.2678                    |
| 10         | 30                     | 1.4890                    |
| 12         | 30                     | 1.5000                    |

Table 2 shows that from 30 grams of fish flour, extraction for 10 hours produced 1.4890 grams of fish oil (4.9% yield), which was not significantly different from the 12-hour extraction. Purification by bentonite produced clearer fish oil, but the color was still brownish even though the fishy smell was
The use of bentonite as an adsorbent is advantageous because bentonite has an interlayer structure that can be modified easily, which will improve its absorption properties [16].

### 3.2. Enzymatic reaction

Before the enzymatic reaction was carried out, an activity test was first performed on a commercial enzyme. The test resulted in enzyme activity of 6,021 unit/mL (1,204 unit/g), which was lower than what was stated on the enzyme package (200,000 unit/g). However, the planned enzyme concentration was calculated based on the activity stated in the package, while the actual enzyme concentration can be seen in Table 1.

Figure 1 shows the TLC results from enzymatic reactions (tests 100, 200, 300 and 400), using n-hexane and diethyl ether (8:2) as eluent.

![Figure 1](image)

**Figure 1.** Results of TLC: 1,2 = commercial omega-3 fish oil; 3,4 = control/without enzyme; 5,6 = test-100 (0.6 units enzyme); 7,8 = test-200 (1.2 units enzyme); 9,10 = test-300 (1.8 units enzyme); 11,12 = test-400 (1.6 units enzyme).

Figure 1 shows that the amount of enzyme added influenced the TLC stain results. Commercial omega-3 fish oil showed the stark difference with Boso fish oil, both not hydrolyzed and hydrolyzed with lipase. This is due to the commercial omega-3 fish oil had higher omega-3 levels compared with Boso fish oil.

### Table 3. Results of enzymatic reactions.

| Test sample | Concentration (ppm) | EPA |  | Methyl linolenic acid |
|-------------|---------------------|-----|---|----------------------|
| Control     |                     |     |   |                      |
| 1000        |                     |     |   |                      |
| 1500        |                     |     |   |                      |
| 2000        |                     |     |   |                      |

Table 3 shows that compared with control, enzymatic reaction increased the concentrations of EPA (Eicosapentaenoic acid) and methyl linolenic acid. The optimum methyl linolenic acid concentration of 8276.6 ppm was obtained from test-1500 containing 9 units lipase concentration, whereas the optimum EPA concentration of 3937.6 ppm was obtained from test-1000 containing 6 units lipase concentration.

### 3.3. Acid value and level of fish oil

Acid value analysis showed that the acid value and acid level of Boso fish oil was 133.2 mgKOH/mL and 13.3%, respectively. The high value of acid value resulted from the formation of a large amount of free fatty acids from oil hydrolysis, which indicated lower oil quality [17].

### 3.4. Value and level of fish oil saponification

Saponification was done in order to determine the percentage of each fatty acid out of total fatty acids. Fish oil having low saponification value is less prone to rancidity. The saponification number obtained...
for the oil sample showed 3.08 mg KOH/mL, while the saponification level was 80%. The lower value of saponification values suggests that the mean molecular weight of fatty acids was lower or that the number of ester bonds was less. This might imply that the fat molecules did not interact with each other [18].

![Figure 2](image.png)

**Figure 2.** Fish oil characterization results using GC-MS (a) not hydrolyzed with lipase, (b) hydrolyzed with lipase.
3.5. Characterization of Fish Oil

GC-MS analysis was carried out to find out the composition of the fish oil (Figure 2). The chromatogram shows that while the unhydrolyzed fish oil (control) had 33 peaks, the hydrolyzed fish oil only had 19 peaks. Each peak gave a mass spectrum that was used to identify the compound. The saturated fatty acids, unsaturated fatty acids and other components detected by GC-MS in Bosh fish oil are listed in Tables 4 and 5.

Table 4. Compounds identified in unhydrolyzed fish oil.

| Peak | Retention time | Compound                          | % area | Type of fatty acid |
|------|----------------|-----------------------------------|--------|-------------------|
| 1    | 1.295          | Oxalic acid                       | 0.36   | -                 |
| 2    | 1.326          | Boric acid                        | 2.59   | -                 |
| 3    | 1.400          | Butanoic acid                     | 0.21   | -                 |
| 4    | 1.647          | n-dodecane                        | 0.10   | -                 |
| 5    | 1.878          | n-undecane                        | 1.18   | -                 |
| 6    | 2.248          | n-dodecane                        | 0.15   | -                 |
| 7    | 2.412          | 2-propenoic acid                  | 0.43   | -                 |
| 8    | 2.816          | Tridecane                         | 0.15   | -                 |
| 9    | 8.630          | Tetradecenoic acid                | 3.62   | Saturated         |
| 10   | 10.670         | Pentadecanoic acid                | 1.31   | Saturated         |
| 11   | 12.380         | 9-hexadecenoic acid               | 5.95   | Unsaturated       |
| 12   | 12.874         | Hexadecanoic acid                 | 22.66  | Saturated         |
| 13   | 14.018         | 9-octadecanoic acid               | 1.52   | Unsaturated       |
| 14   | 14.182         | Hexadecanoic acid                 | 0.43   | Saturated         |
| 15   | 14.477         | Cyclopropionateoctanoic acid      | 0.38   | -                 |
| 16   | 14.875         | Octadecanal                       | 0.30   | -                 |
| 17   | 14.989         | Heptadecanoic acid                | 1.60   | Saturated         |
| 18   | 16.473         | 9,12-Octadecadienoic acid         | 1.27   | Omega-6           |
| 19   | 16.618         | 9-Octadecenoic acid               | 8.84   | Omega-9           |
| 20   | 16.730         | 9-Octadecenoic acid               | 3.66   | Omega-9           |
| 21   | 17.185         | Octadecanoic acid                 | 10.69  | Saturated         |
| 22   | 19.277         | Nonadecanoic acid                 | 0.35   | Saturated         |
| 23   | 19.958         | Methyl arachidonate               | 5.71   | Saturated         |
| 24   | 20.105         | Tricyclo[10.2.1.0(2,11)]pentadeca-4,8-Diene | 4.39 | - |
| 25   | 20.476         | Hexadecatrienoic acid             | 0.30   | Omega-6           |
| 26   | 20.700         | 9,12-Octadecadienoic acid         | 0.37   | Omega-6           |
| 27   | 20.807         | 11-Eicosenoic acid                | 0.31   | Omega-9           |
| 28   | 21.348         | Eicosanoic acid                   | 0.29   | -                 |
| 29   | 23.695         | 5,8,11,14-Eicosatetraenoic acid   | 2.81   | Omega-6           |
| 30   | 23.695         | Tricyclo[10.2.1.0(2,11)]pentadeca-4,8-diene | 14.94 | - |
| 31   | 23.967         | 5,8,11,14-Eicosatetraenoic acid, Tricyclo[10.2.1.0(2,11)]pentadeca-4,8-diene | 1.56 | Omega-6 |
| 32   | 24.125         | Tricyclo[10.2.1.0(2,11)]pentadeca-4,8-diene | 2.27 | - |
| 33   | 24.482         | 11-(5-Pentyl-3,4-dimethyl-2-furyl)undecanoic acid | 0.32 | - |
Production of fish flour from wet fish had a yield of 20.6%, while Soxhlet extraction of fish flour yielded 4.9% of fish oil. Fish oil hydrolyzed by lipase produced 10.76% of omega-6 and 13.84% of omega-9 (Table 5). Omega-3 was not found in either sample.

The results indicated the presence of enzymatic activity of lipase. Addition of enzyme could increase the acquisition of omega fatty acids because the enzyme acted as catalysts. Lipase has an active side closed by lid. Lipase lid tends to be hydrophobic. Therefore, in this study n-hexane was used to increase contact between organic solvent and the active side of lipase which would increase the active side of lipase [19]. Addition of organic solvent also increases thermostability, enantio-selectivity, and lipase activity [20]. Besides that, the addition of n-hexane as a medium caused the enzyme to have a higher catalytic ability when compared to the addition of other solvents. It could increase the acquisition of hydrolysis products of free fatty acid. Hoshino et al showed that the hydrolysis of fish oil by lipase was determined by several interrelated factors, namely: 1) specific substrate differences, including fatty acids and specific positional lipases; 2) differences in the level of reactions that occur during the hydrolysis process; 3) differences in the composition of fatty acids and oils used and the reactivity of each lipase in converting oil to partial acyl glycerol [21]. Enzyme technology can be applied in all of the processing steps to increase yields, decrease energy and chemical consumptions and improve product purity [22].

4. Conclusion

Production of fish flour from wet fish had a yield of 20.6%, while Soxhlet extraction of fish flour yielded 4.9% of fish oil. Fish oil hydrolyzed by lipase produced 10.76% of omega-6 and 13.84% of omega-9. The results indicated the presence of enzymatic activity of lipase. Addition of enzyme could increase the acquisition of omega fatty acids because the enzyme acted as catalysts. Lipase has an active side closed by lid. Lipase lid tends to be hydrophobic. Therefore, in this study n-hexane was used to increase contact between organic solvent and the active side of lipase which would increase the active side of lipase [19]. Addition of organic solvent also increases thermostability, enantio-selectivity, and lipase activity [20]. Besides that, the addition of n-hexane as a medium caused the enzyme to have a higher catalytic ability when compared to the addition of other solvents. It could increase the acquisition of hydrolysis products of free fatty acid. Hoshino et al showed that the hydrolysis of fish oil by lipase was determined by several interrelated factors, namely: 1) specific substrate differences, including fatty acids and specific positional lipases; 2) differences in the level of reactions that occur during the hydrolysis process; 3) differences in the composition of fatty acids and oils used and the reactivity of each lipase in converting oil to partial acyl glycerol [21]. Enzyme technology can be applied in all of the processing steps to increase yields, decrease energy and chemical consumptions and improve product purity [22].

\[ \text{Table 5. Compounds identified in fish oil hydrolyzed with 2.4 U lipase.} \]

| Peak | Retention time | Compound | % area | Type of fatty acid |
|------|----------------|----------|--------|--------------------|
| 1    | 1.291          | Methane  | 1.41   | -                  |
| 2    | 1.325          | Boric acid | 9.57  | -                  |
| 3    | 8.628          | Tetradecanoic acid | 3.34 | Saturated |
| 4    | 10.669         | Pentadecanoic acid | 0.91 | Saturated |
| 5    | 12.369         | 9-hexadecenoic acid | 3.41 | Unsaturated |
| 6    | 12.831         | Hexadecanoic acid | 23.94 | Saturated |
| 7    | 13.845         | Pentadecanoic acid | 6.04 | Saturated |
| 8    | 14.014         | 9-octadecenoic acid | 1.23 | Omega-9 |
| 9    | 14.991         | Heptadecanoic acid | 1.27 | Saturated |
| 10   | 16.470         | 9,12-Octadecadienoic acid | 2.33 | Omega-6 |
| 11   | 16.599         | 9-Octadecenoic acid | 9.09 | Omega-9 |
| 12   | 16.719         | 9-Octadecenoic acid | 3.52 | Omega-9 |
| 13   | 17.162         | Octadecanoic acid | 10.69 | Saturated |
| 14   | 17.704         | Octadeca-9,12-Dienoic Acid Methyl Ester | 7.24 | Omega-6 |
| 15   | 19.945         | Methyl arachidonate | 3.17 | Saturated |
| 16   | 20.090         | Tricyclo [10.2.1.0(2,11)] Pentadeca-4,8-Diene | 2.38 | - |
| 17   | 23.682         | 5,8,11,14-Eicosatetraenoic acid | 1.19 | Omega-6 |
| 18   | 23.844         | Tricyclo [10.2.1.0(2,11)] Pentadeca-4,8-Diene | 8.04 | - |
| 19   | 24.113         | Tricyclo [10.2.1.0(2,11)] Pentadeca-4,8-Diene | 1.23 | - |

Omega-6 present in both unhydrolyzed and hydrolyzed fish oil were 9,12-octadecadienoic acid (linoleic acid), hexadecatrienoic acid and 5,8,11,14-eicosatetraenoic acid. Omega-9 present in both unhydrolyzed and hydrolyzed fish oil was 9-octadecenoic acid (oleic acid). The content of omega-6 and omega-9 in the unhydrolyzed fish oil was 6.31% and 12.81%, respectively (Table 4). Fish oil which was hydrolyzed by lipase (2.4 U) contained 10.76% of omega-6 and 13.84% of omega-9 (Table 5). Omega-3 was not found in either sample.
omega-9, higher than without lipase (6.31% of omega-6 and 12.81% of omega-9). The optimum enzyme concentration to produce methyl linolenic acid and EPA was 9 and 6 units, respectively. The results indicate that enzymatic reaction can increase the acquisition of omega-3, 6 and 9 fatty acids from Boso fish.

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