Sains Malaysiana 50(8)(2021): 2271-2282
http://doi.org/10.17576/jsm-2021-5008-11

Production of Omega-3 Fatty Acids by Enzymatic Hydrolysis from Lemuru Fish By-Products

(Penghasilan Asid Lemak Omega-3 melalui Hidrolisis Enzim daripada Produk Sampingan Ikan Lemuru)

WAWAN KOSASIH*, R.TINA ROSMALINA, CHANDRA RISDIAN, ENDANG SAEPUDIN & SRI PRIATNI

ABSTRACT

Production of omega-3 fatty acids from lemuru fish by-products was studied by enzymatic hydrolysis using a lipase enzyme in one liter of the batch reactor. The hydrolysis temperature of fish oil was set at 45 to 55 °C for 0 to 24 h, whereas agitation from 50 to 150 rpm. RSM-Box Bhenken was used to study the effect of these parameters on omega-3 (EPA, docosahexaenoic acid (DHA), and α-linolenic acid (ALA)) content. The % free fatty acid (FFA), acid index, peroxide index, iodine index, and saponification index of lemuru fish oil was 0.925, 2.52, 42.5, 97.28, and 160.11%, respectively. GC-MS analysis results showed that unsaturated fatty acids content (62.34%), which are consisted of omega-3 (EPA, DHA, and ALA), omega-6 and omega-9, was much higher than saturated acids (12.97%). The experiment data showed that the highest EPA (1.221%) and DHA (0.312%) content were reached at 50 °C and 24 h with 150 rpm of agitation. However, through the RSM-Box Bhenken analysis and 3D surface plot, it was suggested that the optimum condition was obtained at 45 °C and 24 h with 150 rpm of agitation with the content of EPA, DHA, and ALA were 1.709, 0.49, and 1.237%, respectively.

Keywords: By-product; lemuru fish oil; lipase; omega-3; response surface method

ABSTRAK

Pengeluaran asid lemak omega-3 daripada produk sampingan ikan lemuru dikaji dengan hidrolisis enzimatik menggunakan enzim lipase dalam reaktor kelompok satu liter. Suhu hidrolisis minyak ikan ditetapkan pada suhu 45 hingga 55 °C selama 0 hingga 24 jam, kemudian diadukkan dengan kelajuan pengadukan 50 hingga 150 rpm. RSM-Box Bhenken digunakan untuk mengkaji pengaruh parameter ini terhadap kandungan omega-3 (EPA, DHA dan ALA). Peratus asid lemak bebas (FFA), indeks asid, indeks peroksida, indeks iodin dan indeks saponifikasi minyak ikan lemuru masing-masing 0.925, 2.52, 42.5, 97.28 dan 160.11%. Hasil analisis GC-MS menunjukkan bahawa kandungan asid lemak tak tepu (62.34%), yang terdiri daripada omega-3 (EPA, DHA dan ALA), omega-6 dan omega-9, jauh lebih tinggi daripada asid tepu (12.97%). Data uji kaji menunjukkan bahawa kandungan EPA tertinggi (1.221%) dan DHA (0.312%) dicapai pada suhu 50 °C dan 24 jam dengan penggoncangan 150 rpm. Walau bagaimanapun, melalui analisis RSM-Box Bhenken dan plot permukaan 3D, dicadangkan bahawa keadaan optimum diperoleh pada suhu 45 °C dan 24 jam dengan penggoncangan 150 rpm dengan kandungan EPA, DHA dan ALA masing-masing adalah 1.709, 0.49 dan 1.237%.

Kata kunci: Kaedah permukaan; lipase; minyak ikan lemuru; omega-3; produk sampingan; tindak balas

INTRODUCTION

Sardinella lemuru or lemuru fish has been known as a coastal schooling and a greatly migratory species. This group of fish can be found in the Eastern Indian Ocean (Thailand, southern coast of East Java and Bali, Western Australia) and the western Pacific (Java Sea north to the Philippines, Hong Kong, Taiwan, and southern Japan) and is the primary species in the pelagic fishery of Bali strait (Sartimbul et al. 2010).

Lemuru fish is one of the important groups in the fish canning industries in Indonesia. The canned fish product only weighs around half of the weight of the raw fish and the rest becomes by-products or waste. One of the by-products from this fish industry is the fish oil, which is still valuable because it contains a significant amount of fatty acids (Purwanto et al. 2015).

Byun et al. (2008) and Khoddami et al. (2012) reported that fish oil is a source of essential fatty acids or long-chain polyunsaturated fatty acids (PUFAs), mainly of eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) which are called omega-3 fatty acids (Iberahim et al. 2018a). PUFAs are considered essential fatty acids due to they are not metabolized by animals, including fish, so PUFAs should be supplied in their diet (Nascimento et al. 2015). These fatty acids were reported to have beneficial health in the prevention of some diseases, for example, hypertension, arthritis, autoimmune disorders, cancer, and heart diseases (Taati et al. 2018). EPA and DHA are also potentials for the treatment of arteriosclerosis, hyperlipidemia, and cardiovascular disease (Byun et al. 2008). DHA is used for the brain health of babies and older people. EPA formulated with RNA and L-arginine is beneficial for recovery of patients after surgery (Lauritzen et al. 2016). Another example of omega-3 fatty acid is α-linolenic acid (ALA; C18:3 ω-3) which is needed for biosynthesis of EPA and DHA (Shahidi et al. 2018).

Some methods of fish oil extraction have been studied to enrich the essential fatty acids content. The innovative and environmentally or green methods have been studied, such as supercritical fluid extraction, enzymatic hydrolysis, microwave-assisted extraction, and ultrasonic extraction. The green extraction method is a promising alternative to organic solvent extraction. Homayooni et al. (2014) reported the fractionation of triacylglycerol (TAGs) from sardine oil using supercritical CO_2. The extraction was continued with urea complexation to obtain fractions rich in omega-3 fatty acids. Enzymatic hydrolysis is the ideal method to recover oil from fish and fishery processing waste (Ivanovs et al. 2017). The refining process of fish oil from crude fish oil obtained from industry was carried out to produce the unsaturated fatty acids include DHA, EPA, and monounsaturated fatty acid (MUFA). The process comprises a semi-refining by the neutralization and bleaching process with the addition of activated clay. This study suggested that the hydrolysis process of oils from fish-waste is the alternative to the production and preservation of omega-3 (Taati et al. 2018).

Immobilized lipases have been used for the hydrolysis of sardine oil to release of omega-3 fatty acids. The limitation of this technique is that if the porous support is used, the enzymes work only on oil molecules partitioned into the aqueous phase. This study suggested the use of immobilized lipases through adsorption on hydrophobic supports strategies, such as very mild covalent immobilization on CNBr-activated sepharose cyanogen bromide lipase derivatives (CNLD) and lipases physically adsorbed on hydrophobic porous supports hydrophobic lipase derivatives (HLD) (Fernández-Lorente et al. 2011). Enzymatic hydrolysis method has been studied with catfish oil by using alcalase then continued with lipase to concentrate the omega-3 polyunsaturated fatty acids. The result showed that two types of omega-3 can be found in catfish oil like eicosapentaenoic acid (EPA) and linoleic acid (ALA) (Khoddami et al. 2012).

Lipase enzyme has specificity to cleave the triglyceride bond sn-1,3 resulting in polyunsaturated fatty acids (PUFAs) in acyl glycerol form. With such mechanism, previous report suggested that lipase could be used to concentrate the omega-3 fatty acid from fish oil (Raharja et al. 2011; Yadwad et al. 1991). The aim of the recent study was to characterize and optimize the production of omega-3 by enzymatic hydrolysis of lemuru fish oil from by-product of the fish canning industries.

Materials and Methods

Materials

Lemuru fish oil sample was obtained from company CV. Biji Sesawi, Banyuwangi, East Java Indonesia. The other materials used in this study include lipase enzyme (200,000 units/g) obtained from Xi'an Lyphar Biotech Company, phosphate buffer, N2 (approximately 100% in purity), 14% of BF3 in methanol, omega-3 standards (methyl all-cis-5,8,11,14,17-eicosapentaenoic, cis-4,7,10,13,16,19-docosahexaenoic acid methyl ester and methyl cis,cis,cis-9,12,15-octadecatrienoate).

Oleo-chemicals characterization of lemuru fish oil

The fish oil sample was analyzed for the index of free fatty acid, acidity, iodine, saponification, and peroxide value by the American Oil Chemists Society (AOCS) (AOCS 2002, 1995) method.

FREE FATTY ACID

Free fatty acid content was determined by adding 10 g of fish oil into 50 mL 95% ethanol. The mixture was boiled for 10 min and titrated by 0.1 N potassium hydroxide (KOH) solution using phenolphthalein as an indicator. The value was calculated using the molecular weight of oleic acid.

ACID VALUE

For calculating acid value, 1 g of fish oil was added to 10 mL hexane and 25 mL ethanol. The mixture was titrated by 0.1 N KOH using a thymol blue indicator.

IODINE VALUE

The iodine value analysis was conducted by dissolving 0.3 g fish oil in 10 mL CCl_4 and 20 mL iodine bromide solution. The mixture was left to react for 30 min and was added with 10 mL 15% KI solution and boiled 100 mL distilled water. The solution was titrated by 0.1 N sodium thiosulfate ($Na_2S_2O_3$) by using starch solution as indicator.

SAPONIFICATION VALUE

Saponification value was measured by adding 0.5-1.0 g of fish oil to 50 mL 0.5 N alcoholic KOH solution. The mixture was refluxed until clear and diluted until 250 mL. Afterward, 25 mL of the solution was titrated by 0.1N hydrochloric acid (HCl) using phenolphthalein as an indicator.

PEROXIDE VALUE

Peroxide value was determined by dissolving 5 g of fish oil in 30 mL acetic acid-chloroform mixture (3:2), 0.5 mL KI, and 30 mL distilled water. The solution was titrated afterward by 0.01 N $Na_2S_2O_3$ by using starch solution as an indicator.

OPTIMIZATION OF OMEGA-3 PRODUCTION IN BATCH REACTOR

One hundred sixty grams of fish oil was placed in a 1L reactor and mixed with 133 mL of hexane, 133 mL lipase (2000 U/mL), and 374 mL of phosphate buffer 0,1 M pH: 5.7. The reactor was then closed and dinitrogen (N_2) was added for one min at low pressure (1 psi) to purge the oxygen in the reactor. The enzymatic reaction was carried out with the parameters such as reaction time, temperature, and agitation process (rpm).

METHYLATION PREPARATION

During fatty acid methyl ester (FAME) preparation, a pre-test was conducted to identify the suitable method for the methylation process. The pre-test includes sodium methoxide method, potassium hydroxide method and boron trifluoride (BF3) method. Finally, boron trifluoride (BF3) was chosen for better peak separation. Firstly, 0.125 g of fish oil was added into a test tube. Then, 0.5 mL of boron trifluoride (BF3) in MeOH (14%) was added to the test tube. Next, the test tube containing the fish oil and boron trifluoride (BF3) in MeOH (14%) was incubated in an incubator shaker at 55 ℃, 150 rpm for 1.5 h. Afterward, 0.5 mL of saturated sodium hydrogen carbonate ($NaCHO_3$) and 1.0 mL of n-hexane were added to the test tube. The mixture was mixed and shaken well using a vortex for about 30 s and was stored in the freezer for 10 min until two layers is formed. Lastly, 0.5 mL of the upper layer which contains hexane was transferred carefully into a vial for gas chromatography (GC) analysis.

GAS CHROMATOGRAPHY (GC) ANALYSIS

Fatty acids composition of fish hydrolysate samples was analyzed using gas chromatography (GC) Agilent Technologies 7890B equipped with a split injector and detector flame ionization detection (FID) system. FAMEs were separated using HP-5 column (30 m × 0.2 mm i.d, 0.25 μm). The oven temperature was held at 100 ℃ for 2 min, increased to 240 ℃ at 10 ℃/min, and held for 1 min. Temperatures for injector and detector were set at 250 and 300 ℃, respectively. One microliter of sample volume was injected with a split ratio of 100:1 at column temperature 100 ℃. Carrier gases that were used for the system are helium gas 3.0 mL/min controlled at 15.726 psi, whereas hydrogen and air used for FID was held at 30 and 400 mL/min, respectively (Table 1).

GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS

Gas chromatography-mass spectrometer (GC-MS) was used to identify the fatty acid compounds in lemuru fish oil before and after hydrolysis. FAMEs were separated using the HP-5 MS column (30 m × 0.25 mm; 0.25 μm film thickness). Chromatography data were recorded and integrated using MassHunter software. The oven temperature was held at 40 ℃ for 1 min, increased to 300 ℃ at 10 ℃/min and held for 5 min. Temperatures for injector and MSD (mass selective detector) transfer

lines were set at 250 °C. One microliter of sample volume was injected with a split ratio of 0:50. Carrier gases that were used for the system are helium gas, 1.0 mL/min controlled at 7.0699 psi (Table 1).

TABLE 1. Experimental condition of GC and GC-MS

No.	Parameter	GC analysis	GC-MS analysis
1.	Column	HP-5 column (30 m × 0.32 mm; 0.25 µm film thickness)	HP-5 MS column (30 m × 0.25 mm; 0.25 µm film thickness)
2.	Oven temperature	100 °C for 2 min, then increased to 240 °C at 10 °C/min, and held for 1 min	40 °C for 1 min, increased to 300 °C at 10 °C/min and held for 5 min
3.	Temperatures for injector	250 °C	250 °C
4.	Temperatures for detector	300 °C	-
5.	Temperature for MSD (mass selective detector) transfer lines	-	250 °C
6.	Volume of sample	1 µl	1 µl
7.	Split ratio	100:1	100:1
8.	Carrier gas	Helium; flow rate: 3.0 mL/min; pressure: 15.726 psi	Helium; flow rate: 1.0 mL/min; pressure: 7.0699 psi
9.	Flow rate of hydrogen for FID (flame ionization detector)	30 mL/min 400 mL/min	-
10.	Flow rate of air for FID (flame ionization detector)	400 mL/min	-

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Response surface methodology (RSM) was used to determine the optimum conditions for the yield of omega-3 (EPA, DHA, and linolenic acid) of the hydrolyzed lemuru fish oil. The experimental design and statistical analysis were carried out using the Minitab V. 17 statistical package (Minitab Inc., PA, USA). Box-Behnken method was selected to evaluate the effects of three independent variables (temperature, time, and agitation), coded as X1, X2, and X3, respectively, on the yield EPA, DHA, and linolenic acid of the hydrolyzed lemuru fish oil. The minimum and maximum values for the hydrolysis temperature were set at 45 to 55 °C, for 0 to 24 h, whereas agitation from 50 to 150 rpm.

RESULTS AND DISCUSSION

The sample of lemuru fish oil obtained from the fish processed industry in Banyuwangi, Indonesia has been

determined for the oleo-chemicals index, and the data is presented in Table 2. The quality of fish oil is important that can be indicated by its oleo-chemical indices such as free fatty acids content, acidity index, peroxide index, iodine index, and saponification index. The indices of lemuru fish oil determined in this study were lower than those found by Nascimento et al. (2015) in crude oil obtained from the fish industry which has FFA 4.4%, peroxide index 3.3, iodine index 117.7 and saponification index 174.9. On the basis of iodine, lemuru fish oil is considered as non-drying oil which has an iodine index less than 110 g I_2/100g. However, the peroxide index of lemuru fish oil was much higher (42.50 mEq/Kg) than the standard value of IFOS (International Fish Oil Standard) due to possibly the removal of the glycerol molecules, which were attached to the molecules of fatty acids during fish oil extraction. Oleo-chemicals index of fish oil is important data to address suitable material for producing PUFA as the preliminary screening of the materials (Byun et al. 2008).

TABLE 2. Oleo-chemicals index of lemuru fish oil

Oleo-chemicals index	Result	Quality standard value
% FFA (free fatty acid)	0.925 ± 0.02	≤ 1.5[a]
Acidity index (mg KOH/g)	2.52 ± 0.00	≤ 0.8[b]
Peroxide index (mEq O_2/kg)	42.50 ± 3.54	<5[a]
Iodine index (g I_2/100 g)	97.28 ± 0.71	≥ 46[b]
Saponification index (mg KOH/g)	160.11 ± 4.25	≤ 253-263[b]

[a]International Fish Oil Standard (IFOS) 2014; [b] Indonesian National Standard (SNI)

GC-MS analysis is used to identify the free fatty acids profile of lemuru fish oil obtained from a fish processing industry in Banyuwangi (Figure 1). Table 2 shows the summarized fatty acids composition of crude fish oil. Lemuru fish oil contained four saturated fatty acids and six unsaturated fatty acids and compounds such as esters. Unsaturated fatty acids in lemuru fish oil comprised of omega-3 (ALA, EPA, and DHA), omega-6 (eicosatetraenoic acid or arachidonic acid), and omega-9 (11-eicosenoic acid). Unsaturated fatty acids content was found higher than saturated acids (62.34% compared to 12.97%). The highest concentration of fatty acids in lemuru fish oil was α-linolenic acid (C 18:3n-3) followed by docosahexaenoic acid (C22:6n−3). GC-MS results show that lemuru fish oil contained a high amount of omega-3. Iberahim et al. (2018b) previously studied fatty acid content in Jade Perch fish oil. The authors reported that the Jade Perch fish oil contained 41.89-42.15% of monounsaturated fatty acids (MUFAs), 29.41% of saturated fatty acids (SFAs), and 16.85% of polyunsaturated fatty acids (PUFAs). There was only 1.906% of omega-3 fatty acids consisted of 0.7612, 0.2596, and 0.8851% for ALA, EPA, and DHA, respectively. In another study which is related to the omega-3 fatty acid extraction, it was suggested that the low content of omega-3 fatty acid content may be caused by long reaction time, high temperature, and oxidation of the PUFA (Gedi et al. 2015). Our investigation of omega-3 content in the lemuru fish oil which was greater than that of in Jade Perch fish oil, as shown in Table 3, suggested that lemuru fish oil is one of the promising sources for the production of omega-3 fatty acid.

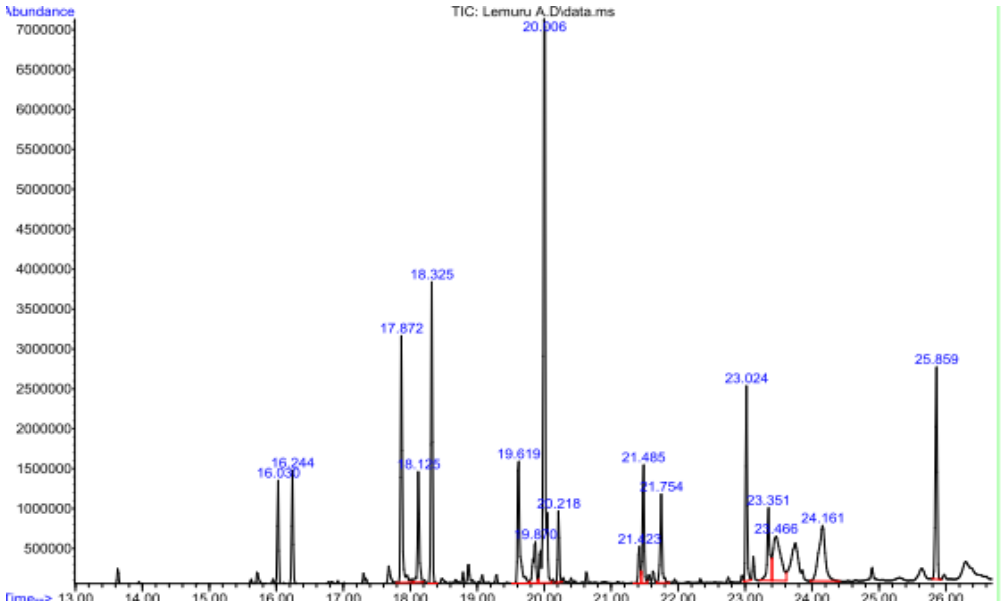

FIGURE 1. GCMS chromatograph of lemuru fish oil

TABLE 3. fatty acids profile of lemuru fish oil

No	Fatty acids	Area (%)
	Saturated fatty acids:	
1	Pentadecanoic acid, C15:0	0.870
2	Hexadecenoic acid, C16:0	6.180
3	Heptadecanoic acid, C17:0	3.520
4	13-Docosenoic acid, C22:0	2.400
	Total	12.97
	Unsaturated fatty acids:	
1	α-Linolenic acid, C 18:3n-3 (ALA)	35.590
2	Eicosapentaenoic acid , C 20:5n-3 (EPA)	6.790
3	Docosahexaenoic acid, C22:6n−3 (DHA)	10.590
4	Eicosatetraenoic acid, 20:4n-6	2.200
5	11-Eicosenoic acid, 20:1n-9	5.810
6	Docosapentaenoic Acid, C22:5	1.360
	Total	62.34
	Other compounds	24.69

Optimization of omega-3 production by enzymatic hydrolysis of lemuru fish oil was determined based on EPA, DHA, and ALA yield. The optimization of enzymatic hydrolysis was analyzed by RSM and Box Behnken method. This method is simple and only limited by the minimum and maximum limits.

Table 4 shows the effect of temperature (X1), time (X2), and agitation speed (X3) to the responses (EPA, DHA, and ALA) that analyzed by GC-FID method. The experiment data shows that the highest EPA and DHA content was reached at 50 ℃, 24 h with 150 rpm of agitation (run 11), whereas the highest ALA content occurred at 45 ℃, 24 h with 100 rpm of agitation (run 8). With the slowest agitation (50 rpm), run 2 and run 4 yielded EPA, DHA, and ALA with the value of less than 0.05%. In the highest temperature and agitation (50 ℃ and 150 rpm, respectively or run 9), the production of EPA, DHA, and ALA dropped to a level lower than 0.05%. The content of omega-3, which was lower than 0.05%, can also be seen in run 17, in which the lowest agitation (50 rpm) and no incubation time was applied. There was no DHA detected on run 13 (55 ℃, 0 h, 150 rpm).

TABLE 4. Experimental data of Omega 3 (EPA, DHA and ALA) obtained by enzymatic hydrolysis of lemuru fish oil

Run	X1 (ºC)	X2 (h)	X3 (Rpm)	EPA (%)	DHA (%)	ALA (%)
1	50	12	100	0.116	0.034	0.104
2	45	12	50	0.034	0.008	0.044
3	55	12	50	0.255	0.087	0.215
4	50	24	50	0.046	0.010	0.051
5	45	12	150	0.936	0.297	0.624
6	45	0	100	0.176	0.107	0.084
7	55	24	100	0.384	0.151	0.384
8	45	24	100	0.843	0.258	0.843
9	55	12	150	0.035	0.005	0.033
10	50	0	150	0.056	0.016	0.074
11	50	24	150	1.221	0.312	0.733
12	50	12	100	0.332	0.104	0.232
13	55	0	100	0.203	0.000	0.111
14	50	12	100	0.270	0.081	0.191
15	50	12	100	0.450	0.014	0.328
16	50	12	100	0.188	0.041	0.147
17	50	0	50	0.028	0.006	0.046

To find out the relationship between Response and Temperature (X1), Time (X2), and Agitation (X3), an analysis of variance (ANOVA) was performed. ANOVA data of the response surface quadratic model for EPA of lemuru fish oil were presented in Table 5. The results of ANOVA analysis show that the Linear and 2-Way Interaction affect the model (p-Value <0.05), except for the interaction between temperature and time (p-Value >0.05), while for all of the square, there was no significantly effect (p-value> 0.05). The results suggest that the models fitted for response variables were empirically adequate due to their high coefficient of determination (R2 >0.93), which means that more than 93% of the response variation could be explained as a function of the three parameters (temperature, time and agitation).

TABLE 5. Analysis of variance (ANOVA) for % EPA

Source	df	Sum of squares	Mean square	F-value	p-value
Model	9	1.85581	0.206201	11.80	0.002
Linear	3	1.11434	0.371447	21.26	0.001
X_1	1	0.15457	0.154568	8.85	0.021
X_2	1	0.51562	0.515620	29.51	0.001
X_3	1	0.44415	0.444153	25.42	0.001
Square	3	0.03880	0.012932	0.74	0.561
X_1X_1	1	0.01218	0.012176	0.70	0.431
X_2X_2	1	0.02466	0.024657	1.41	0.274
X_3X_3	1	0.00042	0.000419	0.02	0.881
2-Way Interaction	3	0.70267	0.234224	13.40	0.003
X_1X_2	1	0.05905	0.059049	3.38	0.109
X_1X_3	1	0.31472	0.314721	18.01	0.004
X_2X_3	1	0.32890	0.328902	18.82	0.003
Error	7	0.12232	0.017475		
Lack of Fit	3	0.05565	0.018549	1.11	0.442
Pure Error	4	0.06668	0.016669		
Cor Total	16	1.97813			

Table 5 shows that the square variable is not significant (p-value> 0.05). The equation for EPA and the response variable (Y) of lemuru fish oil was derived using the regression coefficient of intercept, linear, temperature, time, agitation, and quadratic terms to fit a full response surface model. The equation was given in (1):

$$Y = 0.10 - 0.106\,X1 + 0.0619\,X2 + 0.0559\,X3 + 0.00251\,X1X1 + 0.000531\,X2X2 - 0.000004\,X3X3 - 0.00202\,X1X2 - 0.001122\,X1X3 + 0.00478\,V2V3 \quad (1)$$

This equation was then used to predict the EPA content and compared with laboratory data, which prepared at 50 ℃ for 12 h with agitation 100 rpm. The data shows that the predicted EPA content (0.295%) was higher than EPA content (0.272%) obtained from laboratory data. However, analysis data by RSM shows that the optimum condition of enzymatic hydrolysis lemuru fish oil was reached at 45 ℃ for 24 h with agitation 150 rpm. At this condition, the predicted EPA content was 1.709 ± 0.154%.

Table 6 shows ANOVA data of the response surface quadratic model for the DHA content of lemuru fish oil. The results show that the linear and 2-way interaction affect the model except for the interaction between temperature and time; this is indicated by the P-Value < 0.05 with R2 > 0.96. The data show that all parameters had a significant effect on the model. According to the model regression analysis, the best explanatory model equation was given as in (2):

$$Y = 3.08 - 0.1483\,X1 - 0.0110\,X2 + 0.01833\,X3 + 0.001747\,X1X1 + 0.000211\,X2X2 + 0.000000\,33 - 0.00000\,X1X2 - 0.00371\,X1X3 + 0.000122\,X2X3 \quad (2)$$

TABLE 6. Analysis of variance (ANOVA) for % DHA

Source	df	Sum of squares	Mean square	F-value	p-value
Model	9	0,170109	0,018901	19,53	0,000
Linear	3	0,101722	0,033907	35,04	0,000
X_1	1	0,022786	0,022786	23,55	0,002
X_2	1	0,045266	0,045266	46,78	0,000
X_3	1	0,033670	0,033670	34,80	0,001
Square	3	0,012660	0,004220	4,36	0,050
X_1X_1	1	0,008035	0,008035	8,30	0,024
X_2X_2	1	0,003900	0,003900	4,03	0,085
X_3X_3	1	0,000002	0,000002	0,00	0,961
2-Way Interaction	3	0,055726	0,018575	19,20	0,001
X_1X_2	1	0,000000	0,000000	0,00	0,999
X_1X_3	1	0,034410	0,034410	35,56	0,001
X_2X_3	1	0,021316	0,021316	22,03	0,002
Error	7	0,006773	0,000968		
Lack of Fit	3	0,001379	0,000460	0,34	0,798
Pure Error	4	0,005395	0,001349		
Cor Total	16	0,176882			

Based on the calculation of predicted DHA content by equation (2) (at 50 ℃, for 12 h with agitation 100 rpm), the data showed that the predicted of DHA content were have no significant results (0.055%) with DHA content that analyzed by GC. RSM analysis of DHA content as the response showed that the optimum condition of enzymatic hydrolysis of lemuru fish oil was also reached at 45 ℃ for 24 h with agitation 150 rpm. At this condition, the predicted DHA content was 0.49 ± 0.036%.

Table 7 shows ANOVA data of the response surface quadratic model for the ALA content of lemuru fish oil. According to equation (3), the predicted ALA content (0.228%) was higher than ALA content (0.200%), which obtained from laboratory data (at 50 ℃, for 12 h with agitation 100 rpm). RSM analysis of ALA content at optimized condition (45 ℃, for 24 h, agitation 150 rpm) was 1.237 ± 0.107%.

TABLE 7. Analysis of variance (ANOVA) for % ALA

Source	df	Sum of squares	Mean square	F-value	p-value
Model	9	0,97553	0.108392	12.88	0.001
Linear	3	0.60391	0.201303	23.92	0.000
X_1	1	0.09064	0.090635	10.77	0.013
X_2	1	0.35949	0.359493	42.73	0.000
X_3	1	0.15378	0.153780	18.28	0.004
Square	3	0.06073	0.020242	2.41	0.153
X_1X_1	1	0.02622	0.026218	3.12	0.121
X_2X_2	1	0.02433	0.024329	2.89	0.133
X_3X_3	1	0.01067	0.010666	1.27	0.297
2-Way Interaction	3	0.31089	0.103631	12.32	0.004
X_1X_2	1	0.05903	0.059026	7.02	0.033
X_1X_3	1	0.14513	0.145128	17.25	0.004
X_2X_3	1	0.10674	0.106739	12.69	0.009
Error	7	0.05890	0.008414		
Lack of Fit	3	0.02920	0.009733	1.31	0.387
Pure Error	4	0.02970	0.007425		
Cor Total	16	1.03443			

The effect of interaction between two factors on the predicted omega-3 (EPA, DHA, and ALA) content of lemuru fish oil was presented in 3D curves (Figure 2). The 3D surface plot for all responses shown a similar pattern. These curves were confirmed the optimum condition for omega-3 production by enzymatic hydrolysis of *Sardinella lemuru* oil in 1 L of the batch reactor was at 45 °C, for 24 h and agitation 150 rpm. According to RSM analysis, it is suggested that the temperature and agitation of enzymatic hydrolysis were responsible for the omega-3 content of lemuru fish oil. ANOVA data for EPA, DHA, and ALA content shows that the interaction between temperature and agitation (X1X3) shows a significant effect with $p<0.05$.

Lipase is the enzyme that has specificity with a stereochemical numbering 1.3, so it can hydrolyze the ester bonds of fish oil in the form of triacylglycerol at the primary position resulting in the PUFA fatty acids (Ivanovs et al. 2017). The results suggested that temperature was one of the important parameters in releasing free omega-3 from its complex triglyceride structure in the fish oil. The lowest temperature used in this experiment (45 °C) could be the optimum temperature of the lipase enzyme employed in this study to digest the oil. Time for the enzymatic reaction was also essential based on the results. In this investigation, 24 h was suggested as the best time for lipase to yield the optimum amount of omega-3. The data also explained that agitation had a contribution to the production of omega-3 from lemuru fish oil. The highest agitation in this experiment (150 rpm) was preferred to yield an optimum concentration of omega-3. Agitation in the enzymatic hydrolysis of oil using lipase is crucial because it can increase the surface area of the oil globules, making them more sensitive to lipase action and yielding more lipase adsorption on the oil globules (Jandal et al. 1996).

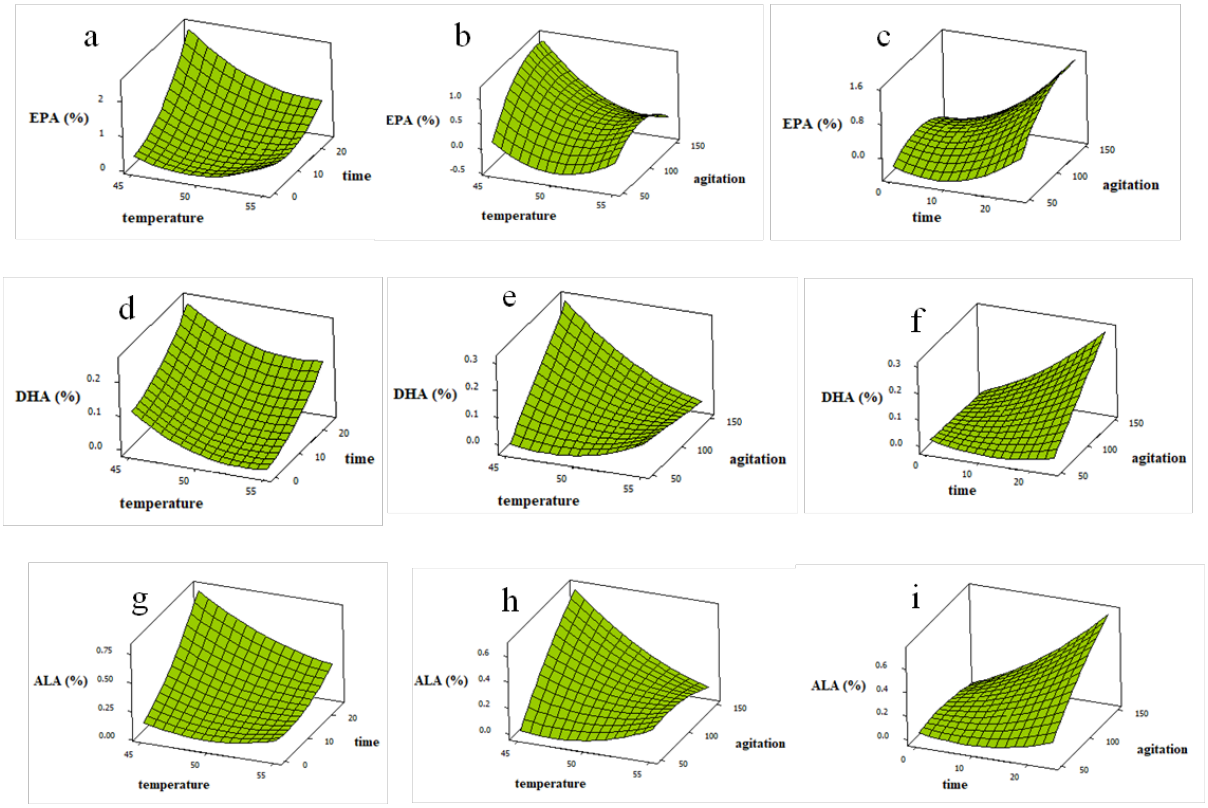

FIGURE 2. 3D surface plot of interaction between two factors on EPA (a, b, c), DHA (d, e, f) and ALA (g, h, I) content of lemuru fish oil hydrolysis products

Conclusion

The optimization of omega-3 production of lemuru fish oil from industrial by-product has been successfully studied using lipase enzymatic hydrolysis technique prepared in a 1 L batch reactor. The parameters chosen were temperature, time, and agitation, which were important in regulating the enzymatic hydrolysis process. The hydrolysis process optimum at 45 °C for 24 h and agitation was 150 rpm yielding EPA 1.709%, DHA 0.49%, and ALA 1.237%, respectively. The study suggested that temperature and agitation had a significant effect on the enzymatic hydrolysis of lemuru fish oil.

Acknowledgements

We acknowledge financial support from the Insinas Ristek-Dikti and Research Unit for Clean Technology, Indonesian Institute of Sciences (LIPI).

References

AOAC. 2005. *Association of Official Analytical Chemist*. Official method of analysis of the association of official analytical of chemist. The Association of Analytical Chemist. Inc.

AOAC. 1995. *Association of Official Analytical Chemist*. Official method of analysis of the association of official analytical of chemist. The Association of Analytical Chemist. Inc.

Byun, H.G., Eom, T.K., Jung, W.K. & Kim, S.K. 2008. Characterization of fish oil extracted from fish processing by-products. *Preventive Nutrition and Food Science* 13(1): 7-11.

Fernández-Lorente, G., Betancor, L., Carrascosa, A.V. & Guisán, J.M. 2011. Release of omega-3 fatty acids by the hydrolysis of fish oil catalyzed by lipases immobilized on hydrophobic supports. *Journal of the American Oil Chemists' Society* 88(8): 1173-1178.

Gedi, M.A., Bakar, J. & Mariod, A.A. 2015. Optimization of supercritical carbon dioxide (CO_2) extraction of sardine (*Sardinella lemuru* bleeker) oil using response surface methodology (RSM). *Grasas y Aceites* 66(2): 1-11.

Homayooni, B., Sahari, M.A. & Barzegar, M. 2014. Concentrations of omega-3 fatty acids from rainbow sardine fish oil by various methods. *International Food Research Journal* 21(2): 743-748.

Iberahim, N.I., Hamzah, Z., Yin, Y.J. & Sohaimi, K.S.A. 2018a. Extraction and characterization of omega-3 fatty acid from catfish using enzymatic hydrolysis technique. *MATEC Web of Conferences*.

Iberahim, N.I., Hann, Y.C., Hamzah, Z. & Sohaimi, K.S.A. 2018b. Extraction of omega-3 fatty acid from Jade Perch (*Scortum barcoo*) using enzymatic hydrolysis technique. *Indonesian Journal of Chemistry* 20(2): 282-291.

Ivanovs, K. & Blumberga, D. 2017. Extraction of fish oil using green extraction methods: A short review. *Energy Procedia* 128: 477-483.

Jandal, J.M. 1996. Effects of some thermal, chemical and mechanical lipase activity in Shammi goat milk treatments. *Small Ruminant Research* 20(3): 275-279.

Khoddami, A., Ariffin, A.A., Bakar, J. & Ghazali, H.M. 2012. Quality and fatty acid profile of the oil extracted from fish waste (head, intestine, and liver) (*Euthynnus affinis*). *African Journal of Biotechnology* 11(7): 1683-1689.

Lauritzen, L., Brambilla, P., Mazzocchi, A., Harsløf, L., Ciappolino, V. & Agostoni, C. 2016. DHA effects in brain development and function. *Nutrients* 8(1): 1-17.

Nascimento, V.L.V.D., Bermúdez, V.M.S., Oliveira, A.L.L.D., Kleinberg, M.N., Ribeiro, R. D.T.M., Abreu, R.F.A.D. & Carioca, J.O.B. 2015. Characterization of a hydrolyzed oil obtained from fish waste for nutraceutical application. *Food Science and Technology* 35(2): 321-325.

Purwanto, M.G.M., Maretha, M.V., Wahyudi, M. & Goeltom, M.T. 2015. Whole cell hydrolysis of sardine (*Sardinella lemuru*) oil waste using *Mucor circinelloides* NRRL 1405 immobilized in poly-urethane foam. *Procedia Chemistry* 14: 256-262.

Raharja, S., Surdarma, P. & Oktavia, T. 2011. Enzyme hydrolysis of fish for production of omega-3 fatty acid using lipase derived from *Aspergillus niger*. *Jurnal Teknologi dan Industri Pangan* 22(1): 64-72.

Sartimbul, A., Nakata, H., Rohadi, E., Yusuf, B. & Kadarisman, H.P. 2010. Variations in chlorophyll-a concentration and the impact on *Sardinella lemuru* catches in Bali Strait, Indonesia. *Progress in Oceanography* 87(1-4): 168-174.

Shahidi, F. & Ambigaipalan, P. 2018. Omega-3 polyunsaturated fatty acids and their health benefits. *Annual Review of Food Science and Technology* 9: 345-381.

Taati, M.M., Shabanpour, B. & Ojagh, M. 2018. Investigation on fish oil extraction by enzyme extraction and wet reduction methods and quality analysis. *Aquaculture, Aquarium, Conservation & Legislation* 11(1): 83-90.

Yadwad, V.B., Ward, O.P. & Noronha, L.C. 1991. Application of lipase to concentrate the docosahexaenoic acid (DHA) fraction of fish oil. *Biotechnology and Bioengineering* 38(8): 956-959.

Wawan Kosasih*, R.Tina Rosmalina, Chandra Risdian & Sri Priatni
Research Unit for Clean Technology
Indonesian Institute of Sciences
Jl. Sangkuriang Bandung, 40135
Indonesia

Wawan Kosasih & Endang Saepudin
Department of Chemistry
Faculty of Mathematics and Natural Sciences
University of Indonesia
Depok 16424
Indonesia

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

Received: 28 September 2020
Accepted: 10 December 2020