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Enzymatic Transesterification of Ethyl Ferulate with Fish Oil and Reaction Optimization by Response Surface Methodology

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Summary

The enzymatic transesterification of ethyl ferulate (EF) with fish oil from cod liver was investigated with Novozym® 435 as catalyst under solvent-free conditions. The purpose of the study is to evaluate the synthesis system for the production of feruloyl fish oil in industry. The modified HPLC method was first set up to characterise the reaction products together with liquid chromatography electrospray time-of-flight mass spectrometry (HPLC--ESI-TOF-MS). The influence of the addition of glycerol to the system on the feruloyl acylglycerol profile was investigated in terms of transesterification performance. The bioconversion rate of EF can be significantly increased with the increased formation of feruloyl fish oil products when appropriate amount of glycerol is present in the reaction. Therefore, an equivalent molar amount of glycerol was added to EF for the practical optimization of the system. The mutual effects of temperature (40 to 70 °C), reaction time (1 to 5 days), enzyme load (2 to 20 %) and molar ratio of fish oil and EF in the substrate (1 to 5) were thus studied with the assistance of response surface methodology (RSM) for the purpose of maximizing the formation of feruloyl fish oil. The models were well fitted and verified. The optimized conditions were found to be: temperature 70 °C, enzyme load 4.3 %, substrate ratio 4.7, and reaction time 5 days. Under these conditions, the maximum conversion of EF reached 92.4 %, and the formation of feruloyl fish oil reached 80.4 %, but the formation of by-product was minimized to 11.4 % only.

Key words: transesterification, ethyl ferulate, fish oil, HPLC, HPLC-ESI-TOF-MS, feruloyl acylglycerol species, RSM

Introduction

Interest in long-chain Ω-3 fatty acids (PUFA) has been increased over the past 20 years due to their potential health benefits. It has been found that Ω-3 fatty acids are essential for normal growth and development. They also play an important role in the prevention and treatment of coronary artery disease, hypertension, diabetes, arthritis, other inflammatory and autoimmune disorders and cancer (*1,2*). However, Ω-3 fatty acids, particularly EPA (C20:5 n-3) and DHA (C22:6 n-3), are very easily oxidized due to the rich content of unsaturated double bonds. Oxidized fish oil or Ω-3 fatty acids can cause a series of problems because they develop undesirable fishy off-flavours, colour deterioration and loss of endogenous antioxidants. Moreover, the free radicals generated from lipid autoxidation can react with proteins and pigments, which together will lead to decreased nutritional quality and the presence of toxic lipid oxidation products (*3*).

For the purpose of preventing or delaying lipid oxidation and increasing the application of fish oil in indus-

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try, one of the popular methods is to add antioxidants. The phenolic acid family are naturally available good antioxidants, as reported (*4–6*). Ferulic acid (E-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid) is a common polyphenolic compound abundant in vegetables. Besides the potent antioxidant property, ferulic acid was also found to have medical effects on age-related diseases such as neurodegenerative disorders, cardiovascular diseases, diabetes and cancer (*7*). However, the application of ferulic acids as antioxidants is limited due to the relatively low solubility in aprotic media. One of the realistic ways to improve its solubility is to modify the functional properties of the original phenolic compounds to obtain an amphiphilic molecule, which is normally achieved by conjugating the carboxylic acid with fatty alcohol or triglycerides through chemical or enzymatic lipophilization. The new amphiphilic molecule will still keep its original functional properties as an antioxidant because the hydroxyl groups associated with phenolic compounds may readily donate an electron or proton to intercept and convert free radicals to a more stable compound (*6*). On the other hand, the new amphiphilic molecule possesses surface activity because it contains both hydrophobic head (fatty acid moiety) and hydrophilic tail (phenolic moiety). It can be located in oil-water interfaces or water-oil interfaces, where the antioxidants can function in a different way in particular emulsion systems. Ideally, better protection/encapsulation through surface active antioxidants should be achieved. Research work is certainly going on to explore the concept as lipid oxidation most frequently occurs in the interface area of the emulsion systems (*6,8*). Initial results have shown certain positive aspects of the lipophilized antioxidants in emulsion systems (*9,10*).

There have been many studies on the synthesis of feruloyl ester using different approaches (*11–18*). The studies showed that, in general, Novozym® 435 from *Candida antarctica* is an efficient enzyme for such synthesis. Sabally *et al.* (*19,20*) reported the transesterification of dihydrocaffeic acid with two oils in organic solvents, but the reaction was long and the yields of structure lipids were low. Thus, a practically mature system is not readily available for the synthesis of conjugated products from ferulic acid and fish oil for the purpose of obtaining a product with high content of feruloyl fish oil.

Therefore, in the present study, a systematic approach was conducted aiming for practical synthesis of the transesterified products from ethyl ferulate and fish oil. The addition of glycerol to the system was first evaluated thoroughly for the purpose of increasing the reaction efficiency. The selected system was further optimized with the assistance of response surface methodology (RSM). The target was to maximize the formation of feruloyl fish oil.

Materials and Methods

Materials

Immobilized lipase from *Candida antarctica* (Novozym® 435) was obtained from Novo Nordisk (Bagsværd, Denmark). EF (ethyl-4-hydroxy-3-methoxycinnamate) was purchased from SYNTHON Chemicals GmbH & Co. KG, Wolfen, Germany (CAS No. 4046-02-0) with 98 % purity. Fish oil (cod liver oil) was a gift donated by Tine Ingredient (TINA BA, Oslo, Norway). Triolein with 90 % purity was purchased from Dr. Frische GmbH (Bremen, Germany). Organic solvents of analytical and HPLC grades were purchased from Sigma-Aldrich, Taufkirchen, Germany.

Transesterification reaction of EF with fish oil

Reactions were conducted in 50-mL jacketed reactor and protected from light. A thermostat water bath was used to maintain the reaction temperature at 60 °C. Fish oil (2 mmol) was mixed with 1 mmol of EF. For the purpose of investigation of the effect of glycerol on transesterification reaction, 0 to 5 mmol of glycerol was supplied. The reaction was started by adding 10 % of Novozym® 435 on the basis of substrate mass and stirred (300 rpm) by a magnetic plate. Ethanol produced during the reaction was removed by vacuum at 5 mbar with a vacuum pump. Samples of the reaction products were withdrawn at various intervals. A reaction mixture of 10 μL was dissolved in 90 μL of acetone, and then further diluted 10 times by methanol. Samples were centrifuged and then subjected to HPLC analysis. The same reaction conditions were also applied for the synthesis of feruloyl mono- and di-oleoyl-glycerols, where 2 mmol of triolein was mixed with 1 mmol of EF, and 1 mmol of glycerol was added to increase the transesterification rate. The reaction was continued for up to 5 days and the reaction mixture was subjected to HPLC-ESI-TOF-MS analysis using the same method as in normal HPLC analysis.

HPLC analysis

The transesterification reaction was monitored by HPLC analysis, using a procedure modified from Sun *et al.* (*21*) and Compton *et al.* (*11*). The analysis of reaction components was performed with an HPLC system purchased from Thermo Fisher Scientific (Copenhagen, Denmark), equipped with a RP C18 column (250×4.6 mm, 5 μm), an LC pumper, an autosampler, and a PDA detector. Data were analysed by ChromQuest v. 5.0 program (Thermo Fisher Scientific, Philadelphia PA, USA). Elution was conducted with solvent A (methanol) and solvent B (containing 0.75 % of acetic acid) at a flow rate of 1 mL/min. The elution gradient was initiated with 50 % solvent A, and increased to 100 % in 10 min, which was maintained for 10 min before reversing to 90 % in another 5 min. The gradient was brought back to the initial condition in 5 min. Injection volume of the sample was 10 μL and feruloyl acylglycerol species were detected under 325 nm of UV absorption. The total bioconversion of EF was calculated by comparing the ratio of EF among the total feruloyl acylglycerol species (including EF and FA) based on the peak area with its ratio before reaction. The formation of feruloyl acylglycerol species was determined by comparing the total peak area of each species with the total peak areas of all feruloyl acylglycerol species. Duplicate analyses were conducted and standard deviations were below 6 %.

HPLC-ESI-TOF-MS analysis

HPLC-ESI-TOF-MS analyses were performed with elution conditions identical to those described previously (*19–21*). Mass spectroscopy was conducted using an electrospray ionization (ESI) coupled to a quadrupole time-of-flight mass spectrometer (TOF-MS, Bruker micrO-TOF-Q, Bremen, Germany). Ionization was performed in the negative mode with a nitrogen flow of 8 L/min, nebulizer pressure of 0.8 bar and temperature of 190 °C. Scan range was from 50 to 1200 *m/z*.

Experimental designs

Response surface methodology (RSM) was used to study the effects of reaction time, temperature, enzyme load, and substrate molar ratio. A three-level and four--factor face-centered design (CCF) was employed in this study. The variables and their levels selected for the study were reaction time (1, 3 and 5 days), temperature (40, 55 and 70 °C), enzyme load (2, 11 and 20 % based on the total mass of substrates) and the molar ratio between fish oil and EF (1, 3 and 5). Table 1 shows the independent factors (x_i), levels, and experimental design. Modde v. 8.0 software (Umetrics AB, Umeå, Sweden) was used for assistance.

Statistical analysis

The mathematical relationship among variables with each response can be calculated by the quadratic polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \qquad /1/$$

where β_0, β_i, β_{ii} and β_{ij} are regression coefficients (β_0 is a constant term, β_i is a liner effect term, β_{ii} is a squared effect term, and β_{ij} is an interaction effect term) and Y is the predicted response value. The models were fitted with multiple regressions with backward elimination.

Table 1. Experimental designs and the corresponding observed and predicted responses

Trial	Variable level				Response value					
	Temp.	Enzyme load	Fish oil/EF molar ratio	Reaction time	Bioconversion of EF/%		Formation of feruloyl-*sn*-glycerol/%		Formation of feruloyl fish oil/%	
	°C	%		day	observed	predicted	observed	predicted	observed	predicted
1	40 (–1)	2 (–1)	1 (–1)	1 (–1)	24.61	21.03	15.17	16.19	9.22	4.73
2	70 (1)	2 (–1)	1 (–1)	1 (–1)	47.61	47.00	25.67	26.03	21.94	20.84
3*	40 (–1)	20 (1)	1 (–1)	1 (–1)	66.38	80.08	44.97	39.95	21.40	40.11
4	70 (1)	20 (1)	1 (–1)	1 (–1)	74.53	74.05	38.70	38.39	35.80	35.69
5*	40 (–1)	2 (–1)	5 (1)	1 (–1)	8.62	–41.46	0.31	–14.33	8.30	–26.83
6	70 (1)	2 (–1)	5 (1)	1 (–1)	41.87	41.14	7.10	6.66	34.70	34.49
7	40 (–1)	20 (1)	5 (1)	1 (–1)	40.95	41.02	1.70	1.58	39.24	39.35
8	70 (1)	20 (1)	5 (1)	1 (–1)	91.62	91.62	11.17	11.17	80.15	80.15
9*	40 (–1)	2 (–1)	1 (–1)	5 (1)	57.30	61.16	46.91	30.23	10.25	30.77
10	70 (1)	2 (–1)	1 (–1)	5 (1)	51.59	51.23	23.15	23.34	28.37	27.91
11	40 (–1)	20 (1)	1 (–1)	5 (1)	87.10	87.54	46.11	46.62	40.96	40.82
12*	70 (1)	20 (1)	1 (–1)	5 (1)	93.16	45.61	46.04	28.34	46.97	17.43
13	40 (–1)	2 (–1)	5 (1)	5 (1)	37.52	37.71	7.70	8.07	29.75	29.52
14	70 (1)	2 (–1)	5 (1)	5 (1)	84.41	84.41	12.33	12.33	71.88	71.88
15	40 (–1)	20 (1)	5 (1)	5 (1)	86.92	87.53	16.97	16.61	69.27	70.37
16	70 (1)	20 (1)	5 (1)	5 (1)	98.93	102.22	10.43	9.48	88.06	92.21
17	40 (–1)	11 (0)	3 (0)	3 (0)	74.18	76.44	21.00	19.59	53.01	56.66
18	70 (1)	11 (0)	3 (0)	3 (0)	97.89	96.78	19.79	20.94	77.90	75.64
19	55 (0)	2 (–1)	3 (0)	3 (0)	47.88	52.96	13.24	11.74	34.52	41.01
20	55 (0)	20 (1)	3 (0)	3 (0)	95.33	91.40	20.95	22.19	73.97	68.87
21	55 (0)	11 (0)	1 (–1)	3 (0)	82.50	87.08	42.66	40.89	39.72	46.03
22	55 (0)	11 (0)	5 (1)	3 (0)	87.58	84.15	14.69	16.20	72.56	67.64
23	55 (0)	11 (0)	3 (0)	1 (–1)	59.87	65.19	16.59	16.08	43.23	49.03
24	55 (0)	11 (0)	3 (0)	5 (1)	94.73	90.56	22.00	22.25	72.49	68.08
25	55 (0)	11 (0)	3 (0)	3 (0)	86.40	88.43	21.26	22.05	65.04	66.13
26	55 (0)	11 (0)	3 (0)	3 (0)	90.70	88.43	22.19	22.05	68.05	66.13
27	55 (0)	11 (0)	3 (0)	3 (0)	91.64	88.43	21.91	22.05	69.47	66.13

0.184 g (2 mmol) of glycerol was supplied to each trial
*runs were eliminated during model fitting

Results and Discussion

Identification of feruloyl acylglycerol species

Transesterification of EF with triacylglycerols results in a mixture of feruloyl acylglycerol species due to the multiple fatty acid composition of fish oil (Fig. 1). In order to simplify the analysis, transesterification of EF with triolein was first investigated following the method used by Compton *et al.* (*11*). Using a similar analysis method, reaction products of transesterification of EF with triolein were qualified by HPLC and LC-MS. Compared to the HPLC method used by Compton *et al.* (*11*), in which a tertiary solvent system was applied to achieve the separation of the different feruloyl acylglycerol species (Fig. 1), we developed a binary solvent system using methanol/water (containing 0.75 % acetic acid) gradient based on a modified method developed by Sun *et al.* (*21*), and similar results were achieved (Fig. 2). The chromatogram in Fig. 2a was obtained for the reaction products of transesterification of EF with triolein, which was carried out by mixing the substrate in the ratio of 2:1:1 of triolein/EF/glycerol. The elution sequence is listed in Table 2 and each peak was identified by HPLC-ESI-TOF-MS according to the corresponding mass. The elution order in the chromatography is in agreement with the previous study (*11*).

According to the present sequence, the first eluted peak (Fig. 2a) was identified as monoferuloyl-*sn*-glycerol (F1-MAG), and the next peak as ferulic acid (FA). The third and fourth peaks were found to be diferuloyl-*sn*-glycerol (F2-DAG) and EF, respectively. Monoferuloyl-monoolein (F1-DAG) is an ester product which contains one feruloyl moiety and one oleic acid on the backbone of glycerol, and the eluted peak of this product was found to be adjacent to the peak of diferuloyl-monoolein (F2-TAG), which contains two feruloyl moieties and one oleic acid on the backbone of glycerol. The last eluted peak was identified as monoferuloyl-diolein (F1-TAG).

The sequence in Fig. 2a was then used to identify the ester products of the transesterification of EF with fish oil. The transesterification of fish oil/EF/glycerol resulted in a series of complex mixtures (Fig. 2b) due to multiple fatty acid composition of fish oil, which makes the classification of each peak in Fig. 2b difficult and ambiguous. However, as suggested by Compton *et al.* (*11*), we assumed that feruoyl acyglycerols with similar structure to feruloyl oleylglycerol would be eluted at similar times. They were classified into different groups based on the number of feruloyl moieties and fatty acids on the backbone of glycerol following the classification in Fig. 2a.

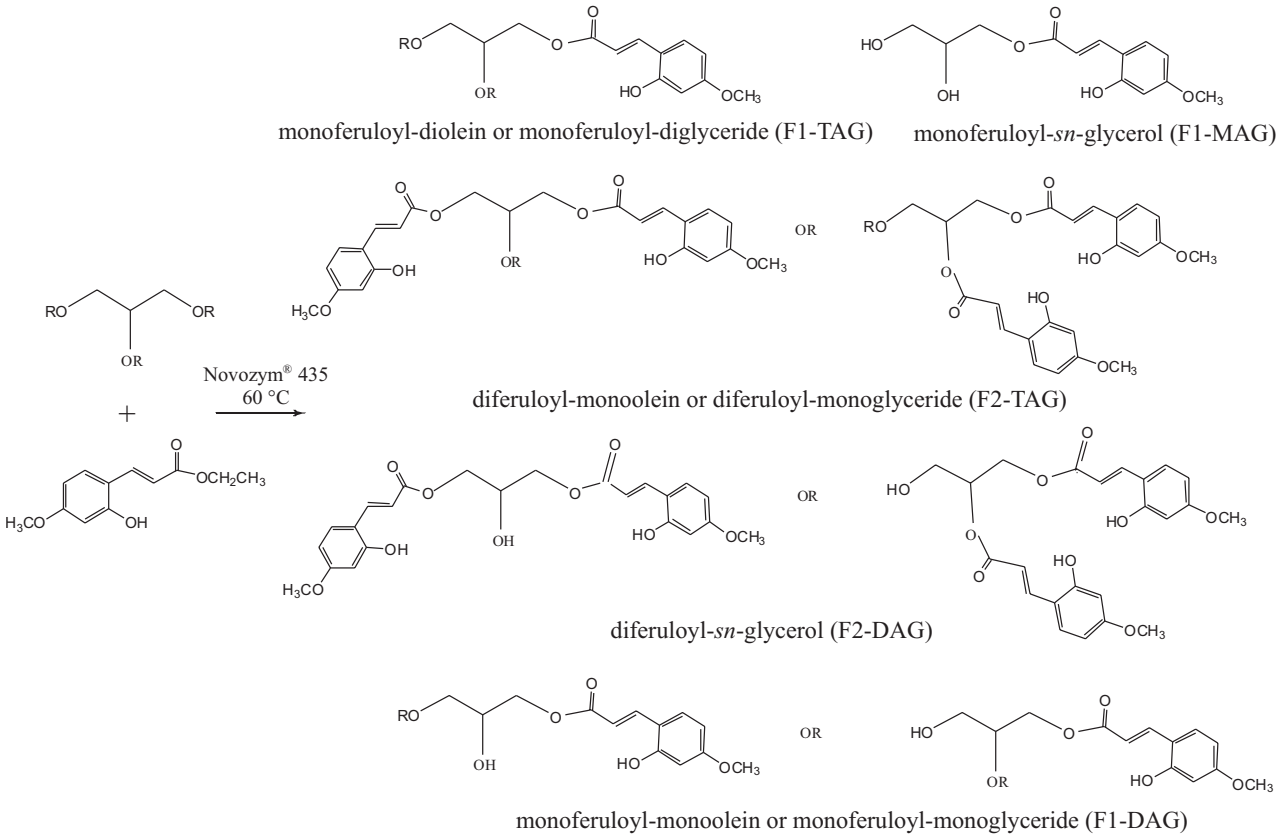

Fig. 1. Scheme of a possible product of transesterification of EF with triolein or fish oil. R=oleic acid or fatty acids from fish oil. Feruloyl acylglycerol species were defined according to the amount of feruloyl moieties or fatty acids on the backbone of glycerol: F1-MAG contains one feruloyl moiety on the backbone of glycerol; F2-DAG contains two feruloyl moieties on the backbone of glycerol; F1-DAG contains one feruloyl moiety and one oleic acid or one fatty acid from fish oil on the backbone of glycerol; F2-TAG contains two feruloyl moieties and one oleic acid or one fatty acid from fish oil on the backbone of glycerol; F1-TAG contains one feruloyl moiety and two oleic acids or two fatty acids from fish oil on the backbone of glycerol. FA and EF are abbreviations for ferulic acid and ethyl ferulate, respectively

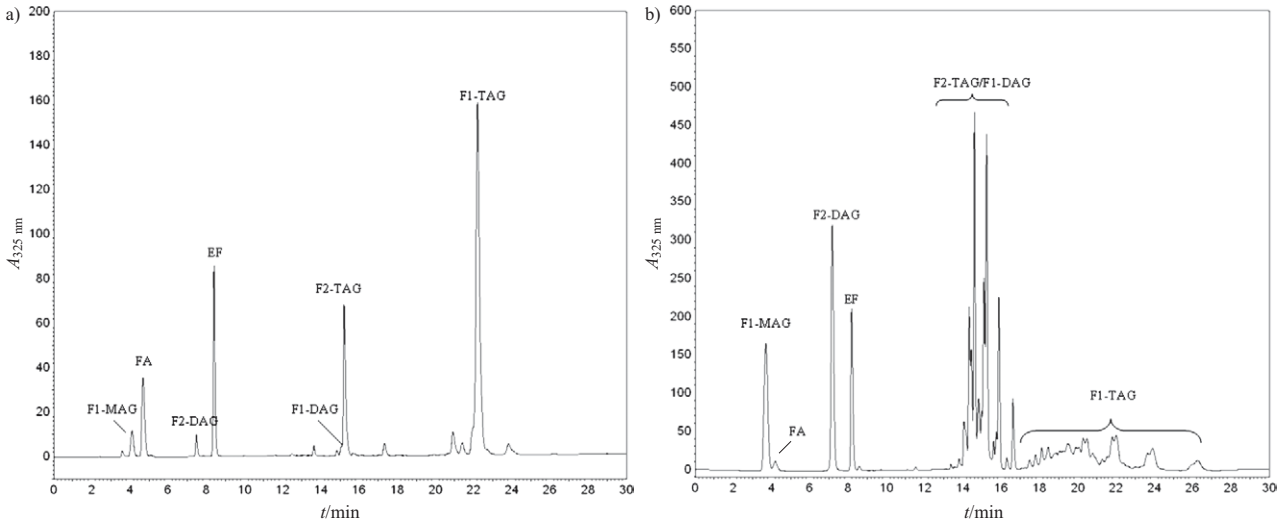

Fig. 2. Analytical reversed-phase HPLC chromatograms of the products of transesterification of: a) EF/triolein/glycerol or b) EF/fish oil/glycerol (2:1:1). The reactions were catalyzed by 10 % of Novozym® 435 (on the basis of substrate) at 60 °C and stirred at 300 rpm for 5 days. See Fig. 1 for acronym definitions

Table 2. Feruloyl acylglycerol species from transesterification of triolein and EF qualified by HPLC-ESI-TOF-MS

Species	R_t/min	M_r/kDa	Major ion (*m/z*)
F1-MAG	4.2	268	267 $[M-H]^-$
FA	4.5	194	193 $[M-H]^-$
F2-DAG	7.6	444.4	443 $[M-H]^-$
EF	8.3	222	221 $[M-H]^-$
F1-DAG	15.2	532	531 $[M-H]^-$
F2-TAG	15.8	708	707 $[M-H]^-$
F1-TAG	22.5	797.2	796 $[M-H]^-$

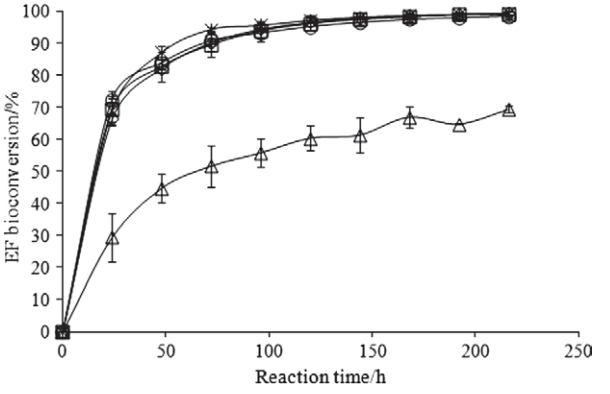

Fig. 3. Effect of glycerol on the bioconversion of EF. Reactions were catalyzed by 10 % of Novozym® 435 (on the basis of substrate) at 60 °C, stirred at 300 rpm and under 5 mbar for 216 h. The molar ratio of fish oil/EF/glycerol was: 2:1:0 (Δ), 2:1:1 (◊), 2:1:2 (□), 2:1:3 (○), 2:1:5 (∗)

Influence of glycerol on the formation of feruloyl acylglycerol species

It has been reported that glycerol can increase the bioconversion rate of EF and the formation of feruloyl acylglycerol species (*11*,*12*,*22*). However, too much glycerol has a negative effect on the transesterification reaction. The reason could be that glycerol extracts the water layer from the enzyme, and the water layer outside the enzyme is essential to maintain its activity (*23*). Therefore, we are interested in studying how glycerol will affect the transesterification of EF with oil. For this purpose, 0 to 5 mmol of glycerol was added into the reaction mixture, which consisted of 2 mmol of fish oil and 1 mmol of EF.

The results in Fig. 3 show that the effect of glycerol on the bioconversion of EF was significant. When no glycerol was present in the reaction, the maximum conversion of EF was only 67 %. On the other hand, when a small amount of glycerol (1 mmol) was supplied, the bioconversion of EF reached above 80 % within 50 h of reaction and finally almost 99 % when the reaction was terminated after 216 h (8 days). However, continuous increase of glycerol amount in the reaction did not seem to affect the increase of the conversion rate.

Considering the formation of different feruloyl acylglycerol species (Fig. 4), glycerol had a positive effect in general, except on the production of F1-TAG (Fig. 4d), where F1-TAG content decreased from 42 to 9 %, following the increase of glycerol from 0 to 5 mmol in the reaction. When there was no glycerol supplied, the highest production of F1-MAG (Fig. 4a), F2-DAG (Fig. 4b) and F2-TAG/F1-DAG (Fig. 4c) was only 6, 2 and 23 %, respectively, which was much lower than when 1 mmol of glycerol was added. In Fig. 4a, it is also shown that the production of F1-MAG increased following the increase of glycerol. However, too much glycerol seems to have adverse effect on the formation of F2-DAG and F2-TAG/F1-DAG (Figs. 4b and 4c), especially when glycerol amount was increased to 5 mmol.

In general, the yield of feruloyl acylglycerol species can be classified into two groups depending on whether they contain fatty acids from fish oil on the backbone of glycerol or not. The first group is the feruloyl-*sn*-glycerol (F1-MAG and F2-DAG), which does not contain fatty acids of fish oil, while the second group is the feruloyl fish oil (F1-TAG, F1-DAG and F2-TAG), which contains

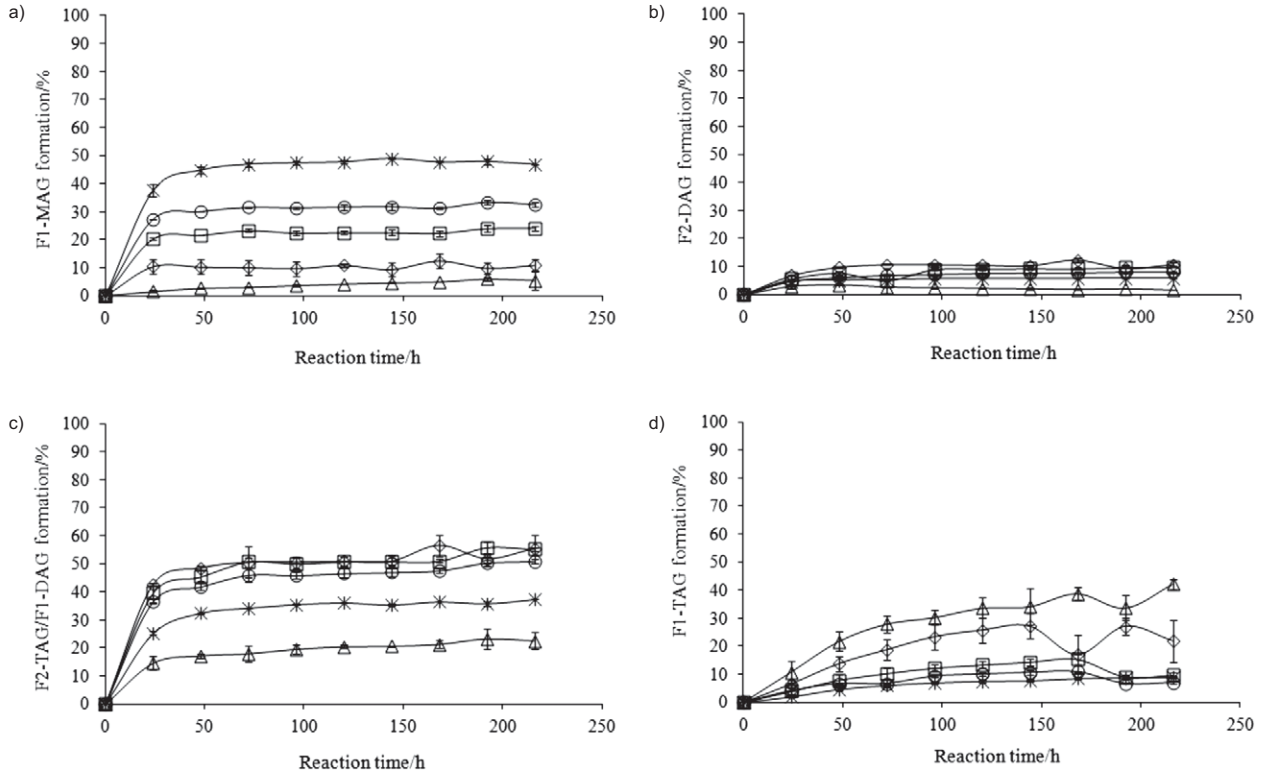

Fig. 4. Effect of glycerol on the formation of feruloyl acylglycerol species. Reaction conditions and corresponding samples were the same as in Fig. 3

one or two fatty acids from fish oil on the backbone of glycerol. The first group is basically the result of the transesterification of EF with glycerol and can therefore be treated as a by-product of the reaction. On the other hand, the second group contains the desired products because they have hydrophobic heads (fatty acid moieties) and hydrophilic tails (feruloyl moieties). The production of the two groups of products is summarized in Table 3. There is a linear correlation between the first group (feruloyl-*sn*-glycerol) and the amount of glycerol added. The total production of this group increased from 8 to 52 %, following the increase of glycerol amount in the reaction from 0 to 5 mmol. On the other hand, the highest yield of feruloyl fish oil (77 %) was found when the molar ratio of fish oil/EF/glycerol was 2:1:1. However, the yield of feruloyl fish oil decreased to about 65 % when the molar ratio of substrate was changed to 2:1:2, which was almost equal to the yield when there was no glycerol supplied to the reaction, and the yield of this product decreased further if more glycerol was added.

RSM model fitting

Response surface methodology (RSM) is a collection of statistical and numerical optimization techniques used to optimize process and product designs (*24*). This methodology is based on the fitness of a polynomial equation to the experimental data and the best optimization of the levels of variables as well as the best system performance are obtained when a response or set of responses of interest are influenced by several variables (*25*). In this study, a four-factor and three-level central composite face-centred design (CCF) was chosen to investigate the effect of four variables (temperature, reaction time, molar ratio of fish oil/EF, and enzyme load). The whole experimental design is shown in Table 1, which includes the experimental results and predicted values. For each factor, the conventional level was set at zero as a coded level and the quadratic model (Eq. 1) was chosen to fit the experimental data. In addition, the significant level to each term in the selected model was set as 0.05.

Table 3. Formation of feruloyl-*sn*-glycerol and feruloyl fish oil in different substrate molar ratios

Fish oil/EF/glycerol molar ratio	Feruloyl-*sn*-glycerol/%			Feruloyl fish oil/%		
	F1-MAG	F2-DAG	Total	F1-DAG/F2-TAG	F1-TAG	Total
2:1:0	(6.00± 0.61)*	(2.00±0.03)*	(8.00±0.64)*	(22.52±2.97)*	(42.16±1.75)*	(64.68±4.72)*
2:1:1	(10.81±2.14)*	(10.64±0.66)*	(21.45±2.79)*	(55.24±5.11)*	(21.89±7.50)*	(77.13±2.38)*
2:1:2	(24.09±0.78)*	(9.70±0.01)*	(33.79±0.76)*	(55.18±0.71)*	(9.63±0.88)*	(64.81±1.59)*
2:1:3	(32.54±0.72)*	(8.11±0.18)*	(40.65±0.54)*	(50.30±0.68)*	(7.07±0.45)*	(57.37±1.12)*
2:1:5	(46.82±0.02)*	(5.75±0.04)*	(52.07±0.06)*	(37.39±0.10)*	(8.86±0.37)*	(46.25±0.47)*

*relative standard deviation was calculated from the duplicate results from different experiments

Among the different trials of the experimental designs (Table 1), the greatest bioconversion of EF and the formation of feruloyl fish oil were achieved in experiment 11, while the highest content of feruloyl-*sn*-glycerol was found in experiment 9. The effect of the four variables as well as their interaction was evaluated based on the coefficients of the full model by regression analysis and tested for their significance on the basis of the p-value. The predicted coefficient values of each variable that has a significant effect on bioconversion of EF are presented in Table 4. The independent variables (x_1, x_2 and x_4), interactions ($x_1 \cdot x_2$, $x_1 \cdot x_3$, $x_1 \cdot x_4$, $x_2 \cdot x_3$, $x_2 \cdot x_4$ and $x_3 \cdot x_4$), and quadratic terms (x_2^2 and x_4^2) are the most significant factors affecting the bioconversion of EF (with $p<0.05$). The quadratic terms x_1^2 and x_3^2 were excluded from the original model by backward elimination since they did not show significant effect. Variable x_3 was kept in the model despite its insignificant effect ($p=0.32$) because it showed significant interaction with other independent variables.

Similarly, independent variables (x_2, x_3 and x_4), quadratic terms (x_2^2, x_3^2 and x_4^2), and interaction variables ($x_1 \cdot x_2$, $x_1 \cdot x_3$, $x_1 \cdot x_4$, $x_2 \cdot x_3$, $x_2 \cdot x_4$ and $x_3 \cdot x_4$) were the significant variables for the formation of F1-MAG and F2-DAG. The independent variables (x_1, x_2, x_3 and x_4), quadratic terms (x_2^2 and x_3^2), and interaction variables ($x_1 \cdot x_3$, $x_2 \cdot x_3$ and $x_3 \cdot x_4$) were the significant variables for the formation of F1-TAG and F2-TAG. Other insignificant variables were excluded from the prediction models except those that have significant interaction effect with other variables.

The three models were further analyzed by ANOVA. The analysis shows that the coefficients of determination (R^2) of the three models for the bioconversion of EF and the formation of feruloyl-*sn*-glycerol and feruloyl fish oil were 0.96, 0.983, and 0.933, respectively, which indicates that these models were suitable for representation of the real relationships among the selected reaction parameters. ANOVA analysis also shows that the probabilities for regression of the model were significant ($p<0.0001$) and the lack of fit was insignificant ($p>0.05$). This means that these models were statistically good and they had no lack of fit at 95 % level of significance. Therefore, these models were successfully established.

Reaction optimization

Figs. 5–7 are contour plots which predict the mutual effect of temperature, enzyme load, fish oil/EF molar ratio and reaction time on the bioconversion of EF (Fig. 5), the formation of feruloyl-*sn*-glycerol (Fig. 6), and feruloyl fish oil (Fig. 7). Like in the previous studies, 2 mmol of glycerol were added to increase the conversion rate.

Fig. 5a represents the effects of temperature and enzyme load, and their mutual interaction on the reaction conversion in three days and substrate molar ratio (fish oil/EF) of 3. Both temperature and enzyme load had positive effect on the bioconversion of EF. The optimal temperature was found to be above 50 °C, when the bioconversion of EF was able to reach above 90 % if the enzyme load was increased to 16 %. The same high conversion could also be achieved at lower enzyme load when temperature was increased further. The positive effect of temperature on the bioconversion of EF is in agreement with a previous finding (*26*). At the same time, a slight decrease of conversion occurred if the enzyme load is increased above 17 % at 50 °C.

Fig. 5b shows mutual relationships between substrate molar ratio and temperature when the enzyme load was 11 % and reaction time was three days. The conversion rate decreased as the substrate molar ratio increased at low temperature (below 55 °C). This situation was not the same when the reaction temperature was higher, when the bioconversion of EF could reach above 90 % if

Table 4. Regression coefficients and significance ($p<0.05$) after backward elimination for bioconversion of EF and formation of feruloyl-*sn*-glycerol and feruloyl fish oil

Variable	Bioconversion of EF		Formation of feruloyl glycerol		Formation of feruloyl fish oil	
	coefficient	p-value	coefficient	p-value	coefficient	p-value
constant	88.43	3.05909E-007	22.05	1.29E-10	66.13	1.21E-09
x_1	10.17	0.000188904	0.68	0.152135	9.49	0.000522
x_2	19.22	0.000236505	5.23	1.87E-06	13.93	3.68E-05
x_3	–1.47	0.000116675	–12.34	2.24E-09	10.80	0.000219
x_4	12.68	2.11735E-005	3.09	9.11E-05	9.52	0.000509
$x_1 \cdot x_1$	–1.82	0.0057857	–1.78	0.082846	0.02	0.995761
$x_2 \cdot x_2$	–16.24	0.0154269	–5.08	0.000484	–11.19	0.014108
$x_3 \cdot x_3$	–2.81	0.000145708	6.50	9.13E-05	–9.30	0.031798
$x_4 \cdot x_4$	–10.55	0.000282499	–2.88	0.012556	–7.58	0.067250
$x_1 \cdot x_2$	–8.00	0.00073279	–2.85	0.001593	–5.13	0.066919
$x_1 \cdot x_3$	14.16	0.00687478	2.79	0.003989	11.30	0.003558
$x_1 \cdot x_4$	–8.98	0.101996	–4.18	0.000129	–4.74	0.085994
$x_2 \cdot x_3$	5.86	0.0225241	–1.96	0.012204	7.70	0.013007
$x_2 \cdot x_4$	–8.17	0.759813	–1.84	0.029771	–6.33	0.051853
$x_3 \cdot x_4$	9.76	0.0485351	2.09	0.008939	7.58	0.014047

x_1=temperature, x_2=enzyme load, x_3=substrate molar ratio, x_4=reaction time

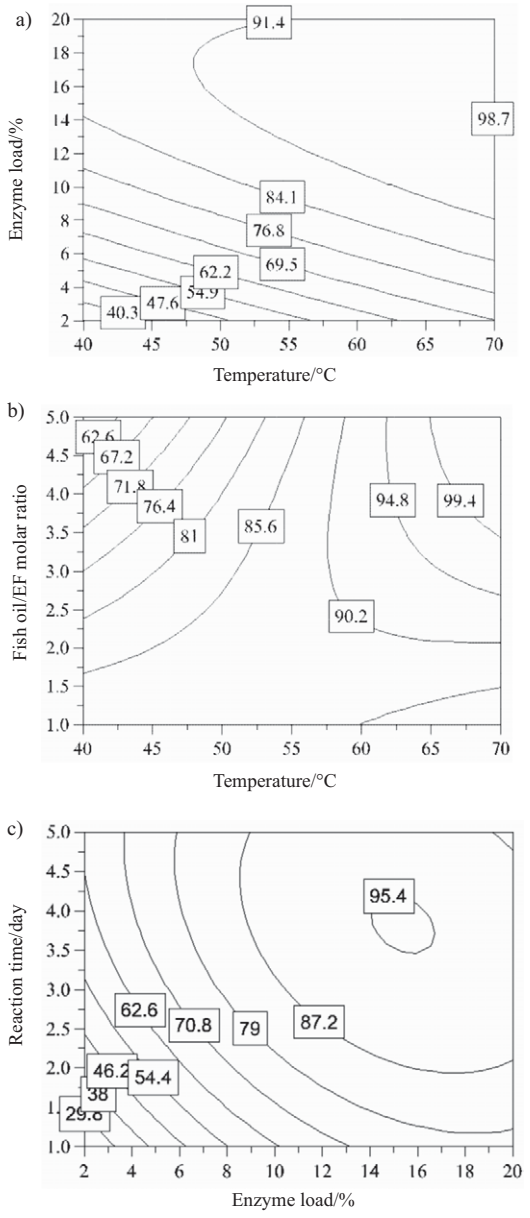

Fig. 5. Contour plots of prediction effect of any factor on the bioconversion of EF. Conditions: a) substrate molar ratio of fish oil/EF=3, reaction time=3 days; b) enzyme load=11 %, reaction time=3 days; c) enzyme load=11 %, substrate molar ratio of fish oil/EF=3

the reaction temperature was above 60 °C. Such phenomenon is probably due to the viscosity of fish oil, where higher substrate molar ratio inhibits the contact of the substrate with the enzyme, but higher temperature can reduce the viscosity of fish oil and, consequently, lead to the increase of the bioconversion of EF.

In Fig. 5c, the bioconversion of EF increases following the reaction time on the first four days, but then there is a slight decrease after five days. The effect of reaction time on the bioconversion of EF with fish oil in this study is in agreement with the previous reports (*9,27*), where it was speculated that this could be due to the formation of hydrolysis products during longer reaction time.

The effect of factors on the formation of feruloyl-*sn*--glycerol can be evaluated with a similar approach. Fig. 6a describes the mutual effects between the reaction time and temperature, where the molar ratio of fish oil/EF was 3 and enzyme load was 11 %. Both reaction time and temperature had positive effect on the formation of feruloyl-*sn*-glycerol, but when the temperature was increased above 60 °C, there was a slight decrease. Therefore, low temperature and shorter reaction time are preferred if we expect to reduce the formation of feruloyl--*sn*-glycerol.

Fig. 6b presents the mutual effect of substrate molar ratio of fish oil/EF and enzyme load, where the reaction temperature was 55 °C and reaction time was three days. The formation of feruloyl-*sn*-glycerol was able to reach 39.6 % when the enzyme load was above 10 %. On the other hand, the formation of feruloyl-*sn*-glycerol reduced as the substrate molar ratio of fish oil/EF increased. Therefore, feruloyl-*sn*-glycerol can be minimized through the increase of substrate molar ratio and decrease of enzyme load.

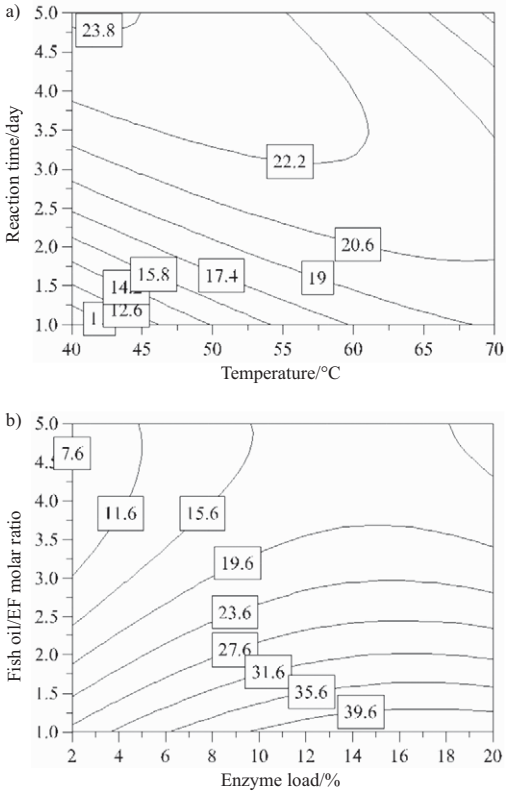

Fig. 6. Contour plots of prediction effect of any factor on the formation of feruloyl-*sn*-glycerol. Conditions: a) enzyme load= 11 %, substrate molar ratio of fish oil/EF=3; b) temperature=55 °C, reaction time=3 days

Feruloyl fish oil is the key product of the reaction. The evaluation of the factor effects, as well as their interactions, is presented in Fig. 7. Fig. 7a shows mutual effects of temperature and molar ratio on the formation of feruloyl fish oil, where enzyme load was 11 % and reaction time was three days. Temperature had no effect on the formation of the product when the substrate molar ratio of fish oil/EF was below 2. However, when

the substrate molar ratio was further increased, temperature had positive effect, following the increase of substrate molar ratio.

Fig. 7b shows the interactive effects of substrate molar ratio of fish oil/EF and enzyme load on the formation of feruloyl fish oil, where temperature was 55 °C and reaction time was three days. There was no obvious effect of substrate molar ratio of fish oil/EF on the formation of feruloyl fish oil when enzyme load was lower than 9 %. However, the formation could be increased following the increase of enzyme load beyond 14 % and substrate molar ratio of fish oil/EF beyond 3.5.

Fig. 7c shows the interactive effects of substrate molar ratio of fish oil/EF and reaction time on the formation of feruloyl fish oil at 55 °C and enzyme load of 11 %. There was no obvious effect on the formation of the products either through the increase of substrate molar ratio of fish oil/EF or reaction time. However, the amount of feruloyl fish oil could be increased through the increase of the substrate molar ratio of fish oil/EF and the prolongation of reaction time.

Optimal conditions for a lower formation of by-product and higher bioconversion of EF as well as the formation of feruloyl fish oil were predicted within the experimental design ranges (Table 5). Two sets of predicted conditions were generated. The predicted values from the models were found to be reasonably in agreement with the experimental values (observed), which confirmed the validity and adequacy of the predicted models.

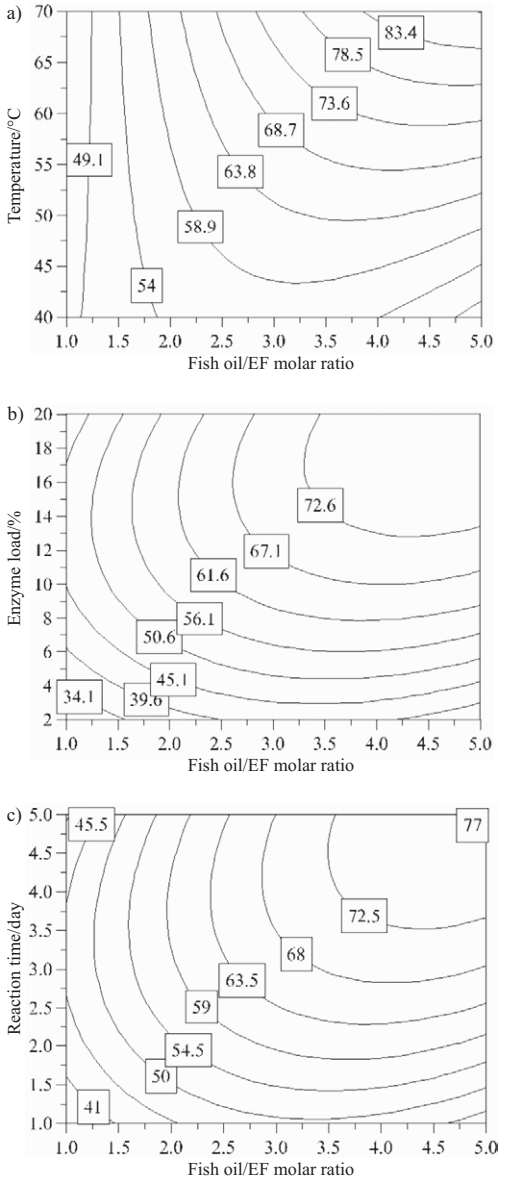

Fig. 7. Contour plots of prediction effect of any factor on the formation of feruloyl fish oil. Conditions: a) enzyme load=11 %, reaction time=3 days; b) temperature=55 °C, reaction time=3 days; c) temperature=55 °C, enzyme load=11 %

Table 5. Optimum conditions generated and verification of the models

Condition	Model	Run 1	Run 2
temperature/°C		70	70
enzyme load/%		4.3	14.5
fish oil/EF molar ratio		4.7	2.7
reaction time/day		5	4.9
bioconversion of EF/%	predicted	92.3	85.6
	observed±SD*	92.4±0.0	99.7±0.1
formation of by-products/%	predicted	13.5	18
	observed±SD*	11.4±0.6	21.9±1.4
formation of feruloyl fish oil/%	predicted	78.6	67.6
	observed±SD*	80.4±0.4	76.8±2.2

*relative standard deviation was calculated from duplicated results from different experiments

Under the predicted conditions, the bioconversion of EF and selectivity towards desired product were greatly improved, except for the reaction time, which still needed five days and could not decrease further. Nevertheless, these reaction conditions are still preferable compared to previous studies (*11–17*,*19–23*,*26*,*27*), where most of the reactions took place for more than six to ten days with lower yield of phenolic lipids than in this study.

Conclusions

The effect of glycerol on the bioconversion of EF and the formation of feruloyl-*sn*-glycerol and feruloyl fish oil was investigated. The results show that the best molar ratio of fish oil/EF/glycerol was 2:1:1. Under these conditions, the bioconversion of EF was almost complete and feruloyl fish oil reached 77 %, while the formation of by-product (feruloyl-*sn*-glycerol) was only 21.4 %. Three quadratic models were generated using RSM for the study of the interactive effect of temperature, enzyme load, substrate molar ratio of fish oil/EF and reaction time. Three responses were selected for the bioconversion of EF, as well as the formation of feruloyl-*sn*-glycerol and feruloyl fish oil. Two sets of optimal conditions were eventually established. According to the optimum conditions, the bioconversion of EF and formation of feruloyl fish oil were able to exceed 92 and 80 % respectively,

while the by-product (feruloyl-*sn*-glycerol) production was less than 12 %.

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