

ENRICHMENT OF POLYUNSATURATED FATTY ACIDS FROM CATFISH OIL WITH IMMOBILIZED *RHIZOPUS ORYZEA* LIPASE

LY THI MINH HIEN

Ho Chi Minh City Open University, Vietnam – hien.ltm@ou.edu.vn

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ABSTRACT

Nowadays, *Pangasius hypophthalmus* fillet is one of the main marine products in Vietnamese Mekong River Delta with over three hundred thousand tons per year. One of the byproducts of this industry is fat, about one hundred thousand tons per year. That byproduct has been only used in animal foodstuff industry after oil extracting or it becomes waste. In order to increase the quality of *Pangasius hypophthalmus* oil, we have enriched PUFA in the oil by hydrolysis with immobilized *Rhizopus oryzae* lipase. Lipase has been immobilized on celite by absorption method. Immobilization experiments have been designed in a two-level full factorial model with three factors. The fish oil has been hydrolyzed with immobilized lipase to enrich PUFA with optimized parameters found out: the ratio of enzyme to support of 1:10 g/g, the ratio of phosphate buffer volume to support weight of 30.55 ml/g, incubation time of 5.15 hours. The result has showed that 60-minute hydrolysis is the most appropriate. Additionally, the oil iodine value has increased from 71.4 to 97.4, and DHA concentration has risen from 1156mg/kg to 1916mg/kg.

Keywords: Celite immobilized enzyme; Lipase; *Pangasius hypophthalmus*; PUFA; *Rhizopus oryzae*.

1. Introduction

In recent years, *Pangasius hypophthalmus* fillet has become the famous marine product of Vietnam. In Mekong River Delta, the growing up of catfish fillet output has increased from 250 thousand to million tons. The byproducts of this industry namely head, tail, skin, bone and especially, fish oil have become waste. Every year, in this area, there is at least 30 thousand tons of fish oil releasing to the environment because the reuse of this byproduct in other industries is not efficient.

Catfish crude oil contains about 12.72% of PUFAs (D.N. Hoang, 1997) being much more than animal fat or coconut oil.

Polyunsaturated fatty acids (PUFAs) are essential for human beings, which we often could not synthesize. PUFAs like DHA or EPA would be enriched by many methods such as: isolating with supercritical solvent, fractionating with urea complexation, selective hydrolysis with lipase,...

Among these, lipase method is the safest for environment and offers a product with the

best quality.

Shimada et al. (1997) hydrolyzed tuna oil with microorganism lipases. *Rhizopus delemar* lipase acted moderately on the DHA ester, and the hydrolysis extent was only 25.7%. DHA could not be recovered efficiently in the FFA fraction by hydrolysis with *Candida* lipase, so the percentage of DHA in this fraction was 36.8%. The results showed that all *Pseudomonas* lipases acted strongly on the ester bond of DHA, and that the hydrolysis extents were high from 59.3 to 71.9% DHA.

Oil hydrolysis with lipase is a complicated process. Triglycerides (oil) do not dissolve in the water phase, so the reaction has to take place at the interface of the water and lipid. Therefore, in order to increase the interfacial area of the oil-in-water emulsion, emulsifiers are added. Additionally, Murty et al., 2002 used immobilized enzyme instead of free enzyme combined with emulsion makes the reaction more efficient.

Lipase could be immobilized on support in some ways: absorption, cross-linking, binding,

entrapment. The research of Soares et al., (1999) showed that *Candida rugosa* lipase was immobilized by covalent binding on controlled pore silica (CPS) with glutaraldehyde. Protein retention and immobilization efficiency was in turn 60% and 18%.

Rakshit et al. (2000) enriched PUFA from tuna oil by *Pseudomonas fluorescens* immobilized lipase with 3 different methods: hydrolysis, esterification of fish oil FFA with alcohols (ethanol, propanol, octanol, glycerol) and transesterification between ethanol and fish oil. In the hydrolysis process, the time was changed from 2.25 to 48 hours. Generally, the weight percentage of DHA and EPA increased in FFA fraction and decreased in glyceride fraction throughout the hydrolysis process. After 48 hours, 80% DHA was transferred to FFA fraction.

In 2005, Linder hydrolyzed salmon oil with *Aspergillus oryzae* lipase. Lipolysis of the oil was carried out with Novozym SP398 to obtain a mixture of free fatty acids and acylglycerols (24 hours, 45% hydrolysis). The mixture was filtered on a hydrophobic membrane to discriminate between high melting saturated fatty acids and low melting acylglycerols. The sum of polyunsaturated fatty acids increased from 41.6% in the crude oil to 46.5% in the permeate.

Fernández-Lorente et al. (2011) immobilized lipase from *Yarrowia lipolytica* and *Pseudomonas fluorescens* on hydrophobic porous supports in order to enrich DHA in sardine oil. The most effective overactivation obtained in the work ranged from 6- to 20-fold.

In another report of Fernández-Lorente et al. (2011), absorption immobilized lipases from *Candida antarctica*, *Rhizomucor miehei* and *Thermomyces lanuginose* were researched on the capability of increasing the concentration of PUFA in sardine oil.

2. Material and method

Material

Pangasius hypophthalmus oil was bought

from Thien Phat Company in Tien Giang province, Vietnam. Fatty acid of the crude fish oil was neutralized by 4% sodium hydroxide solution to get the acid value of 3.75mgKOH/g oil. The iodine value was 71.4 g iodine/100g oil, DHA content was 1156mg/kg oil.

Rhizopus oryzae lipase from Sigma-Aldrich ($\text{pH}_{\text{opt}} = 7.0$; $\text{T}_{\text{opt}} = 37^\circ\text{C}$, 1000U/g enzyme). Celite 545 from Sigma-Aldrich was used as the support for enzyme immobilization.

Immobilization of lipases on celite (Kumar A., 2010)

Celite was washed three times with Tris buffer 0.05 M pH 7.0 to remove soluble impurities. The celite-545 (1g), preequilibrated in a volume of Tris buffer (0.05 M, pH 7.0), was incubated with commercial lipase (*Rhizopus oryzae*). The celite-bound lipase (1g) was tested the activity and then used to hydrolysis catfish oil.

Determination of lipase activity (Soares et al., 1999)

The activities of free and immobilized lipase were tested with the soya oil emulsion as the substrate. The substrate was prepared by mixing 50 mL of the soya oil with 50 mL of emulsification reagent. The reaction mixture consisting of 5 mL of the emulsion, 2 mL of 100 mM sodium phosphate buffer, pH 7.0, and either free (1 mL) or immobilized (500 mg) lipase was incubated for 30 min at 37°C . The reaction was stopped by addition of 10 mL of acetone-ethanol solution (1:1). The liberated fatty acid was titrated with 25 mM potassium hydroxide solution in the presence of phenolphthalein as indicator.

One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free fatty acid/min under the assay conditions.

Two-level full factorial model for optimization of lipase immobilization (Yemata T.A., 2014)

The levels of each factor were chosen so as to cover a range of values of practical interest.

Table 1

Experimental design layout

Order	Factor 1 (Z1)	Factor 2 (Z2)	Factor 3 (Z3)
1	+	+	+
2	+	+	-
3	+	-	-
4	+	-	+
5	-	-	-
6	-	+	-
7	-	-	+
8	-	+	+

To minimize the effect of variability in the response due to extraneous factors, the experiments were performed in a random order.

The analysis of results was carried out using the statistical software Design Expert 7 (DX7).

After run data from Table 2 with DX7, the polynomial model was derived

$$y = \alpha_0 + \sum_{i=1}^3 \alpha_i x_i + \sum_{i=1}^3 \sum_{j=i+1}^3 \alpha_{ij} x_i x_j + \alpha_{123} x_1 x_2 x_3 \quad (1)$$

The statistically significance of the model and coefficients were also determined in DX7.

Hydrolysis of catfish oil and purification glyceride fraction (Shimida et al., 1997)

Some lipase, especially lipase from organism, could enrich PUFAs in oil because they de-esterify firstly the saturated fatty acids. When the PUFA concentration decreases, the hydrolysis continues with the monounsaturated fatty acids, at last, the PUFAs. Because of that, when control the hydrolysis time, the PUFA-rich oil can get in the glyceride fraction while saturated and monounsaturated fatty acids become free fatty acids.

After finding out the optimum parameters in lipase immobilization, the enzyme was

used to hydrolyze catfish oil. The hydrolysis time was increased from 30 to 120 minutes. The substrate was prepared by mixing 50 mL of the catfish oil with 50 mL of emulsification reagent. The reaction mixture consisting of 100 mL of the catfish oil, 50 mL of 100 mM sodium phosphate buffer, pH 7.0, 50 ml emulsification reagent and 1g immobilized lipase was incubated at 37°C.

After hydrolysis, FFA was neutralized by sodium hydroxide solution. Then, glyceride fraction was isolated by extracting with n-hexane and purified by evaporation. The glyceride fraction at last was determined the iodine value and DHA concentration to evaluate the PUFA enrichment by lipase hydrolysis

Determination of iodine value

Method for the determination of iodine value (g/100g) of catfish oil and glyceride fraction after hydrolysis according to Wijs as indicated by AOAC Official Methods of Analysis (1984), Chapter 28.023

Dissolve the sample in CCl₄, add 25 mL Wijs solution and keep in the dark for 1 hour to complete the reaction. Add deionized water and titrate excess iodine with 0.1N sodium thiosulphate solution.

Determination of DHA

Method for the determination of DHA content in catfish oil is hydrolytic extraction gas chromatography method by AOAC Official Methods 966.06 (2010) for fat analysis in food.

3. Results and Discussion

Optimization of *Rhizopus oryzae* lipase immobilization on celite by absorption

The two-level full-factorial design was used to evaluate the effect of the three main process parameters: the ratio of enzyme to support (g/g), the ratio of phosphate buffer volume to support weight (ml/g) and the incubation time (hours)

Table 2

Factors and levels of the experimental design

Factor	Unit	Level	
		+ 1	-1
The ratio of enzyme to support (Z1)	(g/g)	1:17	1:33
The ratio of phosphate buffer volume to support weight ratio (Z2)	(ml/g)	50	20
Incubation time (Z3)	(hours)	3	1

With 3 factors and 2 levels for each factor, $N = 2^3 = 8$ experiments was done to get the practical activity of immobilized lipase.

The lipase activities of eight experiments

in two-level full factorial design are showed in Table 3. The analysis of results was carried out by the statistical software DX7.

Table 3

Lipase activities obtained at the experimental conditions

Order	Factor 1 (Z1)	Factor 2 (Z2)	Factor 3 (Z3)	Enzyme activity Y (UI/g)
1	+	+	+	8.33
2	+	+	-	12.99
3	+	-	-	13.32
4	+	-	+	11.66
5	-	-	-	9.99
6	-	+	-	12.49
7	-	-	+	5.33
8	-	+	+	6.33

By removing the non-significant terms from the full polynomial model, the simplified expression forms as below:

$$Y(\text{UI/g}) = 7,145 + 110.X_1 + 0,186.X_2 - 3,6.X_3 - 2,867.X_1.X_2 + 54,167.X_1.X_3 - 0,021.X_2.X_3 \quad (2)$$

With $R^2 = 0.9995$; $cv\% = 1.72$ and $p\text{-value of the model} = 0,0416 < 0.05$

A good agreement is found between experimental and calculated yields with $R^2 = 0.9995$ and $p\text{-value of the model} < 0.05$. The cv of regression model is 1.72, this cv value indicate that the factual activity of the experiment does not have too much variation.

The equation 2 also includes the coefficients associated with three main factors. Hence, all of the factors have the effects on enzyme activity. There are the coefficients of $X_1.X_2$; $X_1.X_3$ and $X_2.X_3$ in the model, the existence of those coefficients shows that the three factors not only affect the activity but also interact with each other to change the lipase activity.

Depending on the regression model, the optimized immobilized lipase activity in this situation have been 22.95UI/g with:

- The ratio of enzyme to support (Z1): 1:10 (g/g)

- The ratio of phosphate buffer volume to carrier weight (Z2): 30.55 ml/g

- Incubation time (Z3): 5.15 hours

These optimum parameters have been used to immobilize lipase from *Rhizopus oryzae* in the next step of this research.

Effect of hydrolysis time on PUFA enrichment

Catfish oil was hydrolyzed with immobilized lipase in 0, 30, 60, 90 and 120 minutes. The iodine value of the glyceride fraction was determined by Wijs method. The result was analyzed with Stagraphic 3.0 software. The iodine value of the glyceride fraction was shown in Table 4.

Table 4

Iodine value of glyceride fraction after hydrolysis

Hydrolysis time (minutes)	Iodine value (g iodine/100g glyceride fraction)
0	72.12 ^c
30	89.08 ^b
60	98.30 ^a
90	97.40 ^a
120	99.21 ^a

Note: a, b, c statistically significant difference between the parameters in the column (p -value < 0.05)

The crude catfish oil has had the lower iodine value than the hydrolysis glyceride

fraction. After 30-minute hydrolysis, the iodine value has raised from 27.12 to 89.08. When increasing the hydrolyzing time from 30 to 60 minutes, the iodine value has raised approximately more 10 units. When continuing to last the time, the iodine value has not grown up significantly. This result has showed that this enzyme selectively hydrolyzes the oil in the initial phase. At first, this enzyme catalyzes the hydrolysis reaction with saturated or monounsaturated fatty acids in the oil and the unsaturated fatty acids like DHA, EPA,... are left in the glyceride fraction. Therefore, the iodine value has increased in the first 60 minutes of reacting. But after that, the lipase have lost its selective and all kind of fatty acids has been transferred to the fatty acid fraction and the iodine value of the glyceride fraction has not been raised anymore.

Comparison of crude oil and 60-minute hydrolyzed glyceride fraction

In order to evaluate the PUFA enrichment of the immobilized lipase, all of the process from lipase immobilization to hydrolysis oil has been repeated 3 times. The crude oil and the product of this process has been analysis, the result is showed in Table 5.

Table 5

Iodine value and dha concentration in catfish oil and glyceride fraction

Order	Parameters	The glyceride fraction	The crude oil
1	Iodine value	97.42 g iodine/100g	71.40 g iodine/100g
2	DHA concentration	1916 mg/kg	1156 mg/kg

The iodine value and DHA concentration in the glyceride fraction is higher than in the

crude oil. Generally, this immobilized lipase could enrich the PUFA of the catfish oil.

4. Conclusion

Immobilized process of *Rhizopus oryzae* lipase has been optimized and the immobilized lipase has had the activity about 22 UI/g. When hydrolyzing the crude *Pangasius hypophthalmus* oil in 60 minutes,

both of the iodine value and DHA concentration has been increased in turn 26.02 units and 760mg/kg. This enzyme selectively hydrolyzed oil in the initial phase, so it could be used to enrich PUFA in oil like fish oil■

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