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Optimization of enzymatic fish oil extraction from mackerel viscera by response surface methodology

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Abstract

The used of neutral protease for enzymatic fish oil extraction from mackerel viscera was studied following an experimental design as a statistical problem solving approach. Plackett–Burman design was used in order to select the most important variables from the simultaneous study on influence of operating and enzymatic hydrolysis conditions. The optimization of enzymatic fish oil extraction using the response surface methodology allowed a study on the influence of the variables (pH, neutral protease concentration and temperature). From the obtained results it could be concluded that the yield of fish oil was clearly enhanced at optimum conditions. Model validation showed a good agreement between experimental results and the predicted responses.

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Introduction

Fish sources once appeared to be inexhaustible and by-products arising out of fish processing were looked as worthless garbage and discarded without an attempt of recovery, which creating both disposal and pollution problems (Kristinsson and Rasco, 2000). Fish by-products included viscera, head and skin, which had a lot of unexploited potential for value adding and some of them were being utilized at present (Emna *et al*., 2009; Bhaskar *et al*., 2008; Betty *et al*., 2010). The mackerel *(Scomberomorus commerson)* was one of the most popular marine fish in China due to its abundance, year-round availability, low cost, suitable size. Fish viscera was the by-products/wastes of fish processing industry and were usually being discarded during processing and they had high oil content (Sahena *et al.*, 2010; Tsimidou *et al*., 1995; Samiramis *et al*., 2007).

Fish oils were a rich natural source of omega-3 polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which received great interest in the scientific community because of their positive roles on human health and nutrition (Leila *et al*., 2009). Beneficial health effects of ω-3 PUFAs were well demonstrated and included the prevention of a number of diseases, such as coronary heart diseases, hypertension, arthritis, autoimmune disorders, cancer (Guy *et al*., 2009; Douglas *et al*., 1992; Russell *et al*., 2005). Studies with newborns indicated that DHA was essential for the normal functional development of the retina, nerve and brain, particularly in premature infants (Alexandre *et al*., 2005; Dexuan *et al.*, 2010).

Fish oil could be produced by several methods which included hydraulic pressing, vacuum distillation, urea crystallization, supercritical fluid extraction, which all requiring high temperature or high pressure processing or reduction of moisture content in sample prior to extraction (Betty *et al*., 2010). Then these methods could contribute to the loss, denaturation, or decomposition of the thermally labile compounds. Enzymatic tissue disruption may be a valid alternative technique for releasing natural lipids from fish., which using commercial, low cost food grade neutral proteases provides an attractive alternative as reactions could be carried out under mild conditions for short periods of time. Previous studies had shown that, when compared to classical organic extraction, lipid extraction was enhanced by a pre-hydrolysis step using wide-spectrum neutral proteases and a part of the oil could be obtained after hydrolysis and centrifugation. Furthermore, peptides generated during hydrolysis could also be up-graded.

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There were several factors that affected enzymatic fish oil extraction from mackerel visceras including substrates, neutral protease activity, and reaction conditions (temperature, pH, etc.). To improve yield of the fish oil and rate of the enzymatic hydrolysis, research had focused on the optimization of the enzymatic hydrolysis process and enhancement of neutral protease activity.

Response surface methodology was a statistical technique for the modeling and optimization of multiple variables, which determined optimum process conditions by first- or second- order polynomial equations in a sequential testing procedure. This methodology had already been successfully applied for the optimization of enzymatic hydrolysis of several substrates including fish by-product (Linder *et al.*, 2005).

In this work, the enzymatic hydrolysis of mackerel viscera was studied employing preliminary tests and experimental design as a statistical problem solving approach, as the Plackett-Burman method and a response surface methodology (RSM) of central composite rotatable design (CCRD).

Materials and Methods

Materials

Mackerel viscera were collected from a local market in Zhoushan, China in month of May and were brought to the laboratory in iced condition. The protease employed for the optimization of enzymatic fish oil extraction was Neutral protease (Wuxi enzymic preparations, China; AS1.398 Neutral protease; declared activity of 10,000U/g). All the chemicals used in different analysis were of analytical grade, unless otherwise mentioned.

Sample preparation

The mackerel viscera were ground and homogenized at 4°C using Grindomix-GM 300 grinder to reduce particle size. The homogenates were preserved under nitrogen atmosphere at −20°C until further analysis. This was done to minimize oxidation of long chain fatty acids and minimize endogenous lipolytic activity (Gwendolyn *et al*., 2009).

Chemical analysis

Dry matter content in mackerel visceras was determined gravimetrically after oven-drying the samples at 105°C for at least 16 h. Thereafter, ash content was quantified after heating the sample at 600°Cfor 2 h. Total lipids were extracted from the mackerel visceras according to Soxhlet method. Crude protein content (N × 6.25) in the raw material was determined using the Kjeldahl method using KjeltecTM 8400 .

Enzymatic oil extraction from mackerel viscera

Enzymatic hydrolysis experiments on sample of mackerel visceras was performed in a rotary shaker heated from a re-circulating water bath. The enzymatic hydrolysis was done at desired condition and stopped by immediate chilling on ice. The upper oil phase was collected after centrifugation at 10,000×g, room temperature for 15 min and weighed. The amounts of fish oil recovered were calculated as percentages(Y%) of total oil present in the sample. Different enzymatic hydrolysis conditions were tested according to the either Plackett-Burman or CCRD.

$$\text{Total yield of fish oil: } Y\% = \frac{\text{Total yield extracted by enzymatic hydrolysis}}{\text{Total yield extracted by Soxhlet extraction}} \times 100 \quad (1)$$

Experimental design

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modelling and analyzing problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Susana *et al*., 2009). Many variables may potentially affect the efficiency of enzymatic hydrolysis process. In this study, a central composite rotatable design was employed to determine the effects of independent variables on the response and factor interactions, with a total of 15 runs with different combinations of variables. After examining the process and preliminary experiments, 6 variables or factors were identified to include in a Plackett–Burman design.

Plackett-Burman design

Plackett–Burman design was introduced in this study as a first optimization step to identify which factors have a significant effect on enzymatic hydrolysis. For the selection of these factors, Minitab software, version 14.0 was used to generate and analyze the experimental design of Plackett–Burman. Based on Plackett–Burman factorial design, each variable was examined in two levels: −1 for low level and +1 for high level (Kishor *et al*., 2007; Reddy *et al.*, 2008). This design did not consider the interaction effects among variables and it was used to screen and evaluate the important variables that influence the response. In the present work, 6 assigned variables were screened in 12 experimental designs. All experiments were carried out in triplicate and the averages yield of fish oil were taken as response. The factors that were included in the screening experiment and their settings are given in Table 1.

Table 1. Effects and results of the variables in the Plackett–Burman design

Factors		Level		Importance
	Code Variable	Low(-)	High(+)	P Tese
A	Water/sample	2	2.5	0.550
B	pH	6	7.5	0.013
(C)	dummy	-	-	0.109
D	Time (h)	60	75	0.175
E	Neutral protease concentration (%)	0.8	1.0	0.076
(F)	dummy	-	-	0.146
G	Agitation (rpm)	160	200	0.537
H	Temperature (℃)	35	45	0.065
(I)	dummy	-	-	0.983

Central composite design

The central composite design (CCD) is one of the most commonly used response surface designs for fitting second-order models. In our study, the central composite rotatable design (CCRD) was used to optimize the enzymatic fish oil extraction from mackerel viscera. There independent variables, namely pH (X_1), neutral protease concentration (X_2) and temperature (X_3) were studied at three levels. For each of the three variables studied, high (coded +1) and low (coded −1) set points were selected according to the results obtained with preliminary tests, Plackett–Burman design, taking into consideration the required experimental conditions and literature. The results of each CCRD were analyzed using Minitab software, version 14.0. Both linear and quadratic effects of the three variables under study were calculated, as well as their possible interactions, on yield of fish oil. Their significance was evaluated by variance analysis (ANOVA). Three-dimensional surface plots were drawn to illustrate the effects of the independent variables on the dependent variable, being described by a quadratic polynomial equation, fitted to the experimental data. The fit of the models was evaluated by the determination of R-squared coefficient and adjusted R-squared coefficient. The validation of the models optimum values of the selected variables for mackerel viscera was obtained by solving the regression equation using Minitab software, version 14.0 (Hajar *et al.*, 2009; Lee *et al.*, 2006).

Results and Discussion

Approximate chemical composition of mackerel visceras

Crude mackerel visceras contained (%): 27.21±0.23 dry matter (DM); 1.93±0.03 ash, 9.21±1.4 lipid, and 16.07±0.22 protein. The low DM content in mackerel visceras indicated that above 70% of the mackerel visceras constituted water.

Plackett–Burman design

The Plackett–Burman design for 12 runs was used to screen the variables significantly influencing enzymatic fish oil extraction from mackerel viscera. In this design, each medium constituent and enzymatic hydrolysis condition parameter was treated as a variable and three dummy variables were also added as shown in Table 1. Each variable was studied at two levels, namely the original level and 1.25 times the original level. The experiments were performed in triplicate and the average of the yield of fish oil obtained was taken as the response. The analysis of the results indicated that variables pH, neutral protease concentration and temperature, which had confidence levels 90% ($P<0.1$), were selected as the most important variables influencing enzymatic of fish oil extraction. and were further studied; whereas the other variables that did not have a marked influence on yield of fish oil were retained at the original levels: water/sample, 2; agitation, 160 rpm and incubation time, 60min.

Optimization enzymatic fish oil extraction with central composite rotatable design

The yield of fish oil was directly related to enzymatic hydrolsis condition of mackerel viscera. Optimization of the hydrolysis conditions were accomplished by employing the response surface methodology (RSM) with a central composite rotatable design (CCD). Three different factors (pH, neutral protease concentration and temperature) were employed at three equidistant levels (-1, 0 and +1). The actual set of experiments performed (experimental runs 1–15) were shown in Table 2. A second-order polynomial equation was developed to study the effects of variables on the yield. The equation indicated the effect of variables in terms of linear, quadratic, and cross-product terms:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y% as the response variable was the yield of fish oil(%), X_i and X_j were the levels of variables,

Table 2. Actual levels of independent variables along with the observed values for the response variable, yield of fish oil (Y%)

Run#	Variable levels			Y%	
	X_1	X_2	X_3	Experimental	Predicted
1	0	0	0	78.33	77.65
2	0	1	1	58.08	57.99
3	0	-1	-1	62.44	62.54
4	1	1	0	50.64	50.40
5	1	0	-1	58.97	58.83
6	0	1	-1	57.31	57.70
7	0	-1	1	61.03	60.65
8	-1	-1	0	65.64	65.88
9	-1	0	1	69.62	69.76
10	1	-1	0	56.54	56.59
11	0	0	0	78.72	78.66
12	1	0	1	57.56	57.90
13	-1	0	-1	70.77	70.43
14	0	0	0	77.31	77.65
15	-1	1	0	64.62	64.57

X_1 : pH, X_2: Neutral protease concentrate (%),X_3: Temperature (°C)

β0 the constant term, β_i the coefficient of the linear terms, β_{ii} the coefficient of the quadratic terms, and β_{ij} the coefficient of the cross-product terms. All the experimental data were statistically analyzed by software, version 14.0. The graphical representations of the above equation in the form of surface plots were used to describe the individual and cumulative effects of the test variables on the response.

The yields of fish oil obtained from all the experiments were listed in Table 2 according to RSM design. The predicted response values slightly deviated from experimental data. Multiple regression coefficients, obtained by employing a least squares technique to predict a second-order polynomialmodel for the fish oil yield, were summarized in Table 3. The lack of fit was not significant, which indicated that the models was well adapted to the response and was suitable to predict enzymatic fish oil extraction from mackerel viscera. The significance of each coefficient was determined by F-value and P-value. Corresponding P-values suggest that, among the test variables used in this study, X_1 (pH), X_2 (Neutral protease concentration), $(X_1)^2$ (pH×pH), $(X_2)^2$ (Neutral protease concentration×Neutral protease concentration), $(X_3)^2$ (temperature×temperature) and X_1X_2 (pH×Neutral protease concentration) were significant model terms with P-values of less than 0.1. Other terms were insignificant. The coefficients of independent variables determined for the second-order polynomial model for the fish oil yield is given below:

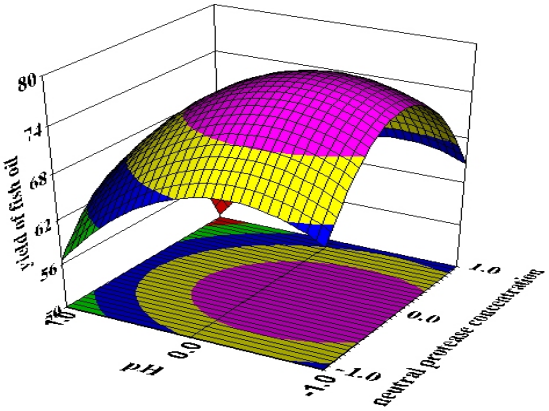

Figure 1. Response surface for yield of fish oil as a function of pH and Neutral protease concentration (at 45°C)

$$Y=78.1200-5.8675X_1-1.8750X_2-0.4000X_3-7.1225X_1^2-11.6375X_2^2-6.7675X_3^2-1.2200X_1X_2-0.0650X_1X_3+0.5450X_2X_3 \quad (3)$$

The analysis of variance and error for the response surfacemodel were given in Table 4. These results as shown that the model was significant at $P < 0.1$ and the linear and square terms were significant at $P < 0.1$. The interaction terms were not significant. The lack-of-fit or adequacy test was significant at $P < 0.1$ showing the adequacy of the quadratic model selected.

Figure 1 shown the response surfaces for the effect of the independent variables on the yield of fish oil. It could be seen in Table 3 that there was an interaction effect between pH and neutral protease concentration on yield of fish oil. At the lowest level of neutral protease concentration, the yield of fish oil was found to increase rapidly with an increase in pH.

Table 3. Regression coefficients of predicated second-order polynomial model for the response variable.

Term	Coef	SE Coef	T	P
Constant	78.1200	0.3431	227.722	0.000
X_1	-5.8675	0.2101	-27.931	0.000
X_2	-1.8750	0.2101	-8.925	0.000
X_3	-0.4000	0.2101	-1.904	0.115
X_1^2	-7.1225	0.3092	-23.034	0.000
X_2^2	-11.6375	0.3092	-37.635	0.000
X_3^2	-6.7675	0.3092	-21.886	0.000
$X_1 X_2$	-1.2200	0.2971	-4.106	0.009
$X_1 X_3$	-0.0650	0.2971	-0.219	0.835
$X_2 X_3$	0.5450	0.2971	1.834	0.126

Table 4. Analysis of the variance (ANOVA) for the fit of the experimental data to response surface model.

Source	DF	Seq SS	Adj MS	T	P
Regression	9	1066.05	118.450	335.51	0.000
Linear	3	304.83	101.608	287.80	0.000
Square	3	754.07	251.356	711.96	0.000
Interaction	3	7.16	2.386	6.76	0.033
R- Error	5	1.77	0.353		
Lack-of-Fit	3	0.71	0.235	0.44	0.748
Pure Error	2	1.06	0.530		
Total	14	1067.82			

At the highest level of neutral protease concentration, the yield of fish oil increase to a certain level and then increase at a slower rate owing to the contribution by the interaction term ($P<0.01$) of pH and neutral protease concentration. During the enzymatic hydrolysis, the combination between lipid and protein was break down, which lead to fish oil release much easier from the mackerel viscera.

The optimum values of the selected variables for enzymatic fish oil extraction by solving the regression equation were X_1= -0.4066 (pH7.3); X_2= -0.0599 (neutral protease concentration 1.0%); X_3= -0.0300 (temperature at 44.8°C). To validate the model, the optimum values for equation (3) were used in triplicate sets of experiments and the maximum response obtained was 78.66%. This values was in good agreement with the predicted value 79.38%.

Conclusion

With the main goal of testing as many factors as possible and selecting those that affected enzymatic fish oil extraction most significantly, a Plackett–Burman design was used. To estimate effects of pH, neutral protease concentrate and temperature on the response and factor interactions, a central composite rotatable design was employed. This experimental design could convert the process factor correlations into mathematical models that predict where the response was likely to be located.

From the results it could be concluded that viscera of mackerel was a potentially useful source of fish oil, which could be a source of economically useful omega-3 PUFAs. The different conditions (pH, enzyme concentration and temperature) for enzymatic hydrolysis revealed that all these variables markedly affected the yield of fish oil. These could be related to the enzymatic hydrolysis conditions by second order polynomials. Using the contour plots, the optimum set of the operating variables were obtained graphically in order to obtain the maximum yield of fish oil. The predicted model fits well with the experimental results. The significant factors: pH7.3; nuetral protease concentration, 1.0%; temperature, 44.8°C and nonsignificant factors: water/sample, 2; agitation, 160 rpm and incubation time, 60min.were found to be the optimum conditions to achieve the maximum yield of fish oil. The model validation provided a good agreement between the experimental results and predicted.

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