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Response surface optimization in determining hydrolysis reaction on Barramundi scales gelatin using the box behnken design

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Abstract

Gelatin hydrolysate is a product with a wide range of benefits in pharmaceutical and food applications. Therefore, this study analyzes the best hydrolysis condition to make Barramundi (*Lates calcarifer*) scales gelatin hydrolysate using ultrasonication and lime juice. The conditions with the best degree of hydrolysis (DH) were optimized using the Response Surface Method (RSM) with Box Behnken Design (BBD). The effects of 3 independent variables, namely time, temperature, and enzyme concentrations, were then examined for model optimization. Based on the extraction method, DH varied between 57.40% and 68.60%. The equation characterizing the variables and DH correlation was DH = 0.01324 – 0.0012A + 0.0013B – 0.0028C -0.0004AB + 0.0019AC – 0.001BC – $0.0004A^2$ – $0,0042B^2$ – $0.0012C^2$, A, B, and C were time, temperature, and E/S concentration. In addition, the optimized hydrolysis was performed at 31.03 mins, 56.84°C temperature, 1.5% E/S concentration, and pH 8.0. At these optimal conditions, alkaline protease hydrolysis yielded a 1.000 desirability value, with a 64.83% DH. Amino acid (AA) with the highest value in Barramundi scales gelatin hydrolysate was lysine (53.03 g/100 g), while isoleucine (0.05 g/100 g) had the lowest value. Based on the results, optimal hydrolysis conditions could be an excellent reference to increase the potential of Barramundi scales gelatin hydrolysate in developing future functional foods with high amino acids, particularly lysine.

1. Introduction

Gelatin is a protein that can be obtained through heat denaturation and hydrolysis of collagen from bones, skin, and white fiber tissue from animals (Bhernama, 2020). Several studies showed that it was typically made from the skin and bones of mammals, such as pigs and cows, leading to the rejection by various communities, including Hindus and Muslims. This rejection has led to the development of alternative sources of obtaining gelatin, including fish (Wangtueai *et al*., 2016). During fish processing, several by-products, such as skin, scales, and bones are often produced as waste, constituting 50-70% of the total weight (Cao *et al*., 2017). Because of the many by-products obtained, there has been an increasing interest in the use (Tkaczewska et al., 2020). A highly demanded fish species by the public is Barramundi (*Lates calcarifer*), which has been reported to contain 20% protein, 5% fat, and omega 3 (Bhernama, 2020). According to a previous study, gelatin extraction has been carried out from Barramundi scales using ultrasonication methods (He *et al*., 2021). In this study, ultrasonication method with a temperature of 60°C and hydrolysis using lime juice (1:3) obtained the best gelatin results with the highest yield (Wonganu, 2020; Hariyanti *et al.,* 2023). Gelatin hydrolysate has a wide range of benefits in pharmaceutical and food applications. Several studies showed that gelatin hydrolysis can be carried out enzymatically, to increase the nutritional value and activity of proteins by converting low-consumption proteins into high-value protein products. The essential factors in enzymatic hydrolysis include pH, temperature, enzyme/substrate ratio, hydrolysis time, enzyme type, and substrate (Tekle *et al*., 2022).

In line with previous studies, gelatin hydrolysis with enzymes has the potential to increase bioactivity (Tekle *et al*., 2022). Optimization procedures have also been carried out to obtain suitable gelatin hydrolysate (Mirzapour-Kouhdasht *et al*., 2020). In a study by

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Mohammad *et al*., (2015), gelatin optimization was carried out at different enzyme concentrations (0.5%, 1%, and 1.5%), temperature (50°C, 57.5°C, and 65°C), and time variations (40 mins, 60 mins, and 80 mins). In this study, the best results were obtained at the ratio of 1.2% enzyme, with a temperature and time of 57.6°C and 80 mins, respectively.

Based on the results, gelatin from Barramundi obtained with ultrasonication method using lime juice was optimized using 3 parameters, namely temperature, hydrolysis time, and ratio enzyme and substrate concentration. The influence of the independent variables was determined using the response surface method (RSM) with box behnken design (BBD).

2. Materials and methods

2.1 Materials

The material applied was Barramundi scales obtained from the Cilincing Traditional Fish Market. Others included surfactant, acetic acid, Bradford reagent NaOH, trichloroacetic (TCA), P5380-100MG Alkaline protease 25 mg Type VIII, lyophilized powder, and 7-15 units/mg solid (Sigma-Aldrich).

2.2 Degreasing and demineralization of fish scales

The degreasing procedure referred to the approach by Hariyanti *et al*. (2022), with few improvements. Waste of Barramundi scales was cleaned of impurities on the surface by soaking with surfactants for 24 hrs and stored in a refrigerator. Subsequently, it was washed with aquadest and dried. After the fish scales were dried, weighing was carried out on the dried fish scales, followed by soaking with acid. The acid used was squeezed lime 1: 3 (w/v) for 24 hrs and stored in the refrigerator. The sample was then rinsed under running water until the pH of the Barramundi scales was neutral (Wonganu, 2020).

2.3 Extraction of gelatin from fish scales

The acquired demineralized fish scales (ossein) were added with aquadest in a ratio of 1:2. Subsequently, the extraction process was carried out using ultrasonication at 60°C for 3 hrs. The extraction result was filtered and laid out on a flat tray. The extraction results were placed on a tray and dried using an oven at 50-60°C for 24 hrs until a gelatin sheet was formed. The formed gelatin sheets were cut into small pieces and mashed with a blender until a gelatin powder formed.

2.4 Response surface methodology using box benkhen design

The study design was based on a previous study (Zhang *et al*., 2022) using RSM design expert program 11. The three-factor and two-response BBD method consisted of 15 randomized trials. Based on the experimental results of each experiment, each model was installed to calculate each response value (Nursal *et al.,* 2022).

The predicted coefficient, with a level >95% (P>0.05), was in the BBD model. The DH could be described as an independent variable function with the second-order polynomial equation as follows:

$$Y=\beta 0+ \sum \beta j\ Xj+ \sum \beta jj\ Xj^2+ \sum \beta ij\ XiXj$$

Where Y is the predicted response; β0, βj, βjj, and βij are the regression coefficients for intercept, linearity, square, and interaction terms. At the same time, Xi and Xj are the coded independent variables.

The responses were statistically assessed, and the model was designed appropriately for variables with a confidence level >95%. Hydrolysis condition optimization was obtained by applying the RSM method, which is divided into four stages (Montgomery 2001), namely (1) formulating and creating a response design; (2) formulation; (3) response analysis; and (4) optimization.

2.5 Formulation preparation and response design

Creating Formulation and response design applies the Design Expert 11.0® program to determine dependent and independent variables. The dependent variable is a variable whose value was kept equal in each treatment due to not impacting the response. Meanwhile, the independent variable is a variable that affected the answer. The dependent variable of this analysis was total Barramundi scale extracts that were hydrolyzed. Meanwhile, the independent variables include enzyme concentration, temperature, and hydrolysis time. This study determined independent variables based on previous studies (Mohammad *et al*., 2015). Trial and error tests obtain the minimum and maximum limits, as shown in Table 1 which were described in the Design Expert 11.0® program, RSM BBD, for randomization. A total of 3 different factors (temperature, time, and E/S concentration) were applied on similar levels (-1 and +1), and DH as the response variable (Y). Each run contained 50 mL protein extract, and gelatin hydrolysis referred to Mohammad *et al*. (2015) with a content of 5% gelatin solution (b/v) in the reaction container. The temperature was set according to the specified temperature, and the pH of the solution was set to pH 8 with the addition of NaOH 2 N. Subsequently, the protease enzyme according to the expected concentration. The reaction was carried out by stirring 300 rpm at the specified time, and the hydrolysis reaction

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was discontinued by setting the temperature to 90ºC for 10 mins. Gelatin hydrolysate was dried using a freeze-dryer and stored in a vacuum container in a dry place.

Table 1. The range of independent variable values

Variable	-1	0	1
Temperature (°C),	45	55	65
Time (mins)	30	60	90
E/S (%v/b)	0.5	1	1.5

After obtaining gelatin hydrolysate products at 15 treatments, the yield value, protein content by the Bradford method, and DH were determined. DH was calculated using the percent trichloroacetic acid ratio (TCA) method. After hydrolysis, 10 mL hydrolysate was mixed with 10 mL of 20% TCA. The mixture was stirred for 1 min and then centrifuged at 10,000×*g* for 15 mins (Wangtueai *et al*., 2016). In addition, the supernatants protein content was obtained by the Bradford method and calculated with the following formula (Hermanto, *et al*., 2021; Amiza *et al*., 2019):

$$\text{Degree of Hydrolysis (\%DH)} = \frac{\text{TCA dissolved protein 10\%}}{\text{the total protein sample}} \times 100\%$$

2.6 Formulation stage

Barramundi scales gelatin hydrolysis with the treatment of enzyme concentration (E/S), temperature, and different hydrolysis times applied to find the best DH.

2.7 Response analysis

ANOVA which were linear, quadratic, special cubic, and cubic was applied to examine each response variable. Models with significance in ANOVA and insignificance in lack of fit are applied to study variables.

2.8 Optimization stage

The Design Expert 11.0® program optimized the response of DH. The program performed the optimization appropriate to the variables and response measurement data. The optimization output recommended optimal new formulas that are appropriate to the program. The best procedure was the variant with the maximum desirability value, a value of the optimization objective function that showed the program's benefit to fulfill desires appropriate to the final product criteria. The value range was from 0 to 1, and the desirability value closer to 1 indicated a better ability to make the desired product (Joyjamras *et al*., 2022).

2.9 Verification for optimum condition

After optimization, the suggested solution for the optimum condition was obtained. From the optimum condition, enzymatic hydrolysis of the implied state was carried out in triplicate. Subsequently, the DH of hydrolysate resulting from hydrolysis was determined. The predicted value given by RSM was then compared with the experimental value obtained using a one-sample t-test.

2.10 Amino acid analysis and chemical score computation

AA compositions were found by applying High-Performances Liquid Chromatography (HPLC). The protein hydrolysate chemical score (CS) was input to analyze the nutritional value of Barramundi scales protein hydrolysate. In addition, the score was correlated to the essential AA profile in a standard protein Joint FAO/WHO/UNU (2007) described. The chemical score was obtained by applying the equation:

$$CS = \frac{\text{EAA in test protein (g/100 g)}}{\text{EAA in standard protein (g/100 g)}}$$

Where CS is the chemical score and EAA is the essential AA content.

3. Results and discussion

The yield was determined by calculating the percentage of dried fish scales and the resulting gelatin powder. The effectiveness of gelatin was shown by the resulting product of gelatin (Wahyuningtyas *et al*., 2019). The greater the yield, the more efficient the treatment method used. Using acid and extraction temperature to manufacture gelatin affected the yield value produced (Hariyanti *et al.,* 2023). In addition, the yield of Barramundi scales gelatin by sonication method at 60°C and hydrolyzed using lime juice obtained a gain of 14.79%.

Based on the 15 treatments, the hydrolysis degrees of each treatment were obtained, as shown in Table 2. BBD had mathematical models which could obtain the best design. Model selection applying Sequential Sum of Squares Models according to total squares and sequence, where the best model was the variant with a probability value <5%. The quadratic model was the best candidate because the probability values <5% (p-value: 0.0021) (Table 3).

Lack of fit test with the Design Expert 11.0® recommended the quadratic model, as shown in Table 4. The lack of appropriate tests was based on model incompatibility, and the parameter applied to describe model incompatibility was p-value. The insignificant lack of fit value should be fulfilled for a good model which shows the DH response suitability with the model (Bekele *et al*., 2022). A squared test selects the best model according to a value close to 1. The adjusted R^2 test recommended the quadratic model as the best result, as shown in Table 5. The interaction between answers

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Table 2. Optimum condition for the degree of hydrolysis in different enzyme concentrations, temperatures and time.

Formula	E/S (%v/b)	Temperature (°C)	Time (mins)	Degree of Hydrolysis (%)
1	1	55	60	57.9
2	1.5	55	90	64
3	1.5	45	60	62.2
4	1	45	30	61.6
5	0.5	65	60	58.2
6	0.5	55	30	61.9
7	0.5	55	90	57.6
8	1	45	90	68.6
9	1	55	60	57.4
10	1.5	65	60	60.8
11	1.5	55	30	64.8
12	1	65	30	63
13	0.5	45	60	63.2
14	1	55	60	65.9
15	1	65	90	61.6

Table 3. Sequential model sum of squares analysis

Squares	Sum of Squares	df	Mean square	F-value	p-value	
Mean vs. total	0.2509	1	0.2509			
Linear vs Mean	0.0001	3	0.0000	3.60	0.0495	
2FI vs. Linear	0.0000	3	6.181×10^{-6}	0.6490	0.5811	
Quadratic vs. 2FI	0.0001	3	0.0000	24.36	0.0021	Suggested
Cubic vs Quadratic	1.611×10^{-6}	3	5.370×10^{-7}	0.3640	0.7901	Aliased
Residual	2.950×10^{-6}	2	1.475×10^{-6}			
Total	0.2511	15	0.0167			

Table 4. Lack of fit test results using Design Expert 11.0® software

Squares	Sum of Squares	df	Mean square
Linear vs. Mean	0.0001	3	0.0000
2FI vs. Linear	0.0000	3	6.181×10^{-6}
Quadratic vs. 2FI	0.0001	3	0.0000
Cubic vs. Quadratic	1.611×10^{-6}	3	5.370×10^{-7}

and hydrolysis process factors was examined using ANOVA. A factor's significance was determined by the likelihood that the p-value should be <5%. This equation had 3 linear terms, 1-factor interaction term, and 1 quadratic effect, namely A, B, C, AC, and B^2 (Table 6). The value of the lack of fit was due to noise since the lack of fit was not as significant as the pure error potential. The model equation that ANOVA suggests was:

$$DH = 0.01324 - 0.0012A + 0.0013B - 0.0028C - 0.0004AB + 0.0019AC - 0.001BC - 0.0004A^2 - 0{,}0042B^2 - 0.0012C^2$$

Where A is temperature, B is time and C is E/S. Bekele *et al.* (2022) stated the regression model was well -defined when $R^2 > 0.80$. A small R^2 indicated a dependent variable's poor relevance in the model. In addition, the model could match the actual data when R^2 approached unity (Bekele *et al.*, 2022).

A 3-dimensional response surface built on the simple linear model could be used to visualize the independent variables' impact on DH (Figure 1A–C). Figure 1A shows the contour plots representing a function of 2 factors, including temperature and time. The figure revealed that DH increased with an increase in temperature. DH increases below the optimum temperature due to the hydrolysis rate increasing when the temperature increases for most reactions. DH also increased with increasing enzyme concentration. DH grew rapidly at the initial reaction and reached the optimum value, as shown in Figure 1B. In this study, enzyme concentration was at the maximum point of 1.5%. DH started to decrease as the time and enzyme concentration were increased, as shown in Figure 1C. The decrease in DH could be because of the decrease in substrate saturation, product inhibition, enzyme activity,

Table 5. Model summary statistics

Source	Sequential p-value	Lack of Fit P-value	Adjusted R^2	Predicted R^2	
Linear	0.0495	0.1396	0.3578	0.0926	
2FI	0.5811	0.1192	0.2994	-0.04039	
Quadratic	0.0021	0.7901	0.9282	0.8178	Suggested
Cubic	0.7901		0.8839		Aliased

Table 6. ANOVA model of the hydrolysis of Barramundi scales gelatin

Source	Sum of Squares	df	Mean square	F-value	p-value	
Model	0.0002	9	0.0000	21.12	0.0018	significant
A-Temperature	0.0000	1	0.0000	11.63	0.0190	
B-Time	0.0000	1	0.0000	13.91	0.0136	
C-E/S	0.0001	1	0.0001	71.09	0.0004	
AB	8.073×10^{-6}	1	8.073×10^{-6}	0.8850	0.3900	
AC	0.0000	1	0.0000	15.30	0.0113	
BC	8.773×10^{-6}	1	8.773×10^{-6}	4.14	0.0976	
A^2	5.082×10^{-7}	1	5.082×10^{-7}	0.5570	0.4890	
B^2	0.0001	1	0.0001	69.87	0.0004	
C^2	5.401×10^{-6}	1	5.401×10^{-6}	5.92	0.0591	
Residual	113.63	5	9.123×10^{-7}			
Lack of Fit	68,13	3	5.370×10^{-7}	0.3640	0.7901	not significant
Pure of error	45.40	2	1.475×10^{-6}			
Cor Total	145.62	14				

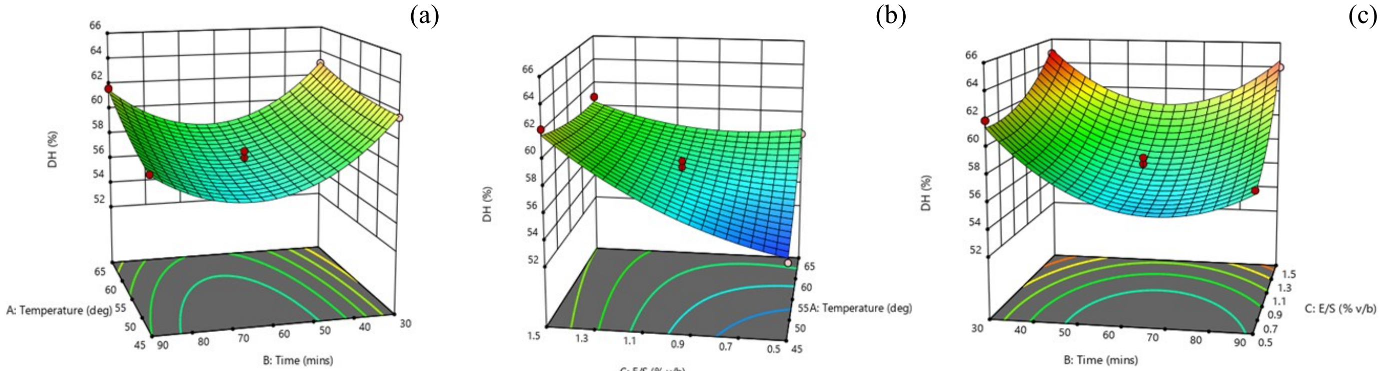

Figure 1. Response surface graph for the degree of hydrolysis as a function of A -temperature and time; B - temperature and E/S, during the hydrolysis of Barramundi scales gelatin with protease enzyme.

or extreme pH concentration that caused enzyme denaturation (Zainol *et al.*, 2021).

DH at the point with temperature parameters of 56.84°C, time 31.03 mins, and enzyme 1.5% was expected to have DH of 64.83% based on the optimization results (Figure 2). The obtained desirability value was 1.000, which indicated how healthy conditions were ideal. A desirability value of 1 denoted an excellent outcome, while 0 showed an unsatisfactory reaction (Prabudi *et al.*, 2018). The success rate for the desirability value of 1 response was 100.0%. In addition, the software could display the best product using RSM, making it possible and economically appealing to produce Barramundi gelatin hydrolysate from fish under ideal conditions, with a 0.664 desirability value. Under the optimum conditions, the predicted value for DH (64.84%) was close to the experimental data (65.02±0.02%) (Table 7).

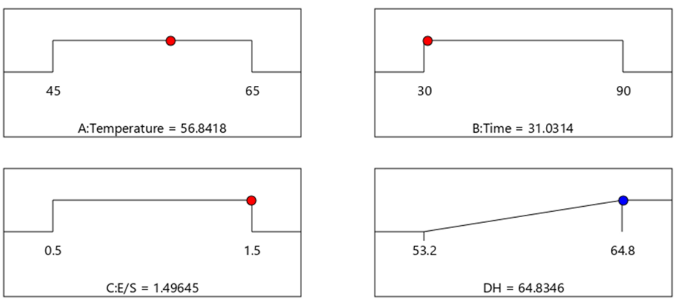

Figure 2. Solution optimization by Design Expert 11.0® software.

AA analysis on gelatin hydrolysate finds the AA proportion contained in essential and non-essential AA. Table 8 showed the presence of 15 types of 23 AA consisting of 9 essential AA, namely histidine, threonine, valine, methionine, arginine, phenylalanine, leucine, isoleucine, and lysine. In addition, there were 6 types of non-essential AA, namely, aspartic acid, serine, glycine, glutamic acid, proline and alanine. AA with the highest concentration was lysine (53.03 g/100 g), while AA with the lowest concentration was isoleucine (0.05 g/100 g) (Table 8). Lysine is an essential AA for any mammal and the basis of protein synthesis (Lise *et al.,* 2021). Glutamate was an abundant AA in fishery products and important in the product taste (Widyastuti *et al.*, 2014).

The chemical score provided a protein nutritional value calculation. This parameter compared the essential AA levels of the analyzed material with standard proteins (Ovissipour *et al.,* 2012). In this analysis, chemical scores were calculated appropriately to goldfish's AA requirements (National Research Council (NRC), 1993). AA composition and comparison with the reference protein indicated AA profile of Barramundi

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Table 7. Formulation obtained from the optimization process

Type	E/S (%v/b)	Temperature (℃)	Time (mins)	DH (%)
Predicted	1.5	56.84	31.03	64.84
Experimental	1.5	57	31	65.02

Table 8. Amino acid content

Amino Acid	Quantity (g/100 g)		Chemical score	
	Barramundi scale gelatin hydrolisate	Reference protein*	Reference protein**	Reference protein***
Essential amino acids (62.25 %)				
Histidine	0.37	1.6	0.71	2.1
Isoleucine	0.05	1.3	1.74	2.5
Leucine	0.50	1.9	1.54	3.3
Lysine	53.03	1.6	2.24	5.7
Methionine	5.32	1.7	0.67	3.1
Phenylalanine	1.46	-	-	6.5
Tyrosine	0	-	-	-
Threonine	0.32	0.9	1.96	3.9
Arginine	0.27	-	-	1.31
Valine	0.59	1.3	1.82	3.6

*Suggested profile of essential amino acid requirements for adults (Joint FAO/WHO/UNU, 2007).
**Chemical score is calculated with the FAO/WHO reference protein as the base.
***Essential amino acid requirements of common carp according to NRC (1993).

scales gelatin hydrolysate was generally comparable in essential AA to AA requirements suggested by FAO/WHO and NRC. The same findings were for the hydrolysate of yellowfin visceral tuna (Ovissipour *et al*., 2012).

4. Conclusion

In conclusion, Barramundi gelatin hydrolysis conditions were optimal with a protease enzyme concentration of 56.84°C, 31.03 mins, E/S concentration of 1.5%, and a desirability value of 1.000. The highest DH 65.02±0.02%, was obtained at E/S 1.5%, 57.0℃, and 31 mins, which was similar to the predicted values in the 95% confidence interval. In addition, optimal hydrolysis could be a better reference for increasing the hydrolysate potential of Barramundi scales gelatin in the development of functional foods in the future.

Conflict of interest

The authors declare no conflict of interest.

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