RESEARCH ARTICLE

Optimization of the Enzymatic Hydrolysis of Yellowfin tuna, (*Thunnus albacares*) Viscera by Response Surface Methodology

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ABSTRACT

Tuna is abundant in the Philippines, but the by-products during processing in various forms may be a source of waste and environmental pollution. To minimize these wastes, they are utilized directly or fermented to serve as food for humans and animals and as a functional food ingredient since they are rich in proteins and polyunsaturated lipids. For this purpose, they are often converted into protein hydrolysates using proteolytic enzymes. This study optimized the hydrolysis of the yellowfin tuna (*Thunnus albacares)* viscera (YFTV) using the enzyme neutrase to produce hydrolysates with a high degree of hydrolysis (DH) and foaming capacity (FC). Using the central composite design of the Response Surface Methodology (RSM), the YFTV protein hydrolysis at varying concentrations (0.5-1.5%, w/v) and hydrolysis time (60 to 180 min) was carried out. From the RSM-generated model, the optimum conditions to obtain the highest DH was 179.50 min hydrolysis time and 1.5% enzyme concentration, and for the highest FC, 176.58 hydrolysis time and 1.5% enzyme concentration. The predicted optimum values using the generated linear and quadratic equations were 17.26% DH and 1.60% FC. The lack of a fit test for both responses yielded an insignificant value ($p > 0.05$) for the model, suggesting that the regression coefficient was sufficient for estimating both responses under any group of variables. The optimized protein hydrolysis conditions of YFTV using Neutrase could be applied in food production systems, especially downstream processing. Furthermore, the utilization of tuna viscera as protein hydrolysates could potentially contribute to the waste management of these processing by-products.

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1. INTRODUCTION

Tuna is abundant in the Philippines and has been one of the top fishery exports in terms of economic value and volume in recent years (Llanto et al. 2018). It continues to be the leading export commodity in 2020, contributing a total volume of 134,412 MT and a value of USD 481 million. Among the tuna products, canned tuna comprised a large percentage, which are being exported (BFAR 2021). Many tuna species are economically important, but the yellowfin tuna receives the highest demand from the country's export market (Nguyen et al. 2011). Tuna is prepared and marketed in various forms, such as raw, frozen, steaks, or processed into canned and ready-to-eat products. Other fish parts, such as the tuna head, tail, and viscera, are minimally utilized as human or animal food, but most are often discarded as waste (Gamarro et al. 2013). Generally, tuna products sold as fresh or chilled whole or headless gutted generate less waste than those from canned tuna processing. Canned fish processing could generate large amounts of solid waste, including viscera. These wastes comprise about 70% of the original material (Guerard et al. 2002). Considering the combined daily industry production, the overwhelming volume of fish processing waste warrants technologies and appropriate measures that can lead to its optimum utilization (Gamarro et al. 2013).

Among these discarded parts, the viscera is potentially useful to be converted into another product with higher value because it is rich in proteins and polyunsaturated lipids. Tuna viscera is found to have a good amount of protein and amino acids that

are used in fish meals and protein concentrates. Many studies have shown that hydrolyzing the viscera using proteolytic enzymes to liberate the amino acids and peptides results in protein hydrolysates that can be utilized as a functional food ingredient because of their biological activities (Ovissipour et al. 2009). In aquaculture, it was found that fish by-products can be a good source of fish meal due to its favorable amino acid profile as well as essential oils (Kim et al. 2019). Tuna canning process waste of about 52-54% has also been used as a feed to Nile tilapia (*Oreochromis niloticus*) to substitute the soybean meals. It resulted in higher growth performance and high feed efficiency (Hernández et al. 2017).

Protein hydrolysis procedures may be carried out using acid or alkaline treatments but were raised with ecological and human health safety issues from the production of secondary by-products (Pasupuleti and Braun 2008). For animal nutrition, alkaline hydrolysis compromises the protein quality of feeds by causing the production of toxic substances such as lysinoalanine, which causes racemization of L-amino acids to D-amino acids and indirectly disrupts arginine and tyrosine during thermal processing (Kristinsson and Rasco 2000). Although acid hydrolysis is a traditional technique, it is challenging to ensure product quality owing to the non-specific peptide bond cleaving and depletion of several essential amino acids during the harsh reaction (Wisuthiphaet et al. 2015). Due to these risks, the enzymatic hydrolysis procedure is preferred as essential amino acids are not lost, while low quantities of enzymes can be conveniently deactivated after mild hydrolysis. In addition, proteolytic enzymes are more selective and accurate in controlling the degree of the peptide bond cleavage during enzymatic hydrolysis (Hou et al. 2017).

Fish protein hydrolysates refers to the product of the degradation of proteins into smaller peptides and amino acids that can be utilized as binders and emulsifiers in food systems and components of nutraceuticals (Venugopal 2016). Due to its free amino acid content, it can also be used as an additive and an important ingredient in animal feeds. Enzyme-aided protein hydrolysates are extensively applied in various processing industries, particularly in food, because of their nutritive value (Han et al. 2020). Specifically, they are used as food binders, emulsifiers, gelling agents, and additives to soups, cereals and desserts, nutraceuticals, cryoprotectants, and aquafeeds (Kristinsson and Rasco 2000; Venugopal 2009; Chalamaiah et al. 2012; Jenkelunas and Li-Chan 2018; Ananey-Obiri et al. 2019; Han et al. 2020). Several studies showed the successful use of enzymes with plant, animal, and microbial origins to produce hydrolysates from fish wastes and underutilized species (Fallah et al. 2015; Gajanan et al. 2016; Klomklao and Benjakul 2016; García-Moreno et al. 2017; Ananey-Obiri et al. 2019; Petrova et al. 2021). Usually, microbial enzymes such as alcalase, neutrase, protamex, and flavourzyme are used, while there are some documented effects of plant proteases such as papain and bromelain. Animal-derived enzymes such as pancreatin and pepsin are also used as proteolytic agents (Tapal and Tiku 2019). Neutrase produces a higher yield and antioxidant and ACE inhibitory activity (Xu et al. 2014; Chen et al. 2016; Mongkonkamthorn et al. 2020). This enzyme also allows protein hydrolysis to yield peptides with C-terminals composed of hydrophobic amino acids such as tyrosine and phenylalanine (Chen et al. 2016).

The physicochemical parameters of the generated hydrolysates are influenced by factors such as the medium and proteolytic enzymes used, as well as the degree of hydrolysis (Jamil et al. 2016). In addition, hydrolysis conditions such as pH, temperature, time, and enzyme concentration influence the properties of the hydrolysates (Ishak and Sarbon 2017). Therefore, optimization of the hydrolysis conditions is needed to produce ideal outcomes in terms of yield and degree of hydrolysis (Jamil et al. 2016). In the optimization of enzymatic protein hydrolysis, the traditional one-factor-at-a-time (OFAT) approach is no longer employed since it is time-consuming and laborious (Zambare and Christopher 2012), and the concurrent effects of multiple factors may not be observed (Zhang et al. 2016).

An alternative approach to optimizing enzymatic hydrolysis is the response surface methodology (RSM), which derives and analyzes multivariable equations using quantitative results where graphical representations defined as response surfaces are generated (Saidi et al. 2013; Ishak and Sarbon 2017). RSM can fill the lapses of OFAT with its mathematical models and cost and time efficiency by minimizing the number of trials and identifying the relationships between the independent variables (Aydar 2018). A study conducted to optimize shrimp waste enzymatic hydrolysis using OFAT and RSM found that the significance of interactions and quadratic effects have been missed in OFAT (Zhang et al. 2016). This emphasizes that the interactions among different factors and discovering their true optimum can be best described by RSM (Chiodza and Goosen 2023).

The study aims to optimize the enzymatic hydrolysis for protein recovery from the yellowfin tuna *(Thunnus albacares)* viscera. The primary objectives

are (1) to determine the hydrolysis conditions through RSM that would yield hydrolysates having the optimum degree of hydrolysis and foaming capacity using the bacterial enzyme, neutrase and (2) to evaluate the crude and soluble protein contents of the hydrolysates produced using the optimum hydrolysis conditions relative to that of the unhydrolyzed yellowfin tuna viscera.

2. MATERIALS AND METHODS

2.1 Sample collection

Yellowfin tuna (*Thunnus* albacares) viscera was obtained from May Bato North, Antique, Philippines, and was immediately placed on an ice box with crushed ice. It was transported to the Seafood Processing Laboratory of the Institute of Fish Processing Technology, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines. The tuna viscera placed in a polyethylene bag was stored at -20 °C until it was used for hydrolysis. The viscera were thawed prior to hydrolysis and further analyses.

2.2 Preparation of the viscera for enzymatic hydrolysis

The viscera was prepared according to the method of Ovissipour et al. (2009) with few modifications on the weight of the viscera, homogenization, and the speed of centrifugation. In the present study, the viscera was minced using a Philips hand blender before heating at 85°C in a water bath for 20 min to disrupt the endogenous enzymes that may interfere during hydrolysis. After heating, distilled water was mixed into the viscera at a ratio of 1:2 (w/v).

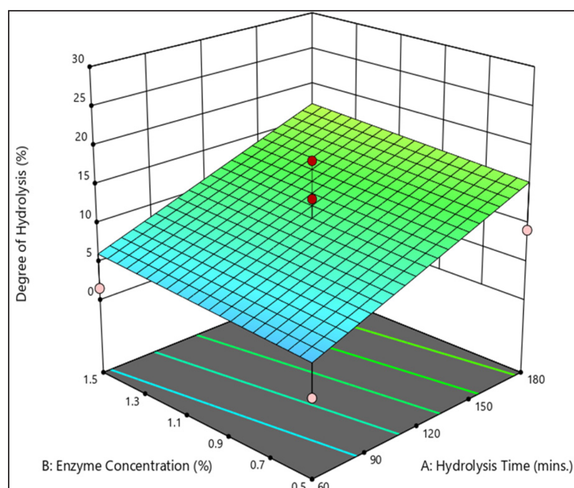

Figure 1. Response surface plot of enzymatic hydrolysis as a function of hydrolysis time and enzyme concentration to the degree of hydrolysis. The plot shows the linear relationship between the degree of hydrolysis and hydrolysis time (A) and the degree of hydrolysis and enzyme concentration (B).

2.3 Optimization of the enzymatic hydrolysis of the yellowfin tuna viscera

The enzymatic hydrolysis reactions were done in triplicates. A 125-ml Erlenmeyer flask was used as a vessel for all reactions containing 10 g of viscera as substrate. The reaction mixture for all replicates was adjusted to pH 7, which is the optimal activity of neutrase by adding 0.2 N NaOH. The samples were placed in a water bath set at 50°C for all experimental conditions. The enzymatic hydrolysis reaction was initiated after adding neutrase to the substrate according to the experimental conditions of each independent variable indicated in Table 1. Following hydrolysis, reactions were deactivated by heating the mixture to 95°C for 15 min. The hydrolysates were then cooled, and the supernatant was collected after centrifugation at 3500 rpm for 20 min.

For the optimization of hydrolysis conditions, the central composite design (CCD) of RSM was used (Table 1). Each independent variable at three levels was coded as -1, 0, and +1. The design provided with 4 center points, 4 factorial points, and 4 axial points for a total of 12 single block runs with two independent variables, hydrolysis time (X1), and

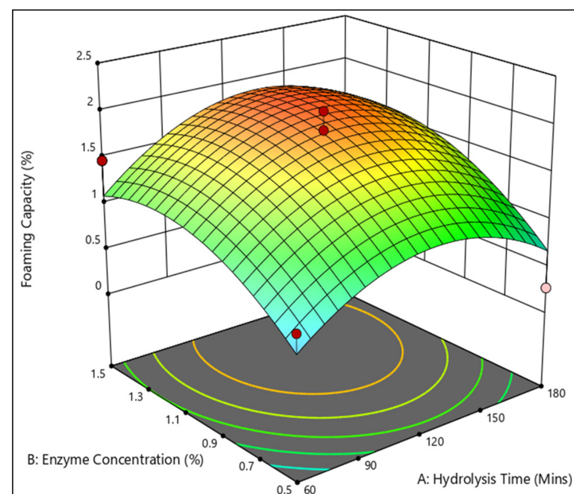

Figure 2. Response surface plot of enzymatic hydrolysis as a function of hydrolysis time and enzyme concentration to foaming capacity. The plot takes a bell-shaped form, suggesting that the foaming capacity increases with increasing hydrolysis time (A) and Enzyme Concentration (B) but to limited values only (120 minutes, 1.0% enzyme concentration). Exceeding these values will reduce the foaming capacity.

Table 1. Hydrolysis conditions of yellowfin tuna visceral protein used in RSM.

Parameter	Factor	Unit	Level of factor		
			-1	0	1
X_1	Hydrolysis Time	min	60	120	180
X_2	Enzyme concentration	%, w/v	0.5	1.0	1.5

The level of factors refers to the range of values tested for the two independent variables (factors): time and enzyme concentration using RSM.

enzyme concentration (X2), all of which were used at three different levels (Ovissipour et al. 2011). The experiments were done randomly to minimize the unexplained variability of the observed responses. The following formula described the method's behavior:

where Y is the dependent variable, which

$$Y = \beta_0 + \sum_{i=i}^{K} \beta_i X_i + \sum_{i=i}^{K} \beta_{ii} X_1^2 + \sum_{i=i}^{n-1} \sum_{i=i}^{n} \beta_{ij} X_i X_j$$

is the degree of hydrolysis and foaming capacity, β_0 is constant and β_i, β_{ii}, β_{ij} are the model's projected coefficients (linear, quadratic, and interaction). While k is the number of factors studied and optimized in the experiment, X_i, and X_j are independent variables of the reactions and $X_i X_j$ and X_1^2 are the interaction and quadratic terms, respectively. The method analyzed the influence of independent components on a reaction (Cao et al. 2008).

2.4 Determination of the protein content of the viscera and hydrolysates

The total crude protein of the viscera and hydrolysates were determined using the Kjeldahl method following AOAC 981.10 for total crude protein analysis (AOAC 2016). Crude protein was determined by multiplying the nitrogen content by a standard conversion factor of 6.25. The soluble protein contents of both the viscera and the hydrolysates were determined using the modified Lowry method described by Mæhre et al. (2018). Bovine serum albumin (BSA) was used as the standard, and the sample maximum absorbances (λ_{max}) were read between 650-660 nm using a UV-Vis spectrophotometer (BMG LABTECH).

2.5 Determination of the degree of hydrolysis

The degree of hydrolysis (DH) was measured using the O-phthaldialdehyde (OPA) assay through the protocol described by Held (2001). The samples and standards were placed in a clear 96-well microplate at 10 µL per well before adding of OPA reagent solution at 200 µL. The mixtures were incubated for 100 seconds at 37°C and the absorbance of the samples was at 340 nm. Leucine was used as the standard sample. Based on the study of Nielsen et al. (2001), the degree of hydrolysis was computed as follows:

$$DH\ (\%) = \left(\frac{h}{h_{tot}}\right) x\ 100$$

where h is the number of hydrolyzed peptide bonds and h_{tot} is the total number of peptide bonds present.

2.6 Determination of the foaming capacity

Foaming capacity (FC) of the yellowfin tuna viscera protein hydrolysates was determined using the method described by Alolod and Nuñal (2018). A 50 mL volume of protein solution (0.5 % v/v) was prepared and homogenized for 1 min using the homogenizer. The mixture was then transferred into a 100 mL graduated cylinder after whipping, and the total volume for each sample was determined and recorded. The method was carried out in triplicate. The following equation was used to calculate foam capacity:

$$FC = \frac{Vt - Vo}{Vo}\ x\ 100$$

where Vt is the volume after whipping (mL) and Vo is the volume before whipping (mL).

2.7 Statistical analysis

RSM using Design-Expert software version 13 was used to generate a completely randomized factorial design for the optimization experiments of tuna viscera enzymatic hydrolysis. Data were subjected to analysis of variance (ANOVA) for mean comparison at 95% confidence level ($p < 0.05$) using the same software. Data for crude and soluble protein contents were also subjected to ANOVA at 95% confidence level ($p < 0.05$) using the IBM SPSS 16 software.

3. RESULTS

3.1 Optimization of hydrolysis conditions to obtain the maximum degree of hydrolysis and foaming capacity

3.1.1 Experimental runs for the optimization of hydrolysis condition

Table 2 shows the experimental runs generated by the Design-Expert software and the corresponding actual and predicted value for the two responses, degree of hydrolysis (DH), and foaming capacity (FC). For the DH, the values ranged from 0 to 26.37 %, and the highest DH was found in the reaction with 1% enzyme concentration and 204 min (Run 11) hydrolysis time. The hydrolysis condition of 0.50 % enzyme concentration and 60 min hydrolysis time (Run 8) yielded the lowest value, with no DH detected.

For the FC, the actual values shown in Table 2 ranged from 0.13% to 2.07%. The hydrolysis time of 120 min and enzyme concentration of 1.0 % (Run 12) yielded hydrolysates with the highest FC, while the hydrolysates with the lowest FC was produced using a hydrolysis time of 35 min and enzyme concentration of 1.0% (Run 3).

3.1.2 Analysis of variance (ANOVA) for the degree of hydrolysis and foaming capacity

The suggested model for DH is the linear model, while for FC, the quadratic model was suggested, as shown in Table 3. The p-value for both responses (DH = 0.0484; FC = 0.0256) was less than 0.05, implying that the model for DH and FC is significant, and this can be used to describe the experimental data.

Table 4 presents the analysis of variance and coefficient of determination (R**2**) of the response surface model. The *p* values of 0.0484 (for DH) and 0.0440 (for FC) indicate that the two independent enzymatic hydrolysis factors significantly affect both responses ($p<0.05$). The R**2** of the DH and FC of the protein hydrolysates were 0.4897 and 0.7951, respectively.

A fundamental explanatory model generated by the software was used to calculate the predicted values obtained in DH and FC shown in Equation 1 and Equation 2, respectively.

Equation 1

$$y= +10.79+5.61A+0.9215B$$

Equation 2

$$y= +1.84+0.1693A+0.3763B+0.0650AB-0.5237A^2-0.3655B^2$$

3.1.3 Response surface plots for the degree of hydrolysis and foaming capacity

Response surface plots of the effects of the two independent factors, hydrolysis time and enzyme concentration to the DH and FC, were generated. For the DH, the surface plot shows that DH is increasing when hydrolysis time is increasing (Fig 1). The same effect is also observed in the relationship between enzyme concentration and DH values. When the two variables are combined, they both showed an effect in the increasing values of DH from the lowest time and enzyme concentration of 60 minutes and 0.5% to its

Table 2. Experimental runs generated by RSM and the actual and predicted value of the degree of hydrolysis and foaming capacity.

Run	Hydrolysis Time (mins.)	Enzyme Concentration (% w/v)	Degree of Hydrolysis (%)		Foaming Capacity (%)	
			Actual Value	Predicted Value	Actual Value	Predicted Value
1	180	0.50	9.24	15.47	0.27	0.67
2	60	1.50	1.50	6.10	1.47	1.09
3	35	1.00	9.54	2.86	0.13	0.55
4	120	1.00	9.75	10.79	1.60	1.84
5	120	1.00	18.23	12.08	1.80	1.84
6	120	1.70	9.40	17.32	1.53	1.65
7	180	1.50	13.32	17.32	1.33	1.56
8	60	0.50	0.00	4.26	0.67	0.47
9	120	0.30	8.17	9.50	0.73	0.59
10	120	1.00	10.61	10.79	1.87	1.84
11	204	1.00	26.37	18.71	1.47	1.03
12	120	1.00	13.30	10.79	2.07	1.84

The experimental runs refer to the combination of time-enzyme concentration values generated by RSM using central composite design (CCD). In the CCD, 4 center points, 4 factorial points, and 4 axial points were inputted that, generated for a total of 12 single block runs. RSM generated predicted values, while the actual values are the results of the actual experiments.

peak value of 120 minutes and 1.0% enzyme ratio. However, the hydrolysis time has a more significant effect on DH than the enzyme concentration.

The contour plot of the FC of the hydrolysates from yellowfin tuna viscera shows a slightly bell-shaped form (Fig 2). This model describes the relationship of FC with enzyme concentration and hydrolysis time, where the FC of the protein hydrolysates from yellowfin tuna viscera is increasing along with the independent factors but to a limited extent. Maximum FC was achieved with a hydrolysis time of 120 min and an enzyme ratio of 1.0%, beyond which FC was reduced.

Table 3. Sequential model sum of squares for the degree of hydrolysis and foaming capacity of yellowfin tuna viscera protein hydrolysates.

Response	Source	Sum of Squares	df	Mean Square	F-value	p-value	
Degree of Hydrolysis (%)	Mean vs Total	1395.98	1	1395.98			
	Linear vs Mean	258.20	2	129.10	4.32	0.0484	Suggested
	2FI vs Linear	1.66	1	1.66	0.0498	0.8291	
	Quadratic vs 2FI	115.00	2	57.50	2.26	0.1851	
	Cubic vs Quadratic	2.75	2	1.38	0.0368	0.9642	Aliased
	Residual	149.66	4	37.41			
	Total	1923.26	12	160.27			
Foaming Capacity (%)	Mean vs Total	18.60	1	18.60			
	Linear vs Mean	1.35	2	0.6753	1.94	0.1994	
	2FI vs Linear	0.0169	1	0.0169	0.0434	0.8402	
	Quadratic vs 2FI	2.20	2	1.10	7.18	0.0256	Suggested
	Cubic vs Quadratic	0.8051	2	0.4025	14.14	0.0154	Aliased
	Residual	0.1139	4	0.0285			
	Total	23.08	12	1.92			

The suggested models by RSM are shown and their corresponding F- and P- values. For the degree of hydrolysis, the linear model is suggested to have an F-value of 4.32, while for the foaming capacity, the quadratic model was suggested with an F-value of 7.12. Both responses have p-values of less than 0.05, implying a significant model used for the study.

Table 4. Analysis of variance and coefficient of determination (R^2) for the response surface model for the degree of hydrolysis and foaming capacity of tuna viscera protein hydrolysates.

Response	Source	Sum of Squares	df	Mean Square	F-value	p-value	R^2
Degree of Hydrolysis (%)	Model	258.20	2	129.10	4.32	0.0484	0.4897
	Residual	269.07	9	29.90			
	Lack of Fit	225.33	6	37.55	2.58	0.2341	
	Pure Error	43.75	3	14.58			
	Total	527.27	11		0.0368	0.9642	Aliased
Foaming Capacity (%)	Model	3.57	5	0.7132	4.66	0.0440	0.7951
	Residual	0.9189	6	0.1532			
	Lack of Fit	0.8060	3	0.2687	7.14	0.0703	
	Pure Error	0.1129	3	0.0376			
	Total	4.48	11		14.14	0.0154	Aliased

R^2 shows the accuracy of the predicted value with the actual value as generated by the RSM. Both responses showed R^2, which lies between 0 and 1 representing an actual value closer to the predicted value.

3.1.4 Conditions for the optimum response for the degree of hydrolysis and foaming capacity

Optimization by response surface modeling gave a combination of hydrolysis conditions for tuna viscera that would yield the optimum DH and FC (Table 5). Hydrolysis using an enzyme concentration of 1.5% and reaction times of 179.50 min and 176.58 min are predicted to yield 17.26% DH and 1.60% FC hydrolysates, respectively.

Table 5. Optimum hydrolysis conditions for the extraction of fish protein hydrolysates.

Response	Independent factors		Predicted Value (%)
	Hydrolysis time (min)	Enzyme Concentration (% w/v)	
Degree of Hydrolysis (%)	179.50	1.5	17. 26
Foaming Capacity (%)	176.58	1.5	1.60

The RSM generated the hydrolysis conditions for the optimum degree of hydrolysis and foaming capacity and the predicted values based on the actual experiments' results.

3.2 Protein contents of the viscera and the hydrolysates

The crude and soluble protein contents of the yellowfin tuna (*Thunnus albacares*) viscera and protein hydrolysates are shown in Table 6. The yellowfin tuna viscera used as raw material for enzyme hydrolysis contained 69.85% protein. The hydrolysates' maximum and minimum protein content produced from the two optimum conditions is 26.97% and 15.52%, respectively. Results also revealed that there are significant differences among ($p < 0.05$) the crude protein content of the raw material and the hydrolysates produced using the two optimum conditions with the significantly highest content ($p < 0.05$) found in the raw material. Comparing the soluble protein content of the three samples showed significant differences ($p < 0.05$) among the hydrolysates produced using the optimum conditions for DH and the other two samples. The significantly highest soluble protein ($p < 0.05$) content of 123.90 mg mL^{-1} was also detected in this sample.

4 . DISCUSSION

RSM with a complete randomized factorial design was used in the present study to optimize hydrolysis conditions such as hydrolysis time and the enzyme concentration on yellowfin tuna viscera by using a microbial enzyme, neutrase. The actual values, as well as the predicted values of the DH in the present study, have a similar range to the study on an eel (*Monopterus* sp.) protein hydrolysate having a DH range of 1.28% - 20.86%. Similar results were also found in the study of Saidi et al. (2013), where the tuna dark-muscle hydrolysates have a DH range of 8.75% - 19.38%.

Table 6. Protein content of the viscera and hydrolysates produced using the optimum hydrolysis conditions for the degree of hydrolysis (DH) and foaming capacity (FC).

Samples	Crude Protein Content (%)	Soluble Protein Content (mg mL^{-1})
Yellowfin Tuna Viscera	69.85 ± 17.35^a	88.43 ± 10.01^a
Hydrolysates from 1.0% neutrase, 204 min hydrolysis	26.97 ± 0.07^b	123.90 ± 7.93^b
Hydrolysates from 1.0% neutrase, 120 mins hydrolysis	15.52 ± 0.92^c	80.38 ± 17.59^a

Values represented are means ± SD. Values in the same column with different superscripts are significantly different ($p \leq 0.05$).

Results of the present study revealed that the FC of the yellowfin tuna protein hydrolysates increased when the hydrolysis time and the enzyme concentration increased but only to a certain level (120 min. hydrolysis time and 1% Neutrase concentration). According to Witono et al. (2016), a characteristic of a higher foaming capacity is directly related to an expansion in surface properties, which is also attributed to incomplete proteolysis wherein a higher number of polypeptide chains are developed, thus enabling more air to be integrated. In addition, the reason for the increasing and the decreasing phenomenon is that the smaller peptides from the lengthy hydrolysis reduce their surface activity, which results in the blocking of a steady film along with the gas bubbles as well as the appearance of hydrophilic proteins throughout the hydrolysis process (Witono et al. 2016).

In the present study, ANOVA was used to illustrate the significance of the regression model for the individual model coefficient and the lack of fit test. In the sequential model sum of squares, the

appropriate model for the experiment is selected based on the highest order polynomial where additional terms are significant, and the model is not aliased. Quadratic was chosen as the appropriate model of foam FC while a linear model for the DH because the p value of both responses was lower than the level of significance ($p < 0.05$) based on Table 3. Having a smaller p value also suggested that the independent variables (hydrolysis time and enzyme concentration) significantly affect DH and FC. In the present study, the best-fit model for the FC is quadratic, which agrees with various studies suggesting the quadratic model is most appropriate for tuna samples (Motamedzadegan et al. 2010; Herpandi et al. 2018; Nguyen et al. 2021). Similarly, the optimization of the foaming capacity of the yellowfin tuna red meat hydrolysates fitted the quadratic model (Parvathy et al. 2018), as does the optimization of the foaming expansion of protein hydrolysates of oneknife unicornfish (Alolod and Nuñal 2018). The F-value, as well as the p-values of both responses obtained in this study, agrees with the reported results by Ishak and Sarbon (2017) for the optimization of enzymatic hydrolysis of shortfin scad waste. For the DH, a linear model was suggested in this study as the best-fit model, similar to the findings of Rasimi et al. (2020) on the yield of the snakehead (*Channa striata*) protein hydrolysates. However, in the studies of Halim and Sarbon (2017) and See et al. (2011), a quadratic model was suggested on eel (*Monopterus* sp.) protein hydrolysates and salmon (*Salmo salar*), respectively.

The R^2 of the response surface models generated in this study were in agreement with the findings of similar studies on visceral proteins (Ovissipour et al. 2010; Ovissipour et al. 2009; Bhaskar et al. 2008). This signifies a better fit between the model and the observations and is also useful for the study to navigate the design model. According to Amiza et al. (2019), there is a more powerful effect on the DH and FC if there is a greater coefficient estimation for a specific term. The positive signs of Equation 1 for DH indicate that each factor gave a linear effect, leading to an increased value of DH. Since factor A (hydrolysis time) showed the highest coefficient estimation, it is the one that has the most effect on the DH, followed by B (enzyme concentration). While for FC, the coefficients A and B also positively affect the response, but the enzyme concentration (B) has the highest value, implying that it has the most effect on the foaming capacity of the hydrolysates.

To further study the effect of the independent variables on the two responses, 3-dimensional response surface graphs were constructed. The observed increasing hydrolysis with increasing enzyme concentration in the present study corresponds to the findings of Jamil et al. (2016) on eel protein, Motamedzadegan et al. (2010) on tuna viscera, and Nurdiyana and Siti Mazlina (2009) on fish waste protein. This indicates that the enzyme-to-substrate ratio and reaction time have partially linear interactions with DH, wherein a relatively high concentration of enzymes will activate more peptide bonds in the material (Mohammad et al. 2015). Similarly, relationships between enzyme activity and hydrolysis time have also been noted in several studies using microbial proteases (Bhaskar et al. 2008; Ovissipour et al. 2010; Molla and Hovannisyan 2011)

Thuy et al. (2004) reported that when the hydrolysates were hydrolyzed for 80 to 92 min, the foaming capacity of the protein hydrolysates from *Pangasius hypophthalmus* increased gradually. However, when the hydrolysis time was extended, its capacity decreased. Findings on the foaming ability of protein hydrolysates as affected by temperature and enzyme concentration by Souissi et al. (2007) and Shaviklo et al. (2012) are in agreement with the results of the present study. It was observed that hydrolysis also increases when the amount of enzyme and the temperature increase, which eventually results in the enzyme becoming saturated. If hydrolysis is extended, more proteins with low molecular weight are released, resulting in a high foaming capacity. However, it was proposed that the foaming ability may be reduced by the generation of amino acids due to protein hydrolysis (Thuy et al. 2004). Further, the low foaming capacity of some protein hydrolysates may be due to the small size of the peptides, which prevents the creation of a secure film across the gas bubbles and the appearance of soluble peptides during extended hydrolysis (Souissi et al. 2007).

The optimum conditions for the highest degree of hydrolysis in the present study are similar to the results of Motamedzadegan et al. (2010) on hydrolyzing the viscera of yellowfin tuna using the enzyme neutrase with the hydrolysis time of 141 mins and enzyme activity of 39.61 AU/kg protein. Also, optimum conditions for the hydrolysis of the viscera of beluga sturgeon were at a similar range of 120 mins and an alcalase concentration of 34 AU/kg crude protein (Ovissipour et al. 2009). Moreover, the results of the present study are also in agreement with Alolod and Nuñal (2018), an enzyme concentration of 2.11% and time of 137.45 mins for the DH, as well as a 2.13% enzyme ratio and 147.73 mins for the foaming capacity, was found to be the optimum conditions for oneknife unicornfish skin gelatin.

Comparing the protein content of the samples with the highest DH and FC values showed that as the hydrolysis time increases, the protein content also increases. This finding agrees with the study of Yathisha et al. (2022), where there was an increase in protein content in the ribbon fish (*Lepturacanthus savala*) as the hydrolysis time increased in the first 3 hours of enzymatic treatment. The period containing the desired protein has a rapid phase during the first hour, preceded by a slow increase once the vital protein has been hydrolyzed. The hydrolysates produced using the optimum conditions for DH were also found to have a higher soluble protein content than the raw material. Protein hydrolysis is the mechanism of breaking down protein peptide bonds into smaller units with the help of some agents. It creates some changes in the proteins, which enhances the solubilization, resulting in the increasing of the ion from the content of the amine and carboxyl groups, leading to a reduced molecular mass of protein as well as the unfolding of the globular proteins, thus resulting in higher soluble protein content than the original raw material (Romadhoni et al. 2016).

Some studies show that hydrolysis time also affects the protein solubility of the viscera and that enzyme concentration may influence solubility (Ramakhrishnan et al. 2013). It was reported by Benjakul and Morrissey (1997) and Gildberg (1993) that an increase in the enzyme concentration of Alcalase also resulted in an increase in the overall proteolysis rate and solubilization of protein. However, it was speculated that the fish tissues contain complex substrates with a high concentration of proteinase inhibitors, making it challenging to illustrate the hydrolysis reaction. Moreover, this increase in protein concentration may also be linked to the possible increase of insoluble nitrogen in fish protein as a result of hydrolysis (Agustin et al. 2021).

5. CONCLUSION

Optimization of the hydrolysis conditions to yield the maximum degree of hydrolysis and foaming capacity was carried out using RSM, where two factors were considered, hydrolysis time and enzyme concentration, that may significantly affect the DH and FC. The range of DH (0 - 26.37%) and FC (0.13% - 2.07%) obtained in the present study coincides with the reported findings of published studies. Results of the present study indicate that RSM is a helpful method for optimizing enzymatic hydrolysis to achieve the desired hydrolysates. For the two hydrolysates produced using the different optimum conditions, the resultant hydrolysate of the conditions for the maximum DH has higher crude and soluble protein content than the hydrolysate from the conditions for maximum FC. However, proper replication of the factorial, axial, and center points is recommended. Other independent variables, such as hydrolysis temperature and pH, can also be used for surface model responses. Moreover, the optimization method may also be used to hydrolyze other processing wastes, such as skins and heads or other processed fishery products.

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AUTHOR CONTRIBUTIONS

De Asis MFDS: Investigation, Formal Analysis, Writing and Editing of the manuscript. **Nuñal SN:** Conceptualization, Resources, Supervision, Formal Analysis, Review and Editing of the manuscript. **Endoma LF:** Formal Analysis, Review and Editing of the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that can be found to influence the work reported in this paper.

ETHICS STATEMENT

No live animal studies were carried out by the authors.

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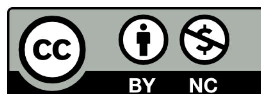

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