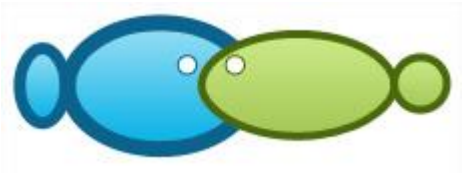

Optimization of protein hydrolysate from visceral waste of Nile tilapia (*Oreochromis niloticus*) by response surface methodology

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Abstract. The present study was targeted to determine the best hydrolysis conditions to produce visceral protein hydrolysates from Nile tilapia (*Oreochromis niloticus*) using alcalase enzymes. Box-Behnken design for response surface methodology was used to optimize the hydrolysis conditions (time, pH and temperature) for preparing protein hydrolysates from Nile tilapia visceral waste. The results showed that the temperature and time are the main factors that influence the degree of hydrolysis. The relationship between the variables and the degree of hydrolysis is characterized by the following equation: $Y=41.46–0.6150A+0.5350B+0.1975C-1.21AB–0.6750AC–0.8500BC–4.64A^2–3.87B^2–3.39C^2$, where A, B and C are pH, temperature and time, respectively. The optimized hydrolysis should be carried out at a pH of 7.9, a temperature of 55.8^0C and with an alcalase enzyme concentration of 1.5% for 1.5 h. At these optimal conditions, alcalase hydrolysis resulted in a desirability value of 0.854, with 41.46% degree of hydrolysis. The amino acid (AA) with the highest value in Nile tilapia viscera hydrolysates is glutamate (5.32 g/100 g), while the AA with the lowest level is glutamine (0.32 g/100 g). The AA analysis indicates that the AA profiles of the Nile tilapia viscera hydrolysates were generally high in essential AAs. The optimal hydrolysis condition can be a good reference for enhancing the potential of visceral protein hydrolysates in future functional food development.

Key Words: amino acids, RSM, Box–Behnken design, Nile tilapia viscera.

Introduction. Nile tilapia (*Oreochromis niloticus*) production in Indonesia has been increasing in recent years. The total production of Nile tilapia was 567078 tons in 2011 and increased to 1084281 tons in 2015 (Ariansyach 2017). The increasing Nile tilapia production has generated a more significant amount of organic waste, including head, skin, fins, tail, frames, viscera and scales. The large quantities of fish waste can create severe pollution, causing problems in the environment, health and economy (Vidotti et al 2003; Arvanitoyannis & Kassaveti 2008). Moreover, viscera fish waste, rich in fat and protein, is more easily degradable.

Twenty percent of the freshwater fish biomass is viscera, a source of high levels of protein and lipids (Bhaskar et al 2008). The content of protein and lipid in fish viscera varies depending on species, sex, age and other factors. The protein content in the viscera of the Persian sturgeon (*Acipenser persicus*) is 15.48%, in catla fish (*Catla catla*) is 8.52%, in yellowfin tuna (*Thunnus albacores*) is 16.72% (Bhaskar & Mahendrakar 2008) and in tuna fish (*Euthynnus affinis*) is 50.18% (Nurhayati et al 2013). For utilizing this protein-rich fish processing by-product waste, several biotechniques have been developed, such as enzymatic hydrolysis (Horn et al 2005), which changes protein waste to hydrolysate. A study by Darmato et al (2017) indicates that fish waste can be considered as alternative healthy diet by adding the fish collagen from bones with Taro (*Colocasia esculenta*) and seaweed (*Eucheuma cottoni*) on rice.

The hydrolysis of proteins is the process of breaking a peptide bond from a protein structure into a simpler bond, by using either enzymes, acids or bases. Enzymatic hydrolysis is more beneficial, because reaction conditions are mild, defective product numbers are low and the quality of the product is high (Horn et al 2005). Enzymatic hydrolysis produces protein hydrolysates with proper nutritional and functional properties (Benjakul et al 2014; Klomklao et al 2013; Ryan et al 2011; Chalamaiah et al 2012). In order to control the enzymatic hydrolysis process, enzyme selection is essential, because different enzymes have different characteristics and abilities. Commercial enzymes are most commonly used to obtain the final product and determine the degree of hydrolysis (Benjakul et al 2014).

Alcalase is known as a highly efficient enzyme in the hydrolysis of fish proteins due to its ability to achieve high levels of hydrolysis in a relatively short time (Horn et al 2005; Villamil et al 2017). Several studies have been employed on enzymatic hydrolysis of fish by-products, such as tuna fins (Lee et al 2010), head and viscera of sardines (Souissi et al 2007), heart (Je et al 2009) and a mixture of skin, viscera, red meat, fins and bones (Muzaifa et al 2012), using alcalase.

The optimum rate of fish protein hydrolysis depends on the value of the degree of hydrolysis (DH) where a percentage of peptide bonds is broken down in smaller peptides (Wang et al 2018). Several methods have been developed to obtain the optimum DH during protein hydrolysis, with the use of trinitrobenzene sulfonic acid (TNBS), O-phthaldialdehyde (OPA), trichloroacetic acid, soluble nitrogen (SN-TCA) and formal titration. Many studies on protein hydrolysates have been performed using various types of enzymes and fish species, and even fish waste, but there has been no research on the utilization of the viscera of Nile tilapia. Therefore, research on the best conditions for tilapia viscera protein (TVP) hydrolysis is lacking.

Response surface methodology (RSM) was first proposed in 1951 and has been widely used in both research and industrial applications. The significant advantages of this program can be used for analysis and modelling a problem with one or more treatments (Raissi & Farsani 2009). RSM is a combination of statistics and mathematical techniques for developing, improving and optimizing processes where responses are influenced by independent variables (Radojkovic et al 2012). RSM not only defines the influence of independent variables but also produces mathematical models, which explain chemical or biochemical processes (Anihouvi et al 2011). In addition, other advantages of using the RSM method are that it does not require large amounts of experimental data and neither a long time. RSM has proven effective in optimizing and monitoring food processes (Wangtueai & Noomhorm 2009). Therefore, this study aims to determine the best conditions to produce protein hydrolysates by treating enzyme levels, acidity (pH), hydrolysis temperature and hydrolysis time, factors that affect the hydrolysis degree, using RSM.

Material and Method

Sample preparation. The visceral waste of Nile tilapia was obtained from processed tilapia from PT Aquafarm Nusantara, Semarang Industrial Estate, located near the laboratory. The study was conducted from September to December 2018. On the way to the laboratory, the viscera of tilapia was put in a cool box container filled with ice to keep it cold (-20^{0}C). After arriving at the laboratory, the viscera was cleaned with water, the fat lining was removed, then the viscera was weighed and the hydrolysis process was carried out. The protease enzyme used in this study was alcalase (Sigma Aldrich) with an activity of ≥0.75 Anson units/mL.

Defatting process. The defatting process was carried out using a modified method proposed by Bhaskar et al (2008). Viscera and Aquadest (1:1) were mixed using a blender until a homogeneous paste was obtained. It was heated at 85^{o}C for 20 minutes to inactivate endogenous enzymes. The homogenate was centrifuged at 10^{o}C for 20 minutes at 5800 rpm to separate fat from protein. The fat phase was discarded and the remaining residues rich in protein were collected. The residues rich in protein were extracted three times with distilled water at 1:1 (w/v), obtaining protein extracts.

Determination of the best condition of hydrolysis using RSM. RSM was statistically analyzed by the Design Expert 11.0® program (Stat-Ease Inc., Minneapolis). Multiple regression analysis was performed to obtain the regression coefficient. The estimated coefficient, with a level higher than 95% (P>0.05), was included in the Box Behnken Design (BBD) model. The degree of hydrolysis can be expressed as a function of the independent variable with the second order polynomial equation (Equation 1):

$$Y=\beta_0+\sum \beta_0 X_j+\sum \beta_{ij} X_j^2+\sum \beta_{jk} X_j X_k$$

Where: Y is the response (DH); $ß_0$ is the interception; $ß_j$ is the linear term, $ß_{jj}$ are the squares and β_{jk} are the interactions.

The obtained responses were statistically evaluated and the model was built based on variables with a confidence level of more than 95%. Optimization of hydrolysis conditions is achieved by using the RSM method, which consists of 4 stages (Montgomery 2001): (1) making a formulation and a response design; (2) formulation; (3) response analysis; (4) optimization.

Formulation preparation and response design. Formulation and response design was performed with the Design Expert 11.0® program to determine dependent and independent variables. The dependent variable was a variable whose value was maintained equal in each treatment, because it is considered not to affect the response, while the independent variable was a variable that affects the response. In this study, the dependent variable was the number of visceral extracts of Nile Tilapia that that was hydrolyzed, while the independent variables were the pH, temperature and time of hydrolysis. The enzyme concentration was 1.5% of the extract (v/w). The determination of independent variables in this study was based on previous studies (Bhaskar et al 2008; Ovissipour et al 2012; Nurhayati et al 2014). Trial and error tests were carried out to determine the minimum and maximum limits (Table 1).

The minimum and maximum limits were introduced in the Design Expert 11.0® program, RSM Box-Behnken Design (BBD) for randomization. Three different factors (pH, temperature and time) were used on the same three levels (-1 and +1). The hydrolysis level (DH%) is determined as the response variable (Y). Each run containd 50 mL of protein extract. Protein extracts were hydrolyzed with 1 N sodium hydroxide using a digital pH meter (Cyberscan 1001, Eutech, Singapore). The solution was inactivated at a temperature of 80-85°C for 20 minutes. After that, the sample was incubated at 4°C for 24 h, centrifuged for 20 minutes and dried using a freeze dryer. The degree of hydrolysis was calculated by the SN-TCA method (Hoyle & Merritt 1994; Amiza et al 2012). A total of 20 mg of protein hydrolysate was added to 20 mL of TCA 10% (b/v). The mixture was incubated for 30 minutes and then centrifuged (7800 rpm, 15 minutes). The supernatant was analyzed for nitrogen content using the Kjeldahl method (Chintong & Pichaiyongvongdee 2016). The degree of hydrolysis was calculated by the following formula (Equation 2):

$$\%DH=\frac{100\%TCA\ N_2\ dissolved\ in\ sample}{N_2\ total}\times 100$$

Where: %DH - hydrolysis level; TCA - trichloroacetic acid.

Formulation stage. The hydrolysis of Nile tilapia visceral waste with the treatment of temperature, pH and different hydrolysis times was performed to obtain the best DH.

Response analysis. Each response variable was analyzed using ANOVA. The following ANOVA models were used in this study: linear, quadratic, special cubic and cubic. The model that gave significance to ANOVA and non-significance on lack of fit was chosen to analyze the variables.

Table 1

The range of independent variable values

Component	*Independent Variable*	*Minimum*	*Maximum*
A	pH	7	9
B	Temperature (Celsius degrees)	45	65
C	Time (hours)	1	2

Optimization stage. The response of the DH was optimized by the Design Expert 11.0® program. The program performs the optimization according to the variables and response measurement data. The output of the optimization is the recommendation of several new formulas that are optimal according to the program. The best formula is the one with the maximum desirability value. The desirability value is the value of the optimization objective function that shows the ability of the program to fulfil desires based on the criteria specified in the final product. The range of values is from 0 to 1. The desirability value that is closer to 1 indicates the better ability to produce the desired product (Raissi & Farsani 2009).

Amino acid analysis and computation of chemical score. The AA compositions were identified using High Performance Liquid Chromatography (HPLC). The chemical score (CS) of the protein hydrolysates was computed to study the nutritional value of protein hydrolysates of tilapia viscera. The score is related to the essential AA profile in a standard protein as described by Joint FAO/WHO/UNU (2007). The chemical score was calculated using the equation:

$$CS = \frac{\text{EAA in test protein (g } 100g^{-1})}{\text{EAA in standard protein (g } 100g^{-1})}$$

Where: CS - chemical score; EAA - the essential amino acid content.

Results and Discussion

Optimum conditions for the degree of hydrolysis. The highest DH was 41.46%, at pH 8, 55°C, 1.5 h and 1.5% enzyme concentration (Table 2). These results were used to construct the model based on several parameters including the Sequential Model Sum of Squares, Lack of Fit Tests and Summary Statistics Models. A possible model for RSM is the Linear Model, 2F1 and Quadratic.

In recent years, studies about hydrolysis of Nile tilapia viscera were limited to fins, head and tail (Shamloo et al 2012; Roslan et al 2015). Most studies revealed that the highest DH was achieved in 15 to 20% after 5 hours of hydrolysis, except from the results of Foh et al (2010), who obtained DH values of almost 25% after hydrolyzing for 120 minutes. The DH obtained in this study was relatively low compared to the results for the viscera protein of Catla (50%) (Bhaskar & Mahendrakar 2008) and Yellowfin Tuna (48.66 %) (Ovissipour et al 2012). However, they were relatively high when compared to Barramundi (*Lates calcarifer*) (23.1%) (Nurhayati et al 2014).

Model selection test using Sequential Sum of Squares Models. The Box-Behnken Design (BBD) has many mathematical models that can be used to determine the best design. Model selection using Sequential Sum of Squares Models based on the number of squares and sequence of models, where the best model is a model that has a probability value of less than 5%. The quadratic model was the best candidate model due to its probability values less than 5% (P value: 0.0001) (Table 3).

Table 2

Optimum condition for the Degree of Hydrolysis in different PH, temperatures and time

Formula	*pH*	*Temperature (^{0}C)*	*Time (hours)*	*Degree of Hydrolysis (%)*
1	9	65	1.5	32.59
2	8	55	1.5	41.46
3	8	55	1.5	41.46
4	7	55	1	33.34
5	7	65	1.5	34.98
6	9	55	2	32.17
7	8	65	1	35.55
8	8	65	2	33.32
9	7	45	1.5	30.89
10	8	55	1.5	41.46
11	8	55	1.5	41.46
12	8	55	1.5	41.46
13	7	55	2	36.01
14	9	55	1	32.20
15	8	45	1	33.38
16	9	45	1.5	33.34
17	8	45	2	34.55

Model selection test based on Lack of fit. Lack of fit test using Design Expert 11.0® recommends the quadratic model (Table 4). Lack of fit test is based on model incompatibility. The parameter used to show the model incompatibility is the P value. The insignificant lack of fit value is a requirement for a good model because it shows the suitability of the response of the degree of hydrolysis with the model (Sin et al 2006).

Table 3

Sequential model sum of squares analysis

Source	*Sum of Squares*	*df*	*Mean Square*	*F Value*	*P value*	
Mean vs Total	21860.97	1	21860.97			
Linear vs Mean	5.63	3	1.88	0.1010	0.9580	
2F1 vs Linear	10.57	3	3.52	0.1526	0.9257	
Quadratic vs 2 F1	225.31	3	75.10	93.77	<0.0001	Suggested
Cubic vs Quadratic	5.61	3	1.87			Aliased
Residual	0.0000	4	0.0000			
Total	22108.09	17	1300.48			

Model selection test based on R Squared. R Squared test was performed to select the best model. The model was selected based on the value close to 1. R Squared test recommends model Quadratic as the best (Table 5).

Table 4

Lack of Fit test results using Design Expert 11.0® software

Source	*Sum of Squares*	*df*	*Mean Square*
Linear	241.49	9	26.83
2F1	230.92	6	38.49
Quadratic	5.61	3	1.87
Cubic	0.0000	0	
Pure Error	0.0000	4	0.0000

Table 5

Model summary statistics

Source	*Standard Deviation*	*R-Squared*	*Adjusted R-Squared*	*Predicted R-Squared*	*PRESS*	
Linear	4.31	0.0228	-0.2027	-0.4337	354.29	
2F1	4.81	0.0655	-0.4951	-1.2573	557.83	
Quadratic	0.8949	0.9773	0.9481	0.6370	89.70	Suggested
Cubic	0.0000	1.0000	1.0000		*	Aliased

Table 6

ANOVA Model of the hydrolysis of Nile tilapia viscera

Source	*Sum of Squares*	*df*	*Mean Square*	*F Value*	*P value*	
Model	241.51	9	26.83	33.50	<0.0001	significant
A - pH	3.03	1	3.03	3.78	0.0930	
B - Temperature	2.29	1	2.29	2.86	0.1347	
C - Time	0.3120	1	0.3120	0.3896	0.5523	
AB	5.86	1	5.86	7.31	0.0305	
AC	1.82	1	1.82	2.28	0.1752	
BC	2.89	1	2.89	3.61	0.0993	
A^2	90.65	1	90.65	113.18	<0.0001	
B^2	63.06	1	63.06	78.74	<0.0001	
C^2	48.39	1	48.39	60.42	0.0001	
Residual	5.61	7	0.8009			
Lack of Fit	5.61	3	1.87			
Pure Error	0.0000	4	0.0000			
Cor Total	247.12	16				

Analysis of variance (ANOVA) test. ANOVA is used to determine the interaction between responses and variables in the hydrolysis process. The significance of a factor is based on the probability of the P value that must be less than 5%. This model equation has one linear term and three quadratic effects, namely AB, A^2, B^2 and C^2 (Table 6). The lack of fit is not significant compared to the possibility of pure error, so the lack of fit value that occurs is caused by noise. The model equation suggested by ANOVA is:

Degree of Hydrolysis = 41.46 – 0.6150A + 0.5350B + 0.1975C -1.21AB – 0.6750AC – 0.8500BC – $4.64A^2$ – $3.87B^2$ – $3.39C^2$

Where: A is pH; B is temperature; C is time. According to Sin et al (2006), the regression model is well defined if the value of R^2 is higher than 0.80. A small R^2 value indicates a poor relevance of the dependent variable in the model. The model can match the actual data when R^2 approaches unity (Sin et al 2006).

The effects of the independent variables on the hydrolysis level can be visualized through a three-dimensional response surface based on the polynomial model (Figure 1A-C). The DH increases with increasing temperature. DH was found to increase rapidly at the beginning of the reaction and reach the optimum value at a temperature of 55.78°C. However, a decrease in DH was observed above the optimum temperature level. High-temperature levels will cause complete inactivation of the enzyme alcalase due to thermal denaturation. These results confirm other findings (Adler-Nissen 2008), where alcalase is active at temperatures ranging from 50 to 70°C.

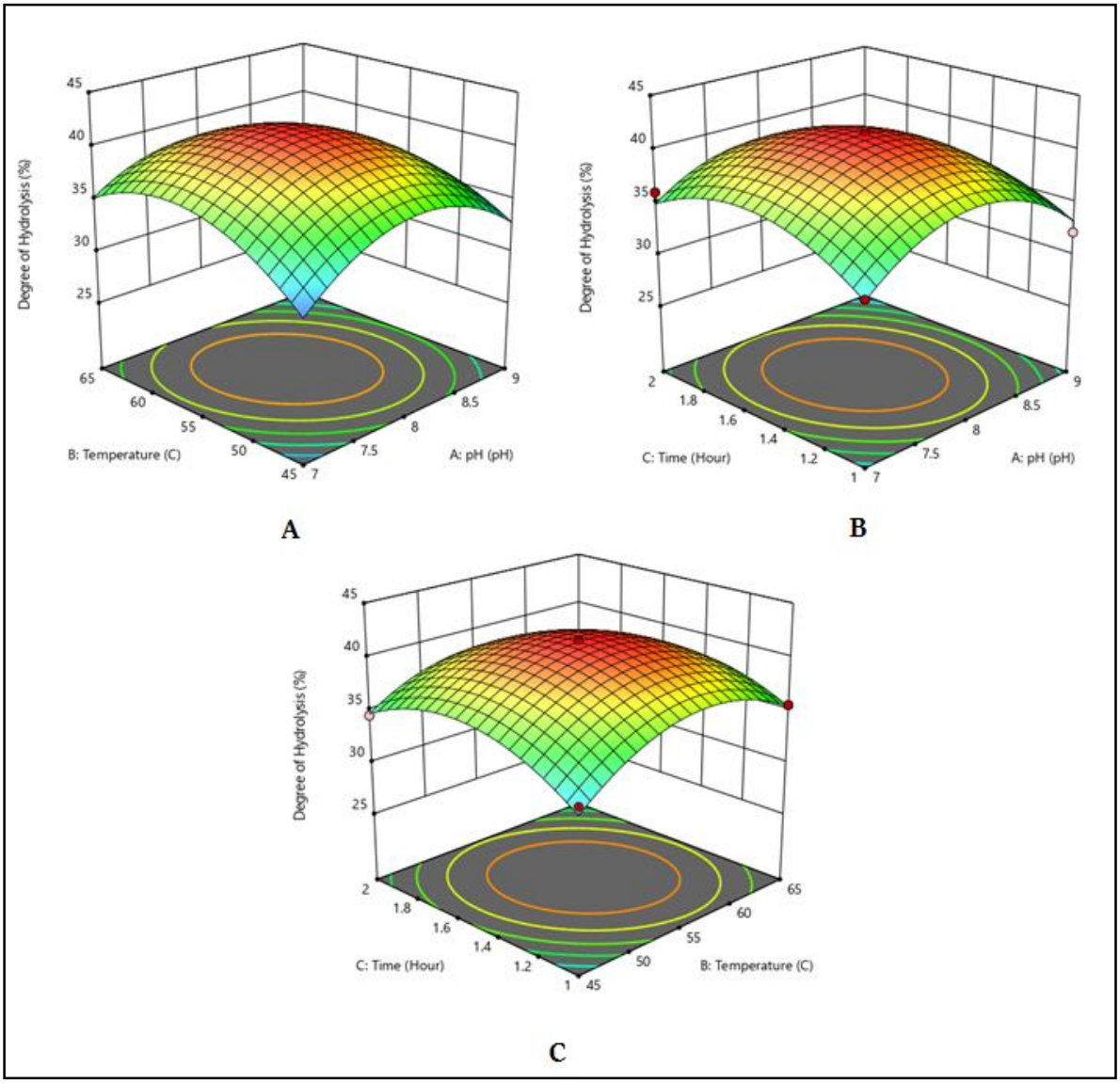

Figure 1. Response surface graph for the degree of hydrolysis as a function of: A - temperature and pH; B - time and pH; C - time and temperature, during the hydrolysis of tilapia (*Oreochromis niloticus*) viscera protein with alcalase enzyme.

DH also increased with increasing pH. The optimum pH for alcalase against TVP hydrolysis is 7.9 and DH begins to decrease when the pH is outside 7.9. The range of pH in which alcalase functions is 6-10 (Bhaskar & Mahendrakar 2008; Ovissipour et al 2012; Barkia et al 2010). DH also increases with increasing time (Figure 1A). DH grew rapidly at the beginning of the reaction and reached its optimum value after 1.5 hours (Figure 1B). The more time passes, the hydrolysis process runs better (Liceaga-Gesualdo & Li-Chan 1999). However, a decrease in DH occurs after reaching the optimum time. The total hydrolyzed protein will increase with increasing hydrolysis time until it reaches a stationary state and shows a linear value (Coligan et al 2002).

DH increases over time with the increase of temperature (Figure 1C). DH increases rapidly at the beginning of the reaction and reaches the optimum value after 1.5 hours and a temperature of 55.78°C (Figure 1C). However, the decrease in DH is clearly seen after reaching the optimum length of time and level of temperature. Some studies about fish by-product hydrolysis using alcalase showed that the optimum temperature is 60°C (Radojkovic et al 2012; Ryan et al 2011; Wangtueai & Noomhorm 2009).

The optimization of the hydrolysis process. The temperature variable is optimized within a range of 45-65^{0}C with an interest level of 3 (+++). pH variable is optimized within a pH range of 7-9, with a level of importance of 3 (+++). The time variable is optimized within a range of 1-2 hours, with the importance level of 3 (+++). Temperature, pH and period of hydrolysis will affect the value of the hydrolysis degree. The response to the degree of hydrolysis is an optimized response with a level of importance of 5 (+++++). Based on the optimization process, the Design Expert 11.0® software provides one optimum formula solution that can be seen in Table 7 and Figure 2.

Table 7

Formulation obtained from the optimization process

Number	*pH*	*Temperature*	*Time*	*Degree of Hydrolysis*	*Desirability*	*Desirability (w/o Intervals)*
1	7.921	55.800	1.514	41.508	0.854	1.000

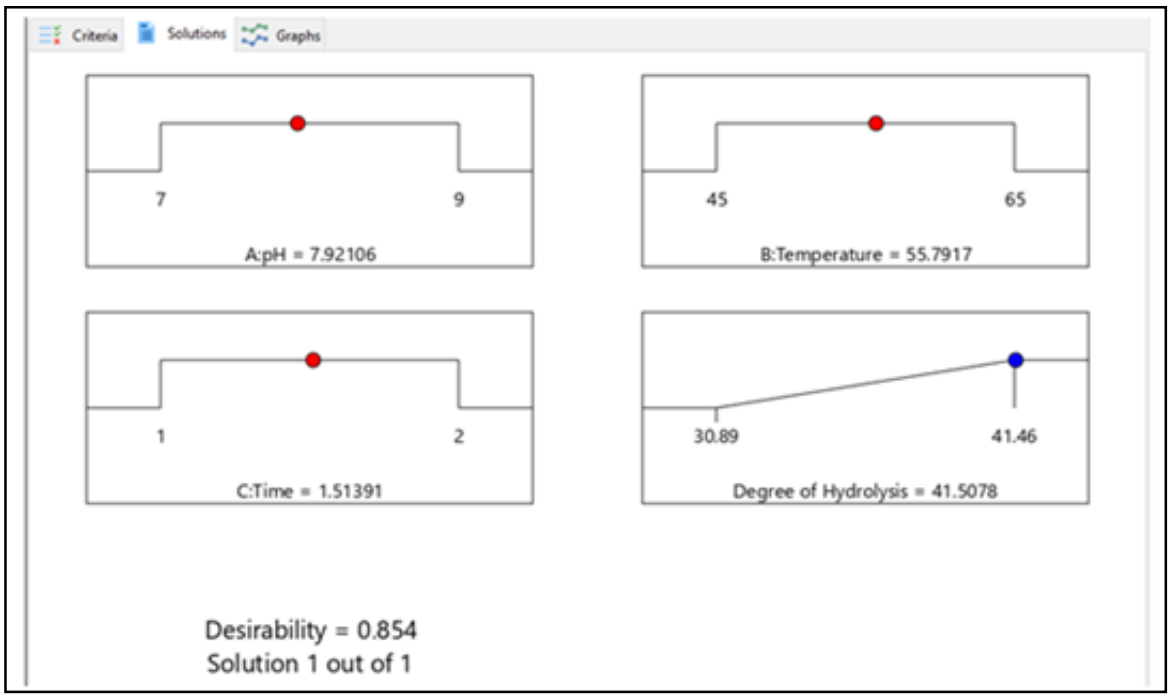

Figure 2. Solution optimization by Design Expert 11.0® software.

Based on the optimization results, the optimum conditions of hydrolysis with alcalase enzyme 1.5% (w/v) were pH 7.9 and 55.8°C, for 1.5 h, for producing the highest degree of hydrolysis, 41.51%. The desirability value was 0.854, which indicates the ability of the software to exhibit the best product with the desirability value as closest to 1.0 as possible. Therefore, with the increase in DH by using RSM, the production of visceral protein hydrolysates from fish in optimal conditions is possible and economically attractive. Further studies on the effectivity of visceral protein hydrolysates, such as *in vitro* and *in vivo* tests using animal model, are necessary to provide a larger perspective on this matter.

Amino acid content. The AA that has the highest level is glutamate (5.32 g/100 g), while the AA with the lowest level is glutamine (0.32 g/100 g) (Table 8). Glutamate is an AA widely found in fishery products and it plays a role in the taste of a product (Widyastuti et al 2014). The results of this study are not different from those of other studies, which indicate that glutamate has the highest content in catla viscera hydrolysate, 15.01 g/100 g (Bhaskar et al 2008), and sturgeon viscera hydrolysate 13.70 g/100 g (Ovissipour et al 2012).

Protein quality can be determined based on the content of essential AAs that construct it. The essential AA content from tilapia viscera protein hydrolysate is quite large compared with the percentage of AAs in the source protein. The essential AA content from the hydrolysate protein of tilapia viscera was 53.08% of the total AA content analyzed. Protein hydrolysate can be used as a source of fish feed protein (Bhaskar et al 2008).

Table 8

The amino acid composition of tilapia visceral protein hydrolysate and chemical score in comparison with the FAO/WHO reference protein

Amino Acid	*Quantity (g/ 100 g)*		*Chemical score*	
	Fish visceral protein hydrolysate	*Reference protein**	*Reference Protein***	*Reference Protein ******
Essential amino acids (53.08 %)				
Histidine	2.04	1.6	0.71	2.1
Isoleucine	1.56	1.3	1.74	2.5
Leucine	2.19	1.9	1.54	3.3
Lysine	2.82	1.6	2.24	5.7
Methionine	0.88	1.7	0.67	3.1
Phenyl alanine	1.07	-	-	6.5
Tyrosine	1.42	-	-	-
Threonine	1.26	0.9	1.96	3.9
Tryptophan	0.42	-	-	-
Arginine	1.93	-	-	1.31
Valine	2.78	1.3	1.82	3.6
Non-essential amino acids (46.92 %)				
Asparagine	0.36	-	-	-
Glutamate	5.32	-	-	-
Serine	1.64	-	-	-
Glycine	2.29	-	-	-
Alanine	2.66	-	-	-
Proline/Hydroxy Proline	1.69	-	-	-
Cysteine	0.47	-	-	-
Aspartate	3.96	-	-	-
Glutamine	0.32	-	-	-

Note: * - 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).

Chemical score. The chemical score provides an estimate of the nutritive value of a protein. This parameter compares levels of essential AAs from the studied matter with those of standard proteins (Ovissipour et al 2012). In the current study, the computed chemical scores were based on the AA requirements of common carp (NRC 1993). The AA composition in this study and the comparison with reference proteins indicates that the AA profiles of the Nile tilapia viscera hydrolysates were generally comparable in essential AAs with the suggested AA requirements recommended by FAO/WHO and NRC. Similar results are reported for yellowfin tuna viscera hydrolysates (Ovissipour et al 2012).

Conclusions. The optimized hydrolysis conditions for Nile tilapia viscera protein hydrolysates with an alcalase enzyme concentration of 1.5% are 7.9 pH, 55.8°C, 1.5 h and a desirability value of 0.854. The optimal hydrolysis conditions could be good references for enhancing the potential of visceral protein hydrolysates in future functional food development.

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