Food Research 5 (4) : 153 - 162 (August 2021)
Journal homepage: http://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Optimization of enzymatic protein hydrolysis conditions of Asiatic hard clam (*Meretrix meretrix*)

Zainol, M.K., Abdul Sukor, F.W., Fisal, A., Tuan Zainazor , T.C., Abdul Wahab, M.R. and *Zamri, A.I.

Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Mengabang Telipot, 21030, Kuala Nerus, Terengganu

Article history:
Received: 3 December 2020
Received in revised form: 21 January 2021
Accepted: 2 April 2021
Available Online: 25 July 2021

Keywords:
Protein hydrolysate,
Response Surface Methodology,
Asiatic hard clam,
Hydrolysis

DOI:
https://doi.org/10.26656/fr.2017.5(4).701

Abstract

This study was aimed to optimise the Alcalase® enzymatic hydrolysis extraction of Asiatic hard clam (AHC) (*Meretrix meretrix*) protein hydrolysate in terms of hydrolysis time, hydrolysis temperature, hydrolysis pH, and concentration of enzyme. Protein hydrolysate produced from AHC (*M. meretrix*) meat was used to determine the optimum hydrolysis conditions. Hydrolysis of AHC meat was optimised using the Central Composite Design Response Surface Methodology (RSM) (CCD). The relationship between four parameters such as temperature (45 – 65°C), enzyme to substrate concentration (1 – 2%), hydrolysis time (60 – 180 mins), and pH (7.5 – 9.5) to the degree of hydrolysis was investigated. The optimum conditions for enzymatic hydrolysis of AHC meat to achieve the maximum degree of hydrolysis (DH) were observed at 65°C, enzyme to substrate concentration of 1%, hydrolysis time of 60 mins, and pH 7.5. The enzymatic protein hydrolysis of AHC meat was predicted using a two factors interaction (2FI) model. Under these optimum conditions, DH's predicted value was 97.41%, which was close to the experimental value (97.89%). The freeze-dried protein hydrolysate powder was characterized concerning the proximate composition. Proximate analysis revealed that the AHC meat contains 7.92±1.76% of moisture, 2.23±0.89% of crude fat, 1.98±0.82 of ash, and 10.53±0.04% of crude protein. While the Asiatic hard clam protein hydrolysate (AHCPH) composed 9.12±0.02% of moisture, 0.80±0.29% of crude fat, and 27.76±0.10% of ash. The protein hydrolysate produced also contained high protein content (50.09±0.88%) and may serve as a good protein source.

1. Introduction

Proteins are one of the most important nutritional components in food production and contribute to foods' functional and organoleptic properties (Toro and Garcia-Carreno, 2002). Protein hydrolysate is the product obtained after the hydrolysis of proteins is achieved by acid, alkali, enzymes, and fermentation methods (Hau *et al*., 2017). Enzymatic hydrolysis is preferred due to its faster reaction rate, mild conditions, and high specificity (Bhaskar *et al.,* 2008). Protein hydrolysates have a high potential to be used as ingredients in healthcare, nutrition, food industry, and cosmetics (Radha *et al*., 2007).

Shellfish are a major but cheap protein source for human consumption and a source of income for small-scale fishermen, providing a livelihood for people in coastal areas and import-export exchange (Wan Norhana *et al*., 2011). Shellfish also offers various taste and texture experiences and adds excitement to the menu and retail display (Roose and Mulier, 2020). Clams and mussels dominate the world bivalve trade in terms of quantity, accounting for around 40% and 38%, respectively (Silva *et al*, 2010). Ryota *et al*. (2012) cited that seafood consumption contributes to good brain development and can also reduce cardiovascular disease deaths and reduce the problem of vision in infants.

Asiatic hard clam (AHC) is a bivalve type mollusc living in freshwater, seawater, intertidal beaches, and deep water (Dore, 1991). In Malaysia, the AHC can be found abundantly in Besut and Setiu, Terengganu, Malaysia. This bivalve feed by extending their siphon (neck) through the sand and collecting microscopic organisms and particulates from the water column to pump water through their digestive systems (Dore, 1991) and tend to accumulate sand in their body tissue (Leung, 2004).

*Corresponding author.
Email: *amir@umt.edu.my*

There are currently a few studies on shellfish hydrolysate, as most researchers favoured the use of protein from fish resources, as it can primarily be obtained from fish waste products (Wangkheirakpam *et al*., 2019). Protein hydrolysate from shellfish is not commercialized even though shellfish enzymes have emerged to accelerate the seafood industry's process or produce other food products, such as fish and shellfish protein hydrolysates and seafood flavourings (Vijaykrishnaraj *et al*., 2016). Few studies have been reported on protein hydrolysate of shellfish. One study has been reported on optimizing the protein hydrolysis of Asian hard clam but using Flavourase and Papain as enzymes (Xie *et al*., 2012). The seafood, especially clam, is available throughout the year because this mollusc is not seasonal.

Moreover, the study of protein hydrolysate for shellfish, especially for bivalves, is uncommon due to the availability of shellfish. The protein hydrolysate from this source can produce a new flavouring agent and be used as a protein supplement. The resulting protein hydrolysates are also cheaper and can have satisfactory organoleptic properties. Furthermore, this data obtained in a later phase will help to provide scientific data which will further support the versatility of the protein hydrolysate obtained. The use of protein hydrolysate from molluscs should be expanded to be more marketable and valuable for the industry. Therefore, this study is aimed to optimize the enzymatic protein hydrolysis conditions of AHC (in terms of hydrolysis time, hydrolysis temperature, hydrolysis pH, and concentration of enzyme) to obtain the highest degree of hydrolysis (DH) value.

2. Materials and methods

2.1 Materials

The Asiatic hard clams (AHC)(*Meretrix meretrix*) were purchased from Kampung Pengkalan Gelap, Setiu, Terengganu, Malaysia. Alcalase® 2.4 L was purchased from Novozymes (Denmark), is a liquid food grade preparation and has a declared activity 2.4 Anson U/g and a density of 1.18 g/ml. Alcalase® is an endoproteinase produced by a selected *Bacillus licheniformis*, whose main component is subtilizing Carlsberg with a molecular mass of 27.3 kDa. All reagents used in this work were of analytical grade chemicals were purchased from the local suppliers.

2.2 Preparation of Asiatic hard clam

The AHC was shucked using a knife to obtain the clam meat. The clam meat then was rinsed several times to remove any soil or mud. The clam meat was then packed and sealed in polyethylene bags and frozen at -40°C for further analysis.

2.3 Preparation of Asiatic hard clam hydrolysate powder

Preparation of Asiatic hard clam protein hydrolysate (AHCPH) was carried out according to Amiza *et al*. (2011) with some modification. The hydrolysis process was done in a water bath (Techne - model TE-10D tempunit). A portion of 82.5 g clam meat was mixed with 60.5 g of distilled water and homogenized (Waring commercial laboratory blender) at low speed for 2 mins. The resulting mixture was then heated at 85°C for 20 mins (to inactivate the endogenous enzymes). After cooling, the mixture was heated until it reached the desired temperature (based on the RSM parameter). At the same time, the pH of the mixture was adjusted to the required pH using Cyberscan pH 510 with an addition of 1 N NaOH solution and 0.1 N HCl solution. Different parameters of enzymatic hydrolysis such as temperature, hydrolysis time, pH and enzyme concentration were also adjusted. After the mixture's temperature and pH reached the intended conditions, 20 g of the Alcalase® enzyme solution was added into the slurry to immediately start the non-enzymatic hydrolysis process. The pH was adjusted to be constant at the selected pH throughout the hydrolysis by adding 1 N NaOH solution and 0.1 N HCl solution. After the hydrolysis was completed, the sample was heated at 85°C for 20 mins to inactivate the Alcalase® enzyme activity. The mixture was then centrifuged at 4000 rpm for 20 mins using a multipurpose centrifuge (Gyrozen 1580R, Korea) to separate the soluble supernatant and precipitate fraction. The supernatant obtained was further processed to AHCH powder using a Labconco Freezone, freeze dryer. The AHCH powder obtained was then kept in a hermetic container and stored at room temperature until further use.

2.4 Optimization by Response Surface Methodology (RSM)

Optimization of the hydrolysis conditions was accomplished by employing the response surface methodology (RSM). Four different independent variables, which were temperature (°C, A), enzyme to substrate concentration (%v/w, B), time (minutes, C), and pH (D), and the levels used for each independent variable were shown in Table 1. The experiment was optimized using a central composite design (CCD) with four independent variables at three levels (+1, 0, -1). It was performed by applying the design expert 6.0.10, Stat -Ease, Inc software order to prepare data for statistical analysis. The experimental design was composed of 30 runs, consisting of 16 factorial points, 8 axial points and 6 replicated points at central points. As four parameters were varied, 15 β-coefficient had to be estimated which

included coefficients for the 4 linear effects, 4 quadratic effects, 6 interaction effects and 1 constant. It was assumed that the estimated behavioural model of both dependent variables was described by a second-order polynomial equation (1):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (1)$$

Where Y represented the estimated dependent variable (Degree of hydrolysis); β_0 represented the constant-coefficient; β_i represented the linear coefficient; β_{ii} represented the quadratic coefficient and βij represented the interaction coefficient. (i = 1-4; and j = 1 -4).

Table 1. Range and level of a parameter that was used in the RSM design

		Range and Level		
Independent Variables		-1	0	+1
Temperature (°C)	A	45	55	65
Enzyme concentration (E/S, %v/w)	B	1	1.5	2
Hydrolysis time (min)	C	60	120	180
pH	D	7.5	8.5	9.5

The analysis of variance (ANOVA) methods was applied to evaluate the developed mathematical model (by applying the lack-of-fit test) and to evaluate the statistical significance of the factors in the model. The ANOVA table for the model concludes the response's analysis and the significant model terms (Pishgar-Komleh *et al*., 2012b). To examine the goodness and evaluate a fitted model's adequacy, the coefficient of the determination (R^2) was calculated. The mathematical model's lack of fit value implies that it is not significant (Pishgar-Komleh *et al*., 2012a). Design expert 6.0.10 software was employed for the regression of analysis and graphical optimization, respectively.

2.5 Determination of degree of hydrolysis (DH).

The degree of hydrolysis was calculated according to the percent of trichloroacetic acid (TCA) ratio method as described by Hau *et al*. (2017). After the hydrolysis, 20 mL of protein hydrolysate was added to 20 mL of 20% (w/v) TCA to produce 10% TCA soluble material. The mixture was homogenized using Velt Scientifica vortex (15Htz, 30s). The mixtures were allowed to stand for 30 mins to allow precipitation, followed by centrifugation (7800 RCF for 15 min). The supernatant was analysed for protein content by the Kjeldahl method (AOAC, 2007). Sample from the hydrolysate was also analysed for protein content. The degree of hydrolysis (DH) was calculated using the formula below:

$$\%DH = \frac{\text{Soluble in TCA 10\% w/v}}{\text{Total N in the sample}} \times 100$$

Where DH = degree of hydrolysis, TCA = Trichloroacetic acid, and N = Nitrogen

2.6 Proximate analysis

The proximate analysis was carried out on AHC meat and on the AHCPH powder prepared using optimum conditions. AOAC (2007) International methods were used to determine the proximate analysis. Moisture analysis, ash analysis, crude protein analysis and crude fat analysis were carried out as part of the analysis.

3. Results and discussion

3.1 Asiatic hard clam hydrolysis

In hydrolysis, all thirty runs of the experiments were done according to the parameters given by the Response Surface Methodology (RSM). After all of 30 runs of hydrolysis were done, the samples were freeze-dried using a Labconco Freezone[6] freeze dryer until dry. After that, the optimum conditions of Asiatic hard clam protein hydrolysate (AHCPH) were obtained and analysed by the RSM. The proximate analysis data were obtained and evaluated based on the optimum conditions suggested: the temperature at 65°C, enzyme to substrate concentration of 1%, hydrolysis time of 60 mins and pH 7.5.

3.2 Optimization of enzymatic protein hydrolysis

The effect of four factors, which were a temperature, °C (A), enzyme to substrate level, % v/w (B), time, min (C) and pH of the substrate (D) on the hydrolysis by Alcalase® was determined using response surface methodology (RSM) with face centered central composite design (CCD). The observed values for the degree of hydrolysis (DH) at different combinations of the independent variables were presented in Table 2. Overall 30 experiments with six replicates in the centre of the design space were carried out to find the best relationship between independent and dependent variables. Center points run interspersed among the experimental setting runs to provide stability of process measurement and inherent variability and check for curvature.

The degree of hydrolysis for each condition of the parameter as shown in Table 2 that depicts the range of the DH was from 53.77% to 99.59%. The value of the degree of hydrolysis for AHCH in this study was higher than DH was given by blue mussel meat (7.02-28.13%) (Silva *et al*., 2010) and Catla viscera (34.23-49.65%) (Bashkar *et al*., 2008).

All the coefficients of linear (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) and interaction (AB, AC, AD, BC, BD,

Table 2. The actual level of independence used in optimizing the hydrolysis conditions using Alcalase® and its dependent values for the degree of hydrolysis

Experiment No.	Run	Independent variable				Dependent
		A	B	C	D	Y
1	30	45	1	60	8	97.61
2	27	65	1	60	8	99.59
3	10	45	2	60	8	83.60
4	14	65	2	60	8	95.31
5	7	45	1	180	8	93.31
6	21	65	1	180	8	53.77
7	20	45	2	180	8	93.22
8	5	65	2	180	8	76.37
9	15	45	1	60	10	70.98
10	25	65	1	60	10	65.13
11	28	45	2	60	10	66.90
12	8	65	2	60	10	95.05
13	17	45	1	180	10	70.04
14	19	65	1	180	10	87.53
15	12	45	2	180	10	87.27
16	24	65	2	180	10	62.67
17	11	45	2	120	9	85.69
18	9	65	2	120	9	91.42
19	2	55	1	120	9	75.69
20	18	55	2	120	9	69.34
21	3	55	2	60	9	91.87
22	6	55	2	180	9	59.23
23	23	55	2	120	8	90.29
24	16	55	2	120	10	85.28
25	4	55	2	120	9	71.23
26	13	55	2	120	9	91.98
27	1	55	2	120	9	95.16
28	29	55	2	120	9	86.78
29	22	55	2	120	9	98.86
30	26	55	2	120	9	71.12

A: Temperature, B: Enzyme concentration, C: Time, D: pH; Y: Degree of hydrolysis (DH)

CD) terms to fit a full response surface model for the response was determined by a multiple regression analysis techniques that are included in RSM. The F-value tests and P-value were performed using variance (ANOVA) to estimate each type of model's significance. In F-value, the highest order model with significant terms shows the parameter relationship well and would normally be chosen. Liu *et al*. (2010) quoted that the larger the value of F-value and the smaller the value of Prob > F, the more significant is the corresponding coefficient term. The model also may be considered statistically significant if the value of Prob > F was lower than 0.05 (Liu *et al*., 2010). However, if the value is greater than 0.100, it indicated that the model terms are not significant. If there are many nominal model terms, model reduction may improve the model (Shamshiry *et al*., 2011). Table 3 also indicates that the Linear model and two-factor interaction (2FI) model were significant, with F-value of 1.36 and 1.27, respectively. In contrast, the Quadratic model was not significant with an F-value of 0.70 and the Cubic model was found to be aliased. Table 3 revealed that the 2FI model was the most suitable model because it was the highest order model with an effective term compared to the other models.

Moreover, the adequacy of the models was evaluated by applying the lack of fit test. The lack of fit test was done to determine the model adequacy. This test is used in the numerator in an F-test of the null hypothesis and suggests that a proposed model fit well or not (Pishgar-Komleh *et al*., 2012a). This test measured the different models' adequacy based on response surface analysis (Pishgar-Komleh *et al*., 2012a). The results in the Table 3 revealed that there is no significant lack of fit for all models due to a large number of Prob>F values, and the 2FI model was suggested because of the highest value of Prob>F value. Data in Tables 3, 4 and 5 indicate that the 2FI model describes the relationship between independent (temperature, enzyme-substrate concentration, time, and pH) and dependent variable (Degree of hydrolysis) well.

Analysis of variance (ANOVA) for the 2FI model was presented in Table 4. As it can be seen, the model's F-value was 2.56 which implies that the model was significant. There was only a 4.8% chance that a "Model F-value" this large could occur due to noise. Furthermore, the value of Prob>F less than 0.05 indicates that the model terms are significant. In this case, all linear model terms (A, C, D) were not significant (P>0.05). For the interaction coefficients (AC, AD, CD), only AC was significant (P<0.05) while AD and CD did not have significant effects (P>0.05). The lack of fit test was used to evaluate the model's fitness (See *et al*., 2011). Table 4 also presented that the lack-of-fit test for the reduced 2FI model was not significant due to the value of Prob>F was 0.63. The Prob>F value for the lack of fit test was large and not significant (P>0.05). The result indicated that the model was sufficiently accurate for predicting the degree of hydrolysis for any combination of experimental independent variables used in enzymatic hydrolysis of AHC meat. This result is similar to another researcher's results (Diniz and Martin 1996; Ovissipour *et al*., 2009; See *et al*., 2011). Several indicators were used to examine the goodness and evaluate the adequacy of a fitted model, as shown in Table 5. The coefficient of determination (R^2), the adjusted determination coefficient (adj. R^2), coefficient variation (CV%) was calculated to judge the adequacy of the model, which this indicator was used by other researchers (Liu *et al*., 2010; Pishgar-Komleh *et al*., 2012a). The R^2 value of 0.4 indicates that the model could explain 40% of the dependent variable's variability. The R^2 value needs to be close to 1 to predict the fitted model's values are near to

Table 3. Analysis of variance (ANOVA) for different models

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F	
Sequential Model Sum of Squares						
Mean	202095.73	1	202095.73			Suggested
Linear	892.51	4	223.13	1.36	0.2774	
2FI	1177.30	6	196.22	1.27	0.3173	Suggested
Quadratic	459.18	4	114.80	0.70	0.6070	
Cubic	1199.37	8	149.92	0.82	0.6093	Aliased
Residual	1278.17	7	182.60			
Total	207102.26	30	6903.41			

DF: Degree of freedom, 2FI: Two-factor interaction

Table 4. Analysis of variance (ANOVA) for reduced 2FI model

Source	Coefficient Estimate	Standard Error	Sum of Squares	*DF	Mean Square	F-Value	Prob > F	
Model	82.08	2.09	2002.85	6	333.81	2.56	0.048	Significant
A	-1.21	2.69	26.35	1	26.35	0.20	0.6575	
C	-4.59	2.69	379.32	1	379.32	2.90	0.1018	
D	-5.12	2.69	472.47	1	472.47	3.62	0.0698	
AC	-6.22	2.86	618.64	1	618.64	4.74	0.04	
AD	3.62	2.86	209.45	1	209.45	1.60	0.218	
CD	4.31	2.86	296.61	1	296.61	2.27	0.1454	
Residual			3003.67	23	130.59			
Lack of Fit			2278.58	18	126.59	0.87	0.6271	Not significant
Pure Error			725.10	5	145.02			
Cor Total			5006.53	29				

*DF: Degree of freedom, A: Temperature, C: Time, D: pH

Table 5. Goodness and adequacy of a fitted model

Std. Dev: 11.4278	R-Squared: 0.400049
Means: 82.07633	Adj. R-Squared: 0.243539
C.V.: 13.92337	Pred. R Squared: -0.1138
PRESS: 5576.256	Adeq. Precision: 5.476049

the actual data. The adjusted determination coefficient (adj. R^2) of 0.24 illustrated less correlation between the independent variables.

The coefficient variation (CV) independent of the unit is defined as the ratio of the standard deviation of an estimate of the mean value of the observed response (Pishgar-Komleh *et al*., 2012b). This factor is a measure of reproducibility and repeatability of the models (Pishgar-Komleh *et al*., 2012b). The results indicated that the CV value was 13.92%, which illustrated that the model could be reproducible because the CV was greater than 10%.

All linear terms (A, C, D) were not significantly different and only AC showed a significant effect. In contrast, AD and CD showed no significant effect on the interaction term (AC, AD, CD) (Table 4). Concerning Table 4 and the coefficient, the final equation, which can show the relationship between factors in terms of coded, is shown in Equation (2):

$$Y = 82.08 - 1.21A - 4.60C - 5.12D - 6.22AC + 3.62AD + 4.31CD \quad (2)$$

The Y, A, C and D value indicates the DH, temperature (°C), time (min) and pH. Zhou and Regenstein (2004), quoted that the examination of fitted models was done to verify that there was no least square regression assumption is violated and to ensure that it gave an adequate approximation to the true system.

3.3 Analysis of response surface

The optimization study was performed to evaluate the optimal experimental parameters (Pishgar-Komleh *et al*., 2012b). The effect of temperature (A), enzyme to substrate level (B), time (C), and pH (D) on the degree of hydrolysis (DH) was determined using response surface methodology (RSM). A desirability profile was employed to establish the optimum level for each condition. Temperature, enzyme to substrate level, time and pH was set in arranged ranges, while DH was expected to respond maximally. The optimum conditions of enzymatic hydrolysis of AHC meat were given in Table 6 with 10 solutions. The conditions selected were 65°C for temperature (A), 1.0% for enzyme and substrate level (B), 60 mins for time (C) and 7.5 for pH (D). The predicted value of response DH was 97.41% obtained from the calculation using the model equation with the highest desirability, which is 0.988. The DH's independent variables' influences can be judged through three-dimensional views of the response surface plot and the respective contour plot (See *et al*., 2011). Figure 1 indicates an increase in hydrolysis temperature led to an increase in the degree of hydrolysis. However, DH was

Table 6. Suggested conditions of Asiatic hard clam (AHC) hydrolysis using Alcalase®

Number	Temperature	Enzyme concentration	Time	pH	DH	Desirability	
1	65	1	60	7.5	97.41	0.988	selected
2	64	1	60	7.5	97.32	0.987	
3	62	1	60	7.5	97.05	0.986	
4	64	1	60	7.5	97.38	0.984	
5	65	1	60	7.7	96.32	0.982	
6	53	1	60	7.5	95.80	0.979	
7	51	1	60	7.5	95.58	0.977	
8	50	1	60	7.5	95.38	0.976	
9	60	1	60	7.5	96.84	0.974	
10	55	1	91	7.5	91.54	0.953	

decreased as the pH increased. Usually, microbial enzymes like Alcalase® operating at alkaline pH, have been reported to be the most efficient in seafood protein hydrolysis Harun *et al*., 2017).

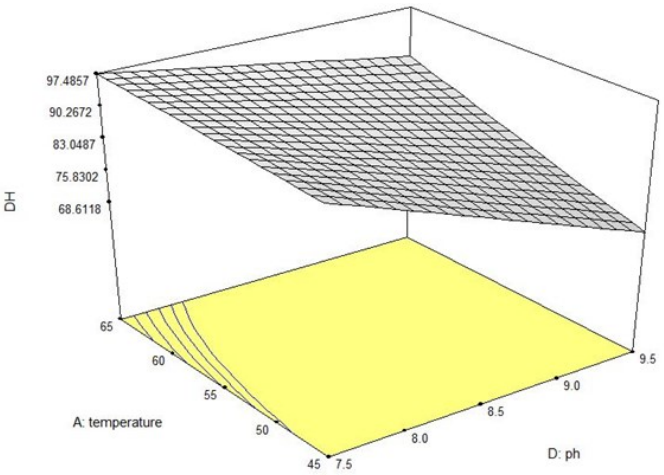

Figure 1. DH as a function of temperature and pH during protein hydrolysis of AHC with Alcalase®

The data also shows that the reaction starts to decrease when the pH was higher than pH 7.5. According to Clemente (2000), most enzymatic hydrolysis are developed under a mild condition of pH 6 to 8 to avoid the extremes required for chemical and physical treatments and minimize the side reactions. The enzyme might be denatured and was inactivated at higher pH than that was stated by Clemente (2000) even though Alcalase® is active in the range of pH from 8 to 10. Changes in pH throughout the hydrolysis might disturb the efficiency of the enzyme capability in hydrolyzing the protein, which most probably the main cause of the result obtained (Salwanee *et al*., 2013) and causing a continuous loss of enzyme activity (Silva *et al*., 2010). Yu and Ahmad (1998) reported that in many enzyme-catalysed reactions, pH could affect the changes in the reactants make the protein structure of the enzyme to be denatured or the ability of the substrate to bind to the enzyme might be less effective due to the disturbance of the ionic character of the substrates. However, the optimal pH may vary according to the substrate and enzyme concentration used in hydrolysis (Salwanee *et al*., 2013).

Figure 1 also showed that DH reached the maximum level when the temperature reached 65°C. The optimum condition of this AHCH was 65°C, which was in the range of optimum condition by Alcalase® (55-70°C). The DH started to increase below the optimum temperature because the rate of hydrolysis increased when the temperature increases for most reactions. The rate of the chemical reaction being catalysed also is increased as the temperature is increased (Illanes, 2008). The data is in concert with the studies by Mukhin and Novikov (2001). They reported that the exposures of peptide bonds during the enzymatic hydrolysis lead to increased DH caused by the heat treatment. Compared to the previous study on the hydrolysis of seafood using Alcalase®, the study reported the optimum temperature for Catla viscera waste (Bashkar *et al*., 2005) was 55°C, salmon skin (See *et al*., 2011) was 55.3°C and silver catfish frame (Amiza *et al*., 2011). It shows that the Alcalase® reacts at a higher temperature on the AHC compared to reacting with other seafood.

Figure 2 shows the effect of temperature and enzyme concentration on the DH of AHC hydrolysis. The DH was increased with the increase of temperature and enzyme concentration by 2% of concentration. The hydrolysis chances to occur were higher as there are more enzyme molecules present in a higher enzyme to substrate ratio (Harun *et al*., 2017). The result obtained might be due to the increase of smaller peptides and amino acids present in the hydrolysate. Salwanee *et al*., (2013), added that some of the peptide released was greater hydrolysed as the Alcalase® concentration was increased. Hence, increasing the enzyme concentration allowed the occurrence of hydrolysis at a higher degree, thus resulting in a higher DH. The finding was in agreement with the previous studies by See *et al*. (2011) and Amiza *et al*. (2011) as the enzyme was constant at a 2% enzyme to substrate ratio. In contrast, a lower enzyme to substrate ratio was 1.25% for Catla viscera (Bashkar *et al*., 2007), and 0.34% for blue mussels (Cha *et al*., 1998), respectively. A different choice of enzymes

exhibits varying specificities rate of reaction and optimal working parameters in the hydrolysis of polypeptide chains (See *et al*., 2011).

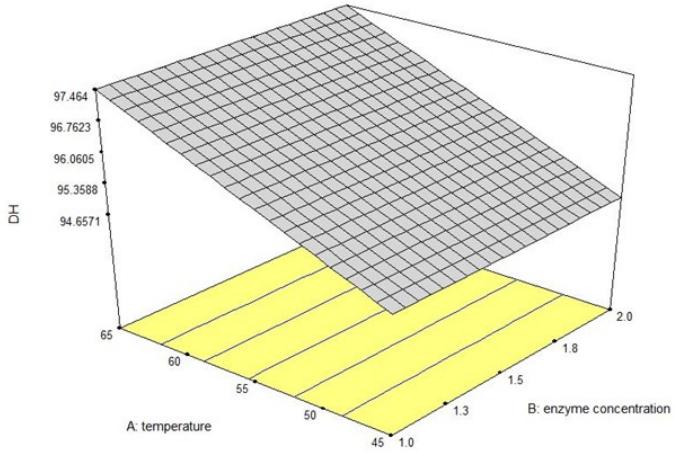

Figure 2. DH as a function of temperature and enzyme concentration during protein hydrolysis of AHC using Alcalase®

Figure 3 shows the response surfaces and their corresponding contours of the combined effect of hydrolysis time and pH on DH using Alcalase®. As it can be seen, DH starts to decrease as the time and pH are increased. Haslaniza *et al*. (2010) stated that a longer incubation time would allow the enzyme to act more extensively on the protein, thus increasing proteolysis, resulting in higher DH obtained. However, the result obtained was different from what the researcher reported. The decrease of DH might be due to the decrease in enzyme activity, substrate saturation, product inhibition or extreme pH concentration that cause the enzyme's denaturation. The optimum value of hydrolysis time for this finding was shorter than the previous study, which was 163 mins for the Silver catfish frame and 135 minutes for Catla (Bashkar *et al*., 2008; Amiza *et al*., 2011).

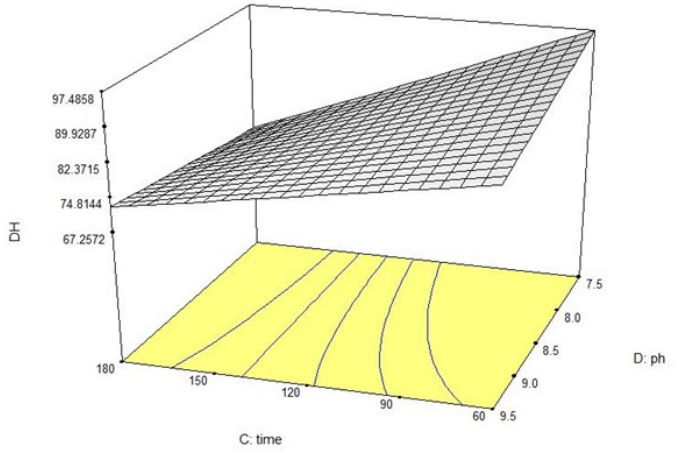

Figure 3. DH as a function of time and pH during protein hydrolysis of AHC with Alcalase®

In order to verify the optimized results, a validation test was performed to determine the adequacy of the suggested models. Under these optimized conditions, the confirmation experiments were conducted. According to the 2FI model suggested, the predicted result of the DH was shown in Table 7. The data showed that the predicted value was 97.41%, respectively under the optimum conditions (65°C, 1% enzyme to substrate concentration, 60 min and pH 7.5) close to the experimental response observed 97.89%.

Table 7. The predicted and experimental value for the degree of hydrolysis in AHCPH

	DH (%)	
	Predicted value	Experimental value
Asiatic hard clam hydrolysate	97.41	97.89

DH: Degree of hydrolysis

One sample T-test was conducted to verify the optimized result with a hypothesis of H_0: $\mu = 97.41$ and H_1: $\mu \neq 97.41$. Table 8, shows that the sample mean was 97.89% degree of hydrolysis. The t-statistic was 0.72 while the p-value was 0.604. As the absolute value of the t-statistic increases, the p-value becomes smaller.

Table 8. One sample T-Test data

N	Means	StDev	SE Mean	95%CI	T	P
2	97.89	0.948	0.67	89.3771;106.4029	0.72	0.604

The 95% confidence interval includes the comparative value of 97.41%. This was equivalent to failing to reject the null hypothesis (H_0: $\mu = 97.41$) for this t-test with α value of 0.05. Thus, according to the t-test and the sample data, the null hypothesis (H_0: $\mu = 97.41$) failed to be rejected at the 0.05 α-level. It also indicated that the DH value for the experiment depicted no significant difference from the predicted value. So, the model's experimental values within a 95% confidence interval, indicating that the model was suitable for the estimation of the experimental value.

3.4 Proximate composition

The proximate analysis was carried out on the AHC meat and freeze-dried AHCPH powder. The freeze-dried AHCPH powder prepared using the hydrolysis conditions gave the highest DH values. The proximate composition of AHC meat and AHCPH is presented in Table 9 (based on the dry matter).

The data shows that protein content in AHC meat was at 10.53%. The protein content of AHC meat ranged from 10.55% to 15.54% (Xie *et al*., 2012). Furthermore, Muyonga *et al*. (2004) stated that the crude protein content of the portentous material was the maximum possible yield of protein hydrolysate that can be isolated. Furthermore, the AHC meat consisted of low ash and fat content, which were 1.98% and 2.23% respectively.

However, the ash content of AHC meat from Xie *et al*. (2012) was higher, in the range of 12.8% to 22.4%. The difference in the ash content of AHC meat could be due to the different habitats of AHC life in the coastal area of China. AHC meat also had lower moisture content which was 7.92%, while AHCH had a moisture content of 9.12%. The increasing value of moisture content in AHCH might be due to the incompleteness of drying in the sample during freeze-drying and moisture absorption in the surrounding during storage.

Table 9 shows the crude protein content of AHCH was the biggest component of the protein hydrolysate compared to ash, fat and moisture content. According to See *et al*. (2011), the crude protein content can be used as an indicator of the protein hydrolysate's purity. The crude protein of AHCH was 50.09%, indicating the high purity of the protein hydrolysate. The ash content of the AHCH was high, which was 27.76%. The ash might be due to the addition of NaOH and HCl during enzymatic hydrolysis.

Table 9. Chemical composition of the AHC meat and AHCPH

	Asiatic hard clam meat (%)	Asiatic hard clam hydrolysate (%)
Moisture analysis	7.92±1.76*	9.12±0.02
Crude protein analysis	10.53±0.04	50.09±0.88
Crude fat analysis	2.23±0.89*	0.80±0.29
Ash analysis	1.98±0.82	27.76±0.10

*Based on dry matter

Fat content in the AHCH was very low compared to AHC meat, which was 0.80%. This was because some oil layers were removed during hydrolysate centrifugation (Halimaton *et al*., 2012). Kristinsson and Rasco (2000), Nilsang *et al*. (2005), and Motamedzadegan *et al*. (2010) agreed that the fat content in protein hydrolysate was reduced compared to the raw material because the fat was excluded with insoluble protein fraction by centrifugal separation. Decreasing fat content in the protein hydrolysate contributes to lipid oxidation stability (Motamedzadegan *et al*., 2010). The low content of fat in the AHCH can ensure sample stability during storage. Higher lipid content in the sample will promote lipid peroxidation thus will lead to oxidative damage to protein causing the reduction in the quality and stability of the protein (Shahidi *et al*., 1995; Diniz and Martin 1997; Kristinsson and Rasco 2000; Nilsang *et al*., 2005; Motamedzadegan *et al*., 2010; See *et al*., 2011).

4. Conclusion

The study shows that the interaction between temperature and time significantly influenced the degree of hydrolysis of Asiatic hard clam (AHC) hydrolysate by Alcalase®. According to the model suggested (2FI), the optimum hydrolysis parameters were 65°C, 1% of the enzyme to substrate concentration, 60 mins, and at pH 7.5. The experimental degree of hydrolysis obtained under these optimum conditions was 97.89%, which was closed to the predicted value of 97.41%. With these optimum hydrolysis conditions, the protein hydrolysate obtained composed of a high percentage of protein (50.09±0.88%), ash (27.76±0.10%) and a lower percentage of fat (0.80±0.29%) and moisture (9.12±0.02%) compared to the original raw material with 10.53±0.04% of protein, 1.98±0.82% of ash, 2.23±0.89% of fat and 7.92±1.76% of moisture. The results indicated that RSM is an effective tool for optimizing hydrolysis parameters to obtain the optimum degree of hydrolysis of AHC. All the data obtained could be adopted to produce Asiatic hard clam protein hydrolysate (AHCPH) at the industrial scale.

Conflict of interest

The authors declare no conflict of interest

Acknowledgement

This research was supported by Universiti Malaysia Terengganu. The authors would like to thank the FPSM for the facilities provided to conduct the study.

References

Amiza, M., Nurul Ashikin, S. and Faazaz, A. (2011). Optimization of enzymatic protein hydrolysis from silver catfish (*Pangasius sp*.) frame. *International Food Research Journal*, 18(2), 775-781.

AOAC International. (2007). Official Methods of Analysis of AOAC International. AOAC Official Method 990.26. 17th ed. Gaithersburg, USA: AOAC International.

Bhaskar, N., Benila, T., Radha, C. and Lalitha, R.G. (2008). Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catla catla*) for preparing protein hydrolysate using a commercial protease. *Bioresource Technology*, 99(2), 335-343. https://doi.org/10.1016/j.biortech.2006.12.015

Cha, Y., Kim, H. and Kim, E. (1998). Development of blue mussel hydrolysate as flavouring. *Journal of Food Science and Nutrition*, 3(1), 4-10.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science and Technology,* 11(7), 254-262. https://doi.org/10.1016/S0924-2244(01)00007-3

Dore, I. (1991). Shellfish: a guide to oysters, mussels,

scallops, clams and similar products for the commercial users. New York, USA: Van Nostrand Reinhold.

Halimaton, S.M., Amiza, M. and Wong, C. (2012). Effect of degree of hydrolysis on the physicochemical properties of blood cockle (*Anadara granosa*), presented at the UMT 11th International Symposium on Sustainability Science and Management, p. 478-484. Terengganu, Malaysia.

Haslaniza, H., Maskat, M., Wan Aida, W.M. and Mamot, S. (2010). The effects of enzyme concentration, temperature and incubation time on nitrogen content and degree of hydrolysis of protein precipitate from cockle (*Anadara granosa*) meat wash water. *International Food Research Journal*, 17(1), 147-152.

Harun, Z., Amiza, M.A., Sarbon, N.M. and Zainol, M.K (2017). Optimisation of enzymatic protein hydrolysis of Mud Crab (*Scylla sp*.) to obtain maximum Angiotensin-Converting Enzyme Inhibitory (ACEI) activity using Response Surface Methodology. *Malaysian Applied Biology*, 46(3), 33-40.

Hau, E.H., Mohd Zin, Z., Nasution, Z., Shaharudin, N.A. and Zainol, M.K. (2018). Physicochemical properties of powdered protein hydrolysate from Yellowstripe scad (*Selaroides leptolepis*) fish. *International Food Research Journal*, 25(6), 2555-2561.

Illanes, A. (2008). Enzyme biocatalysts: Principles and applications. Chile: Springer Science and Business Media B.V.

Kristinsson H.G. and Rasco B.A. (2000). Biochemical and functional properties of Atlantic salmon (*Salmo salar*) muscle proteins hydrolysed with various alkaline proteases. *Journal of Agriculture and Food Chemistry*, 48(3), 657-666. https://doi.org/10.1021/jf990447v

Leung, K.M.Y. and Lui, K.K.Y. (2004). Sand elimination by the Asiatic hard clam (*Meretrix meretrix)* (L.): influences of temperature, salinity and season. *Journal of Shellfish Research*, 23(2), 1-10.

Liu, Y., Zheng, Y. and Wang A. (2010). Response Surface Methodology for optimizing adsorption process parameters for methylene blue removal by a hydrogel composite. *Adsorption Science and Technology,* 28(10), 913-922. https://doi.org/10.1260/0263-6174.28.10.913

Motamedzadegan, A., Davarniam, B., Asadi, G., Abedian, A.M. and Ovissipour, M. (2010). Optimization of enzymatic hydrolysis of Yellowfin Tuna (*Thunnus albacares*) viscera using Neutrase. *International Aquatic Research*, 2, 173-181.

Mukhin, V.A. and Novikov, V.Y. (2001). Enzymatic hydrolysis of proteins from Crustaceans of the Barents Sea. *Applied Biochemistry and Microbiology*, 37(5), 538-542. https://doi.org/10.1023/A:1010218712622

Muyonga, J.H., Colec, C.G.B. and Duodub, K.G. (2004). Extraction and physicochemical characterization of Nile perch (*Lates niloticus*) skin and bone gelatine. *Food Hydrocolloids*, 18(4), 581-592. https://doi.org/10.1016/j.foodhyd.2003.08.009

Nilsang, S., Lertsiri, S., Suphantharika, M. and Assavanig, A. (2005). Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. *Journal of Food Engineering*, 70(4), 571-578. https:// doi.org/10.1016/j.jfoodeng.2004.10.011

Ovissipour, M., Abedian, A., Motamedzadegan, A., Rasco, B., Safari, R. and Shahiri, H. (2009). The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from Persian sturgeon (*Acipenser persicus*) viscera. *Food Chemistry*, 115(1), 238-242. https:// doi.org/10.1016/j.foodchem.2008.12.013

Pishgar-Komleh, S.H., Keyhani, A., Mostofi-Sarkari, M.R. and Jafari, A. (2012b). Application of response surface methodology for optimization of picker-husker harvesting losses in corn seed. *Iranica Journal of Energy and Environment*, 3(2), 134-142. https:// doi.org/10.5829/idosi.ijee.2012.03.02.0027

Pishgar-Komleh, S.H., Keyhani, A., Mostofi-Sarkari, M.R. and Jafari, A. (2012a). Optimization of seed corn harvesting losses applying Response Surface Methodology. *Journal of Applied Sciences, Engineering and Technology,* 4(15), 2350-2356.

Radha, C., Kumar, P.R. and Prakash, V. (2007). Preparation and characterization of a protein hydrolysate from an oilseed flour mixture. *Food Chemistry*, 106(3), 1166-1174. https://doi.org/10.1016/j.foodchem.2007.07.063

Roose, G. and Mulier, L. (2020). Healthy advertising coming to its senses: The effectiveness of sensory appeals in healthy food advertising. *Foods*, 9(1), 51. https:// doi.org/10.3390/foods9010051

Ryota H., Yoshida, M. and Fukunaga, K. (2012). Seafood consumption and components for health. *Global Journal of Health Science,* 4(3), 72-86. https:// doi.org/10.5539/gjhs.v4n3p72

Salwanee, S., Wan Aida, W.M., Mamot, S., Maskat, M.Y. and Ibrahim, S. (2013). Effects of Enzyme Concentration, Temperature, pH and Time on the Degree of Hydrolysis of Protein Extract from Viscera of Tuna (*Euthynnus affinis*) by Using

Alcalase. *Sains Malaysiana*, 42(3), 279–287.

See, S., Hoo, L. and Babji, A. (2011). Optimization of enzymatic hydrolysis of Salmon (*Salmo salar*) skin by Alcalase. *International Food Research Journal*, 18(4), 1359-1365.

Shahidi, F., Han, X.-Q. and Synowicki, J. (1995). Production and characteristic of protein hydrolysates from Capelin (*Mallotus villosus*). *Food Chemistry*, 53(3), 285-293. https://doi.org/10.1016/0308-8146 (95)93934-J

Shamshiry, E., Nadi, B., Mahmud, A.H. (2011). Optimization of Municipal Solid Waste Management. International Proceedings of Chemical, Biological and Environmental Engineering, 1, 119-121.

Silva, V.M., Park, K.J. and Hubinger, M.D. (2010). Optimization of the enzymatic hydrolysis of mussel meat. *Journal of Food Science*, 75(1), 36-42. https:// doi.org/10.1111/j.1750-3841.2009.01414.x

Toro, M.A. and Garcia-Carreno, F.L. (2002). Current Protocols in Food Analytical Chemistry. p. B2.2.1-B2.2.14. United Kingdom: John Wiley and Sons, Inc.

Vijaykrishnaraj, M., Roopa, B.S. and Prabhasankar, P. (2016). Preparation of gluten-free bread enriched with green mussel (*Perna canaliculus*) protein hydrolysates and characterization of peptides responsible for mussel flavour. *Food Chemistry*, 211, 715-725. https://doi.org/10.1016/ j.foodchem.2016.05.094

Wan Norhana, M.N., Nor Ainy, M., Othman, M., Faazaz, A.L., Ismail, I. and Yusri, A. (2011). Establishment of a shellfish model farm: a case study in Sg. Jarum Mas, Perak, Malaysia. *International Food Research Journal*, 18(1), 79-94.

Wangkheirakpam, M.R., Mahanand, S.S., Majumdar, R.K. Sharma, S. Hidangmayum, D.D. and Netam, S. (2019). Fish waste utilization with reference to fish protein hydrolysate – A Review. *Fishery Technology*, 56, 169 – 178.

Xie, W.Y., Chen, C., Liu, X.S., Wang, B., Sun, Y., Ma, Y. and Zhang, X. (2012). *Meretrix meretrix*: Active components and their bioactivities. *Life Science Journal*, 9(3), 756-762.

Yu, S.Y. and Ahmad, R. (1998). Hydrolysis of proteins from *Liza subviridis*. *Asian Fisheries Science*, 10, 251-257.

