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Optimization of enzymatic hydrolysis of tilapia *(Oreochromis niloticus)* by-product using response surface methodology

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Abstract

Fish protein hydrolysate was recovered from tilapia by-product (TB) through enzymatic hydrolysis using alcalase enzyme. Hydrolysis reaction of TB was monitored according to the degree of hydrolysis (DH) by employing O-phtaldialdehyde (OPA) method. Optimization process for obtaining high yield of TB protein hydrolysate was performed using response surface methodology (RSM) by optimizing a combination of four independent variables namely, pH (6.5-8.5), temperature (55-70°C), substrate concentration (10-17.5% w/v), and enzyme concentration (1.5-3.5% w/w) with (DH) as a response. The optimum enzymatic hydrolysis conditions were obtained at pH 7.5, temperature of 60°C, substrate concentration of 15% (w/v) and 2.5% (w/w) of enzyme concentration and yielded about 20.20% of DH after hydrolyzing for 120 min. RSM generated model predicted that 20.42% of DH could be achieved at these conditions and this model was valid based on the DH value obtained from the experimental study (20.31%) which was quite similar with the predicted value. High yield of DH obtained from the optimization process could produce fish protein hydrolysate with good nutritional and functional properties.

Introduction

Red tilapia *(Oreochromis niloticus)* is the most popular and cultured freshwater fish in Malaysia. The production of farmed red tilapia has gradually increased every year. According to Department of Fisheries Malaysia (2011), production of red tilapia contributes about 33,260 tonnes and its production showed an increased when compared to 2010 with 29,014 tonnes. This increase is due to the high demand for food protein that can be supplied by red tilapia. Due to the growing demand of tilapia fillet for producing fish based food products, a large amount of by-product have been generated and discarded without any attempt to recover its protein (Kristinsson and Rasco, 2000). As a result, numerous problems have been created including environmental problem (Arvanitoyannis and Kassaveti, 2008).

Fish by-product was found to contain varies in protein content ranging from 15-60% (Valdimarson and James, 2001; Fahmi *et al*., 2004; Je *et al*., 2004; Sathivel *et al*., 2004; Jung *et al*., 2006). It is believed to possess high nutritional properties and good essential amino acid composition (Venugopal *et al*., 1996). Obviously, it will be a loss to the fish processing industry if such by-products are disposed without any effort to make them valuable. Various approaches have been used to prevent this problem and the most interesting and promising alternative ways is by converting fish by-product into products with good functional or biological properties (Kristinsson and Rasco, 2000).

Enzymatic hydrolysis method using protease enzyme could offer several advantages such as requiring mild reaction condition, lacking undesirable products, and high product quality and yield. The widely utilized commercial enzyme is driven by the ease of degree of hydrolysis (DH) determination in controlling the properties of end products (Benjakul and Morrissey, 1997). In order to control the enzymatic hydrolysis process, the selection of enzyme is a crucial factor that needs to be emphasized because different enzymes have different specificities and capabilities on a substrate use. In particular, alcalase was found to be a highly efficient enzyme in the hydrolysis of fish proteins (Adler-Nissen, 1986; Benjakul and Morrissey, 1997; Aspmo *et al*., 2005) due to its capability in attaining high degree of hydrolysis in a relatively short period

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under mild condition (Aspmo *et al.*, 2005). Generally, alcalase has great ability to hydrolyze fish protein in the temperatures ranging from 50-70°C and pH level ranging from 6-10 (Adler-Nissen, 1986).

Several studies have been conducted on optimizing of enzymatic hydrolysis from fish wastes or by-products using alcalase enzyme for the preparation of fish protein hydrolysate (Bhaskar *et al*., 2008; Amiza *et al.*, 2011; Hou *et al*., 2011; See *et al*., 2011). An optimal working condition for alcalase was found to vary depending on the type of substrate and processing method (Adler-Nissen, 1986; Aspmo *et al.*, 2005). A wide range of alcalase enzyme working conditions indicate that there is a need to determine the optimal condition for each process parameter in order to achieve a good result.

Hydrolysis of fish protein was monitored based on the DH in which the percentage of peptide bond cleaved. Most studies have used pH-stat method to measure the DH (Adler-Nissen, 1986). Basically, the pH-stat method is related to the number of protons released during hydrolysis. The free proton causes a decrease in the pH of the reaction mixture, while the addition of base is required to maintain pH. The amount of base required has a direct relationship with the number of peptide bonds cleaved. However, this method has some drawbacks such as limited in measuring DH value above 30% for single enzyme system, only applicable for pH ranging from 7 to 9 (Adler-Nissen, 1986) and not suitable to be used in a buffering hydrolysis system.

Several methods have been developed to monitor DH during protein hydrolysis including trinitrobenzenesulfonic acid (TNBS), O-phthaldialdehyde (OPA), trichloroacetic acid, soluble nitrogen (SN-TCA) and formol titration. Generally, DH can be determined directly by using the TNBS and OPA methods based on the measurement of free amino groups released from hydrolysis and both methods are comparable. However, the TNBS method possesses several disadvantages such as laborious, required a longer time to obtain results and the use of unstable and toxic chemicals. On the contrary, the OPA method offers several advantages including being more accurate, easier and faster to carry out, the use of more stable and less toxic reagents that are suitable for measurement hydrolysates in buffer solution (Church *et al.*, 1983). The principle of OPA method is based on the reaction of free amino groups released by hydrolysis and O-phtaldialdehyde in the presence of β-mercaptoethanol. Free amino group can be quantified accurately based on the linear relationship between colour intensity and concentration of α-amino groups (amino acid standard) using spectrophotometer at 340nm. However, no research has been reported on the optimization of enzymatic hydrolysis from tilapia by-product using OPA method.

Response surface methodology (RSM) was found to be effective in optimizing and monitoring food processes (Wangtueai and Noomhorm, 2009). Therefore, the objective of this study was to optimize the enzymatic hydrolysis condition on tilapia by-product through optimization process using RSM and the OPA method was used to measure the highest possible degree of hydrolysis (DH) that can be achieved.

Materials and Methods

Sample preparation

Fresh red tilapia *(Oreochromis niloticus)* was purchased from a local wet market in Selangor, Malaysia and transported immediately to the laboratory on ice. The sample was washed, eviscerated and hand filleted. After removing the viscera, tilapia muscle and by-product (head, frames, tail) were separated. Tilapia by-product (TB) was minced using a blender and then packed in polyethylene bags and frozen and stored at -20°C until further use.

Enzymatic hydrolysis reaction

Alcalase enzyme® 2.4 L (Novo Industry, Denmark) was used for conducting enzymatic hydrolysis reaction. Enzymatic hydrolysis was conducted according to the method described by Zarei *et al*. (2012) with a slight modification. Briefly, minced TB was thawed overnight in a cold room (4°C). Then, 10-17.5% w/v of minced TB was mixed with 50 ml of 50 mM buffer solution (pH 6.5-8.5). The mixture was preincubated at the reaction temperature ranging between 55-70°C for 20 min prior to adding alcalase enzyme at a desired enzyme concentration of 1.5.-3.5% w/w to initiate an enzymatic hydrolysis reaction. The enzyme concentration used in this hydrolysis reaction was calculated based on protein content in a substrate. The enzymatic hydrolysis was carried out for 120 min. The reaction was terminated by heating the mixtures in a water bath at 90°C for 15 min. The mixture was immediately cooled and then centrifuged at 10000 rpm for 20 minutes. The supernatant was collected and further determined the degree of hydrolysis (DH).

Degree of hydrolysis (DH)

In protein hydrolysis, determination of the DH is the key factor for monitoring the hydrolysis reaction progress. DH is defined as the percentage of cleaved

peptide bond, as follows:

$$DH = h/h_{tot} \times 100 \quad (1)$$

Where, h_{tot} is the total number of peptide bonds in the substrate studied and h is the number of hydrolyzed bonds. In this study, DH was calculated using the OPA method, as described by Church *et al.* (1983) as well as Nielsen *et al.* (2001) with a slight modification. Briefly, the OPA reagent was prepared daily as follows: 7.62 g sodium tetraborate decahydrate and 200 mg sodium dodecyl sulfate were dissolved in 150 mL deionized water. 160 mg o-phthaldehyde 97% was dissolved in 4 mL ethanol and mixed with the above solution. 400 µL of β-mercaptoethanol was added into the solution and this solution was made up to 200 mL with deionized water. 50 mg serine was diluted in 500 mL deionized water (0.9516 mmole/L). 3 mL OPA reagent was added into 400 µL of serine standard or protein hydrolysate and mixed for 5 s. The mixtures were incubated for 2 min at room temperature and the absorbance (at 340 nm) was measured using spectrophotometer.

Experiment for optimization

Response surface methodology (RSM) was employed to optimize the enzymatic hydrolysis conditions of TB using alcalase enzyme. The influences of four independent variables, A (pH), B (temperature, ºC), C (substrate concentration, %w/v), and D (enzyme concentration, %w/w) at five levels (-2, -1, 0, 1, 2) on the DH were investigated using central composite design (CCD) and the coded and actual values of experimental design are shown in Table 1.

Table 1. Independent variables and their coded and actual levels used in RSM studies for optimizing hydrolysis conditions of tilapia by-product using Alcalase

			Coded Level				
Factor	Unit	Symbol	-2	-1	0	1	2
pH		A	6.5	7.0	7.5	8.0	8.5
Temperature	°C	B	50	55	60	65	70
Substrate Concentration	%	C	10.0	12.5	15.0	17.5	20.0
Enzyme Concentration	%	D	1.5	2.0	2.5	3.0	3.5

The range of each independent variable was determined according to the results obtained from preliminary study (data not shown) in which the minimum and maximum levels were selected based on the highest DH was obtained at that ranges. The experimental values for the degree of hydrolysis (DH) under different combination of independent variables are presented in Table 2. Each experiment was replicated twice and the average of the DH was taken as the response, Y. The CCD consisted of 30 treatments including eight axial point ($\alpha = 2$) and six replicates of the central points.

Statistical analysis

The response surface methodology (RSM) was statistically analyzed by Design-Expert, Version 8.0.11 software (Stat-ease Inc., Minneapolis, Minn., U.S.A.). The multiple regressions analysis was performed to obtain the coefficients. Estimates of the coefficients, with levels higher than 95% ($p > 0.05$), were included in the CCD models. The DH can be expressed as a function of independent variables by a second order polynomial equation:

$$Y = \beta_o + \sum \beta_o x_j + \sum \beta_{jj} x_j^2 + \sum \beta_{jk} x_j x_k \quad (2)$$

Where, Y is the response (Degree of Hydrolysis); β_0 is the intercept; β_j, β_{jj} and β_{jk} are the linear, quadratic and interaction terms, respectively. The responses obtained were statistically evaluated and the model was built based on the variables with the confidence levels of more than 95%.

Results and Discussion

Influences of independent variables on hydrolysis of TB

Central composite design (CCD) was used to optimize the hydrolysis conditions (pH, temperature, substrate concentration and enzyme concentration) of TB by monitoring the DH as the response. The combinations of independent variables and observed values for DH are presented in Table 1 and the highest DH was achieved at pH 7.5, temperature of 60ºC, substrate concentration of 15% (w/v) and 2.5% (w/w) of enzyme with average value of 20.20% after hydrolyzing for 120 min. Until recently, no research was found on the enzymatic hydrolysis of TB to support the finding. Studies on enzymatic hydrolysis of tilapia is only limited to tilapia muscle (Abdul-Hamid *et al*., 2002; Foh *et al*., 2010; Shamloo *et al*., 2012) and most of the studies reported that the highest DH were achieved at 15 to 20% after hydrolysis for 5 h except from the study reported by Foh *et al*. (2010) who obtained the DH value almost at 25% after hydrolyzing for 120 min. Relatively low DH value was obtained in TB when compared with other fish by-products such as catla visceral waste (Bhaskar *et al*., 2008), catfish frame (Amiza *et al*., 2011), and salmon skin (See *et al*., 2011).

Table 2. Actual levels of independent variables used in optimizing the hydrolysis condition of tilapia by-product using Alcalase with the observed values for Degree of Hydrolysis (DH)

Run	pH(A)	Temperature(B)	Substrate(C)	Enzyme(D)	Experimental DH values
1	7.0	65	12.5	3.0	15.99
2	8.0	55	12.5	3.0	18.92
3	8.0	65	12.5	2.0	13.01
4	7.0	55	17.5	3.0	17.21
5	8.0	55	17.5	3.0	16.55
6	8.0	55	17.5	2.0	16.05
7	7.0	65	12.5	2.0	13.90
8	7.0	55	12.5	3.0	18.33
9	7.0	65	17.5	3.0	14.89
10	8.0	65	17.5	2.0	13.80
11	8.0	65	17.5	3.0	13.38
12	7.5	60	15.0	2.5	20.43
13	7.5	60	15.0	2.5	20.75
14	8.0	65	12.5	3.0	12.00
15	8.0	55	12.5	2.0	17.09
16	7.0	55	12.5	2.0	16.07
17	7.5	60	15.0	2.5	19.95
18	7.0	55	17.5	2.0	15.11
19	7.0	65	17.5	2.0	12.66
20	7.5	60	15.0	2.5	19.47
21	7.5	50	15.5	2.5	17.35
22	7.5	60	15.0	3.5	20.09
23	7.5	70	15.0	2.5	9.00
24	7.5	60	15.0	1.5	19.89
25	7.5	60	10.0	2.5	16.97
26	7.5	60	15.0	2.5	20.22
27	7.5	60	20.0	2.5	12.60
28	8.5	60	15.0	2.5	20.20
29	6.5	60	15.0	2.5	19.10
30	7.5	60	15.0	2.5	20.38

Table 3. Analysis of variance for the second order response surface model for degree of hydrolysis

Source	Sum of square	Degree of freedom	Mean square	F value	P value
Model	260.23	14	18.59	19.03	<0.0001
A	0.056	1	0.056	0.057	0.8141
B	74.91	1	74.91	76.70	<0.0001
C	8.64	1	8.64	8.85	0.0100
D	4.15	1	4.15	4.25	0.0583
AB	3.19	1	3.19	3.26	0.0924
AC	0.63	1	0.63	0.65	0.4346
AD	3.78	1	3.78	3.87	0.0692
BC	1.77	1	1.77	1.81	0.1998
BD	0.90	1	0.90	0.92	0.3527
CD	0.036	1	0.036	0.037	0.8503
A^2	4.44	1	4.44	4.55	0.0511
B^2	112.06	1	112.06	114.75	<0.0001
C^2	71.87	1	71.87	73.57	<0.0001
D^2	2.76	1	2.76	2.83	0.1146
Residual	13.67	14	0.98		
Lack of fit	12.72	10	1.2	5.34	0.0604
Pure error	0.95	4	0.24		
Cor. Total	285.20	29			

R^2 = 0.9501, A=pH, B=Temperature (°C), C=Substrate concentration (%), D=Enzyme concentration (%)

The independent and dependent variables were analyzed to obtain a regression equation that could predict the response within the given range. The regression equation for DH which is the response variable (Y) of TB was derived using the regression coefficient of linear, quadratic and interaction terms to fit a response surface model. The response surface equation model obtained through RSM is shown below:

$$Y = - 494.61 + 37.24A + 10.53B + 4.84C + 28.04D - 1.61A^2 - 0.08B^2 + 0.26C^2 - 1.27D^2 - 0.18AB + 0.16AC - 1.95AD + 0.03BC - 0.10BD - 0.04CD \quad (3)$$

Where, Y, A, B, C and D are DH, pH, temperature, substrate and enzyme concentration, respectively. The results of the analysis of variance (ANOVA) for the hydrolysis of TB are presented in Table 2. ANOVA showed that the second-order polynomial model adequately represented the experimental data with a high coefficient of determination value (R^2 = 0.9501). Besides, the regression model is significance with P<0.001. According to Sin *et al.* (2006), the regression model is well defined if R^2 value is higher than 0.80. A small value of R^2 indicates a poor relevance of the dependent variables in the model. The model can fit well with the actual data when R^2 approaches unity (Sin *et al.*, 2006).

The ANOVA results also demonstrate that the linear model terms of temperature (B) has a significant effect (p<0.05), followed by substrate concentration (C); nonetheless, pH (A) and enzyme concentration (D) were not significant (p>0.05). The results indicate that temperature and substrate

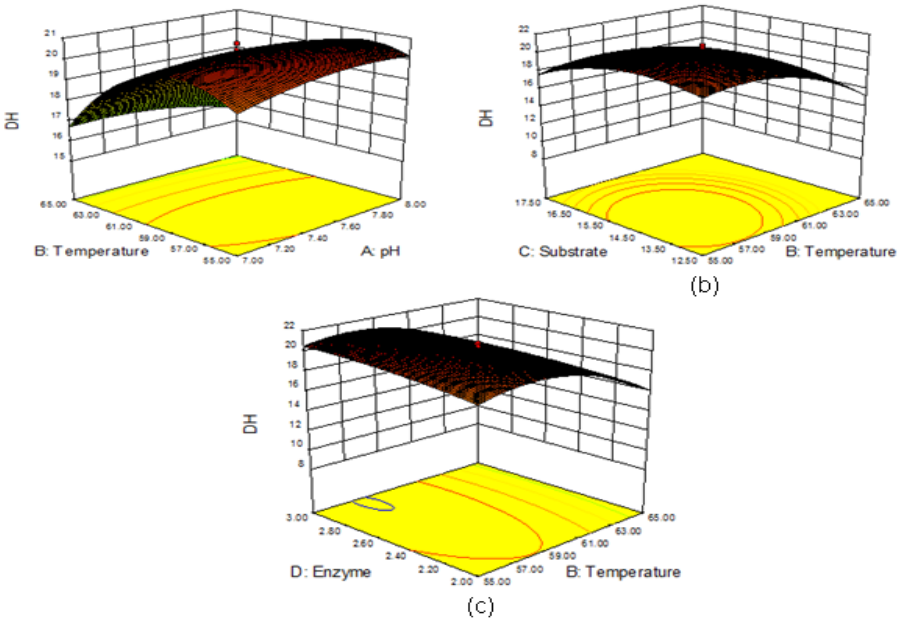

Figure 1. Response surface graph for DH as a function of (a) temperature and pH, (b) substrate and temperature, (c) enzyme and temperature during the hydrolysis of tilapia by-product with alcalase

concentration had a strong influence on DH value. Meanwhile, pH and enzyme had a less effect. Among the quadratic coefficients, (B^2) and (C^2) have shown to have a significant effect ($p<0.05$) but (A^2), (D^2) and the entire interaction coefficients (AB, AC, AD, BC, BD and CD) were not significant ($p>0.05$). This indicated that, interaction between pH, temperature, substrate concentration and enzyme concentration had less effect on DH. The fitness of the model was evaluated through the lack of fit test. The P-values for the lack of fit test was not significant ($p>0.05$) indicating that the model was fit for predicting the enzymatic hydrolysis of TB. A good fit means that the generated models adequately explained the data variation.

The effect of the independent variable on the degree of hydrolysis can be visualized through a three-dimensional response surface which is based on the second order polynomial model (Figure 1a-c). Figure 1a shows the three-dimension graph plot for DH as a function of temperature and pH. The result showed that DH increased with the increase in temperature. DH was found increased rapidly at the beginning of the reaction and reached the optimum value at 60°C. However, a decrease in the DH value was observed above the optimum temperature level. A high temperature level will lead to a complete inactivation of alcalase enzyme due to the thermal denaturation. This result is in agreement with the finding by Adler-Nissen (1986) whereby alcalase is active at temperature ranging from 50-70°C. Several optimization studies on hydrolysis of fish by-product using alcalase have demonstrated that the optimum temperature was achieved at 60°C (Benjakul and Morrissey, 1997; Normah *et al.* 2005; Amiza *et al*., 2011). Similarly, DH was increased with the increase in pH. Meanwhile, the optimum pH value for alcalase to hydrolyze TB was at pH 7.5. Beyond this level, the yield of DH started to decrease. This value is in agreement with the working pH range of Alcalase (pH 6-10) reported in several studies (Benjakul and Morrissey, 1997; Bhaskar *et al.,* 2008; Amiza *et al.*, 2011; See *et al*., 2011).

Figure 1b shows the effects of substrate concentration and temperature on the hydrolysis of TB. Based on the results, increasing the substrate concentration would increase the DH value and the optimum value was obtained at 15% (w/v) of substrate concentration. However, further increases of substrate concentration would cause a decrease in the DH value. The decreases in DH at higher substrate concentrations could be due to the certain phenomena such as the decrease in the concentration of peptide bonds susceptible to hydrolysis by alcalase, possible inhibition of enzyme by the hydrolysis product and enzyme inactivation (Guerard *et al*., 2002).

Figure 1c shows the effects of enzyme concentration and pH on DH of TB hydrolysis. The result revealed that DH increased as the enzyme concentration was increased. The optimum enzyme concentration for hydrolyzing of TB was achieved at 2.5% (w/w) with DH values ranging from 19.47-20.75%. Nonetheless, DH remained constant at the enzyme concentration above this level. This indicated that there would be more chances for hydrolysis to occur at the initial reaction until an optimum level

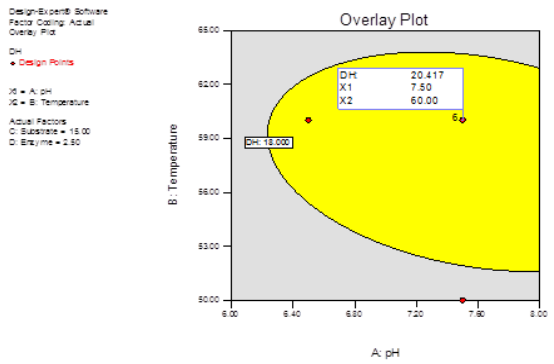

Figure 2. Contour plot of enzymatic hydrolysis on tilapia by- product as a function of temperature and pH

of 2.5% w/w was reached, and when higher level of enzyme was added, there was no change in the DH value. This value is in agreement with the findings obtained by several researchers (Normah *et al.*, 2005; Bhaskar *et al.*, 2008; See *et al.*, 2011; Amiza *et al.*, 2011), whereas, DH was almost constant at 2% of enzyme concentration.

Optimization of hydrolysis and verification

From Figure 2, the optimum enzymatic hydrolysis conditions obtained using superimposed RSM were at pH 7.5, temperature of 60°C, 15.0% (w/v) of substrate concentration and 2.5% (w/w) of enzyme concentration with the DH value at 20.42%. In order to verify the model, RSM suggested the optimum levels of different independent variables based on the desirability profile. If the desirability value is close to 1.0, it means the suggested conditions are suitable to achieve the highest DH. The model predicted the desirability value was 0.968 and the hydrolysis conditions were selected at pH 7.5, temperature of 50°C, substrate concentration of 15.0% (w/v) and enzyme concentration of 2.5% (w/w) to achieve the highest DH at 20.42%. Hydrolysis was carried out using the predicted optimum condition and it managed to obtain DH at 20.31%, which was quite similar to the predicted DH value. This indicates the model used is valid for predicting the DH of TB.

Conclusion

The second order polynomial has been shown to adequately describe and predict the DH value of TB. The hydrolysis of TB using alcalase enzyme is greatly influenced by different operating conditions such as pH, temperature, substrate concentration and enzyme concentration for the production high yield of TB protein hydrolysate. The optimum hydrolysis conditions were obtained at pH 7.5, 50°C, 15.0% (w/v) substrate concentration and 2.5% (w/w) enzyme concentration with DH value of 20.20% after hydrolyzing for 120 min. TB protein hydrolysate produced has potential for applications in food, healthcare and pharmaceutical products.

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