Journal of
Fisheries and Aquatic Science

ISSN 1816-4927

Academic
Journals Inc.

www.academicjournals.com

Journal of Fisheries and Aquatic Science 8 (4): 494-505, 2013
ISSN 1816-4927 / DOI: 10.3923/jfas.2013.494.505

Optimizing the Enzymatic Hydrolysis of Skipjack Tuna (*Katsuwonus pelamis*) Dark Flesh Using Alcalase® Enzyme: A Response Surface Approach

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ABSTRACT

The goal of this study was to optimize enzymatic hydrolysis of the dark flesh of skipjack tuna (*Katsuwonus pelamis*). Alcalase 2.4 L® was used as the hydrolytic enzyme at various concentrations (1, 1.5, 2, 2.5 and 3% w/w) at pHs from 6 to 10, temperatures from 35 to 75°C and times of 2 to 6 h. The experiment was performed following a five-level second-order central composite design with six replications at the center points. The high coefficients of determination for degree of hydrolysis (R^2 = 0.8909) and free tryptophan content (R^2 = 0.9732) indicate the suitability of the design for predicting the responses. The optimum hydrolytic conditions predicted by the response surface methodology were 2% Alcalase® at pH 8.86 and 65.4°C for 5.74 h. These conditions resulted in an experimental degree of hydrolysis of 20.74% and free tryptophan content of 102.23 mg kg^{-1}.

Key words: Skipjack tuna, protein hydrolysate, response surface analysis, free tryptophan, degree of hydrolysis

INTRODUCTION

The canned fish processing industry generates solid wastes that are composed of muscle, viscera, gills, dark flesh/dark muscle, head, bone and skin and this waste can reach up to 70% of the original raw material (Guerard *et al*., 2002). Sutanbawa and Aknes (2006) estimated the wastes of the tuna canning industry to be 450,000 tons annually. They also concluded that methods to add value to tuna processing by-products must be developed.

Dark muscle is a protein-rich part of the tuna. It is considered to be a by-product in the tuna canning industry because of its undesirable characteristics, including unpleasant flavor, dark color and susceptibility to oxidation. Dark muscle is commonly used to produce low-market value products such as fish meal and fertilizer. An alternative use of this by-product is to convert it into Fish Protein Hydrolysate (FPH), which is widely used in food systems. FPH has been made from fish by-products as well as underutilized fish species through the process of hydrolysis (Kristinsson and Rasco, 2000). Hsu (2010) conducted studies of the enzymatic production of hydrolysate from tuna dark muscle and Hsu *et al*. (2011) highlighted the potential use of tuna dark muscle FPH in the

pharmaceutical industry due to its antiproliferative and antioxidative effects. However, optimization of the enzymatic hydrolysis process for skipjack tuna dark flesh especially using Alcalase 2.4 L®, has not been reported previously.

FPH obtained from controlled enzymatic hydrolysis is among the best protein hydrolysates in terms of nutritional properties, balanced amino acid composition and high digestibility (Kristinsson and Rasco, 2000). Several researches had mentioned the nutritional value of hydrolysate product from fisheries product/by-product. Foh *et al.* (2011) mentioned that the FPH from Nile Tilapia have good nutritional properties, whereas Zhao *et al.* (2011) also reported high nutritional value of Shrimp processing byproduct hydrolysate. However, it is not used for human consumption because of its bitter flavor and fishy odor. A general acknowledged problem encountered in the use of food protein enzymatic hydrolysis is the formation of bitter taste. The bitter taste can be ascribed to hydrophobic peptides and result from the degradation of the protein substrate (Kamara *et al.*, 2011). One of the amino acids contributing to the bitter taste is tryptophan (Pedersen, 1994; Dewilche *et al.*, 2001).

Even though the enzyme's manufacturers has stated a range of optimum condition for the enzymes, the determination of more specific condition is needed due to the effect of different substrate and condition eligibility in industries. For example, the optimum conditions for Alcalase® according to the application sheet are in pH value 8 and temperature of 50-60°C (Novozymes, 2001). Nonetheless, Normah *et al.* (2005) found that the optimum condition of hydrolysis of Threadfin bream was at pH 8.5 and temperature of 60°C which are different from suggested condition. Similar pattern has been reported by See *et al.* (2011), which mentioned the optimum condition of Salmon (*Salmo salar*) hydrolysis at pH 8.39 and temperature of 50°C. However, the optimization study of hydrolysis using Alcalase 2.4 L® is not much reported. To address these issues, we conducted tuna dark flesh hydrolysis using Alcalase 2.4 L®, followed by the response surface methodology to identify the optimum parameters for generating high hydrolysis activity and low free tryptophan production.

Today, response surface methodology is popular method that have been used for optimization and found to be effective to be applied in wide range of study such as enzymatic hydrolysis from sesame flour (Kanu *et al.*, 2009), starch hydrolysis by α-amylase (Jaiswal *et al.*, 2011), gelatin extraction optimization (Kasankala *et al.*, 2007), culture medium formulation (Ibrahim and Elkhidir, 2011), defatted corn protein extraction (Kongo-Dia-Moukala and Zhang, 2011), extraction of polysaccharides from Chinese malted sorghum (Claver *et al.*, 2010), cyclodextrin glycosyltransferase production (Ai-Noi *et al.*, 2008), honey candy recipe (Seth and Mishra, 2011), solubilization of palm mill eluent (Chou *et al.*, 2010) and even the tranesterification and biodiesel making (Jeong *et al.*, 2009; Shieh *et al.*, 2003). In this research, we anticipate that the optimization results may lead to the development of the most effective FPH production from tuna dark muscle with acceptable (i.e., low) bitterness.

MATERIALS AND METHODS

Raw materials and chemicals: Skipjack tuna by-product (dark flesh) was obtained from PT. Medan Tropical Canning and Frozen Industry (Medan, Indonesia), transported to the laboratory of the Food Technology Programme in a frozen state and kept at -20°C until use. Alcalase 2.4 L® was obtained from Novo Nordisk (Bagsværd, Denmark) and stored at 4°C until use. Tryptophan standard (5-Fluoro-L-tryptophan = 98.0% (HPLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemical reagents used for the experiments were analytical grade.

Production of protein hydrolysate: Thawed skipjack tuna dark flesh was minced in a blixer (Robot Coupe, Vincennes Cedex, France) and then heated for endogenous enzymes inactivation and fat removal at 85°C for 20 min in a wise-water bath (Daihan Scientific, Seoul, Korea) following Guerard *et al.* (2001). The treated material was then cooled and centrifuged at 3500 rpm for 20 min at 4°C (Union 5KR centrifuge, Hanil Science Industry, Gyeyang-gu Incheon, Korea) to separate the oil. The separated oil was removed and the protein-rich solid was used for the experiments. The protein-rich solid (sample) was mixed with sodium phosphate buffer 1:2 (w/v) and homogenized for 2 min at ambient temperature. The pH of the mixture was adjusted to 6.0, 7.0, 8.0, 9.0 and 10.0 by adding 2 N NaOH. Different concentrations of Alcalase 2.4 L® (1, 1.5, 2, 2.5 and 3% w/w) were added to the protein-rich solid sample. All reactions were performed in 250 mL conical flasks in a shaking incubator (LM-570R Orbital Shaker Incubator, Chemist Scientific Corp., Taipei Hsien, Taiwan) with constant agitation (200 rpm) at 35, 45, 55, 65 and 75°C for 2, 3, 4, 5 and 6 h. After each treatment, the reaction was terminated by heating the solution in a water bath at 85°C for 10 min (JP Selecta SA, Barcelona, Spain) to assure inactivation of the enzyme. The hydrolysate then was cooled in ice to room temperature and centrifuged at 10000 rpm at 4°C for 20 min in a Kubota 6500 centrifuge (Tokyo, Japan) to collect the supernatant.

Degree of hydrolysis: Degree of hydrolysis (DH) was estimated following Hoyle and Merrit (1994). To the supernatant, one volume of 20% trichloroacetic acid (TCA) was added, followed by centrifugation at 10000 rpm at 4°C for 10 min to collect the 10% TCA-soluble materials. Total nitrogen in the 10% TCA soluble material and the substrate was estimated by the Kjeldahl method using a Kjeltec protein analyzer (FOSS analytical AB, Hoeganaes, Sweden). The DH was calculated as:

$$DH\ (\%) = \frac{10\%\ TCA + soluble\ N_2\ in\ the\ sample}{Total\ N_2\ in\ the\ sample} \times 100$$

Free tryptophan content: Free tryptophan (FT) content was determined following Sanchez-Machado *et al.* (2008). A 250 mg aliquot of the freeze dried sample was placed in a volumetric flask and diluted to 25 mL with acidic water (ultra-pure water adjusted to pH 6.3 with 0.1 M HCl) to obtain a concentration of 10 mg mL^{-1}. Afterwards, the samples were sonicated (Elma Hans Schmidbauer GmbH and Co. KG, Singen, Germany) for 2 min to allow complete dissolution to occur. The solution was then analyzed using a HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Waters Alliance 2690 Separation Module, a Waters fluorescence detector and a Waters Millennium 32 workstation version 3.2. The separation was performed with a Hypersil ODS C18 column (250×4.6 mm, 5 μm) (Thermo Scientific, Waltham, MA, USA) fitted with a Hypersil ODS guard column. The mobile phase consisted of a mixture of methanol: 40 mM sodium acetate buffer (adjusted to pH 4.5 with acetic acid; 20:80, v/v) filtered through a 0.22 mm membrane and degassed; a flow rate of 1.00 mL min^{-1} and column temperature of 26°C was used. The fluorescence was recorded at the optimal wavelengths for tryptophan (λ_{ex} = 280 and λ_{em} = 348 nm) for another 15 min.

Statistical analysis: The experiment was performed using the central composite design with six replications of the center points and five levels of each of four variables. The five levels were coded as -2, -1, 0, +1 and +2 using Design Expert® version 8.0.0 (Stat-Ease, Inc. Minneapolis, MN, USA).

Table 1: Level of selected factor in actual and coded form

Factor	Actual conditions	Coded level
Time (h)	2, 3, 4, 5, and 6	-2, -1, 0, +1, +2
Temperature (°C)	35, 45, 55, 65 and 75	-2, -1, 0, +1, +2
Concentration (%)	1.0; 1.5; 2.0; 2.5; and 3.00	-2, -1, 0, +1, +2
pH	6.0; 7.0; 8.0; 9.0; and 10	-2, -1, 0, +1, +2

Table 2: CCD for optimum hydrolysis variables on degree of hydrolysis and free tryptophan of tuna dark muscle

					Result	
	Time (h)	Temp. (°C)	Conc. (%)	pH		
Run	Coded x_1	Coded x_2	Coded x_3	Coded x_4	DH (%)	FT (mg kg^{-1})
1	-1	-1	-1	-1	17.46	112.18
2	-1	-1	-1	-1	17.03	112.36
3	-1	1	-1	-1	18.58	77.42
4	1	1	-1	-1	18.96	98.35
5	-1	-1	-1	1	17.37	111.98
6	1	-1	-1	1	18.14	123.59
7	-1	1	-1	1	18.05	76.90
8	1	1	-1	1	18.96	100.00
9	-1	-1	1	-1	18.71	131.81
10	1	-1	1	-1	17.75	98.05
11	-1	1	1	-1	18.96	93.00
12	1	1	1	-1	19.79	79.91
13	-1	-1	1	1	18.45	130.00
14	1	-1	1	1	18.75	109.00
15	-1	1	1	1	18.84	108.28
16	1	1	1	1	19.60	93.78
17	-2	0	0	0	19.57	144.00
18	2	0	0	0	21.19	138.90
19	0	-2	0	0	16.55	135.46
20	0	2	0	0	20.05	75.99
21	0	0	0	-2	17.34	70.00
22	0	0	0	0	19.63	86.00
23	0	0	-2	0	18.96	99.01
24	0	0	2	0	19.28	115.80
25	0	0	0	0	20.60	116.35
26	0	0	0	0	20.43	121.33
27	0	0	0	0	20.24	117.00
28	0	0	0	0	19.81	112.52
29	0	0	0	0	19.94	117.61
30	0	0	0	0	19.92	117.62

DH : Degree of hydrolysis (value of triplicate), FT : Free tryptophan (value of triplicate)

The independent variables were X_1 (time, h), X_2 (temperature, °C), X_3 (Alcalase 2.4 L concentration, %) and X_4 (pH). Table 1 shows the experimental design for the actual (X) and coded (x) levels of variables, whereas Table 2 shows the results of the analysis. The response function Y for DH and FT was approximated by a second-degree polynomial using Eq. 1:

$$Y = b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_4+b_{12}X_1X_2+b_{13}X_1X_3+b_{14}X_1X_4+b_{23}X_2X_3 +b_{24}X_2X_4+b_{34}X_3X_4+b_{11}X^2_1+b_{22}X^2_2+b_{33}X^2_3+b_{44}X^2_4+\varepsilon \quad (1)$$

Table 3: Sequential model sum of squares of degree of hydrolysis and free tryptophan response

		DH (%)		FT (mg kg^{-1})	
Source of variations	DF	Sum of square	p-value	Sum of square	p-value
Mean	1	10788.47		346500	
Linear	4	15.92	0.0066	4790.05	0.0066
Interaction	6	1.68	0.9463	1513.18	0.4836
Quadratic	4	15.97	<0.0001	4739.46	<0.0001
Cubic	8	1.61	0.7815	149.16	0.5954
Residual	7	2.50		154.68	
Total	30	10826.16		357900	

DH : Degree of hydrolysis, FT: Free tryptophan

The linear, interaction and quadratic effects are represented along with the coefficient (b0) by b1, b2, b3 and b4 (linear effects); b12, b13, b14, b23, b24 and b34 (interaction effects); and b11, b22, b33 and b44 (quadratic effects). The adequacy of the model was checked by accounting of fit summary generated by the Design Expert software (Table 2). The optimum conditions were determined using the Myers and Montgomery desirability method contained in the software, the chosen desired goal were the independent variables were kept within in range, whereas the response for DH was maximized and that for FT was minimized.

RESULTS AND DISCUSSION

Table 2 provides the results of experiments performed using a combination of the variables for five response functions (DH and FT). Table 3 shows the fit summary calculated by the software to determine the most suitable model for the responses. Table 4 shows the results of the analyses of variance (ANOVAs) and the coefficient of the second-order polynomial for the coded level of the variables for all of the response functions. The coefficient of the second-order polynomial for the actual level of the variables for all of responses is shown in Table 5. Finally, Table 6 shows the numerical calculation of the optimum conditions for enzymatic hydrolysis. Figure 1-5 show the response surface graphs. The experimental data shown represent the mean value of triplicate measurements.

Model fitting: Each of the response variables (Y) for DH and FT was fitted by a second-order polynomial response. The sequential model of sum of squares analyses indicated that adding up the quadratic terms significantly improved the model ($p<0.0001$ for DH and FT) (Table 3). Therefore, the quadratic model is the most appropriate model for modeling the DH and FT response. The corresponding coefficients of determination (R^2), adj-R^2 and coefficients of variations (CV) can also indicate the adequacy of the model (Koochecki *et al*., 2009). The high determination coefficients for the DH ($R^2 = 0.8909$) and FT ($R^2 = 0.9732$) response (Table 4) indicate the suitability of the second-order polynomial for predicting the responses. These values are in agreement with Little and Hills (1978), who reported that the R^2 should not be <80%.

The high value of R^2 must be followed by close value of adj-R^2 to make sure that the non-significant terms have not been included in the model (Koochecki *et al*., 2009). In our study, the adj-R^2 values were quite high (0.7891 and 0.9482 for DH and FT, respectively) and the coefficients of variations were low (2.76 and 4.19 for DH and FT, respectively), the latter show enough precision and reliability to assure that the quadratic model can adequately model the

Table 4: Condensed ANOVA for the response surface quadratic model of dependent variables

		DH (%)		FT (mg kg^{-1})	
Source of variations	DF	Sum of square	p-value	Sum of square	p-value
Model	14	33.58	<0.0001	11042.69	<0.0001
X_1	1	1.4	0.0389	56.18	0.1166
X_2	1	9.48	< 0.0001	4273.92	< 0.0001
X_3	1	0.1	0.5523	294.30	0.0017
X_4	1	4.93	0.0007	165.64	0.0119
X_1X_2	1	0.64	0.1480	220.65	0.0049
X_1X_3	1	0.53	0.1850	38.90	0.1861
X_1X_4	1	0.03	0.7434	1193.18	< 0.0001
X_2X_3	1	0.42	0.2341	6.37	0.5832
X_2X_4	1	0.066	0.6316	11.47	0.4634
X_3X_4	1	0.000186	0.9796	42.61	0.1675
X_1^2	1	0.057	0.6556	778.39	< 0.0001
X_2^2	1	8.77	< 0.0001	356.23	0.0008
X_3^2	1	3.56	0.0026	278.11	0.0021
X_4^2	1	7.4	0.0001	3044.39	< 0.0001
Residual	15	4.11		303.84	
Lack of fit	10	3.61	0.0839	263.89	0.0997
Pure error	5	0.5	39.9500		
Total	29	37.69		11346.5200	
R^2		0.8909		0.9732	
Adj-R^2		0.7891		0.9482	
CV (%)		2.76		4.1900	

Variables X_1: Time, X_2: Temperature, X_3: Concentration, X_4: pH

Table 5: Regression equations for the response functions in the actual level of variables

No.	Equation
1	$Y_1 = -45.58074-0.87052X_1+0.72119X_2+6.28253X_3+9.30176X_4+0.019962X_1X_2+0.36371X_1X_3-0.043636X_1X_4-0.032455X_2X_3-0.0066405X_2X_4-0.006818X_3X_4-0.045492X_1^2-0.0056549X_2^2-1.44197X_3^2-0.51924X_4^2$
2	$Y_2 =-653.76963-1.72368X_1+0.2146X_2+12.42673X_3+194.55102X_4+0.37136X_1X_2+3.11838X_1X_3-8.63563X_1X_4+0.12621X_2X_3+-0.084663X_2X_4+3.26375X_3X_4+5.32717X_1^2-0.036038X_2^2-12.73708X_3^2-10.53533X_4^2$

Variables X_1: Time, X_2: Temperature, X_3: Concentration, X_4: pH , Response: Y_1: Degree of hydrolysis, Y_2: Free tryptophan

Table 6: Optimum result condition

				Predicted		Experimental	
Time (X_1)	Temp. (X_2)	Conc. (X_3)	pH (X_4)	DH (%)	FT (mg kg^{-1})	DH (%)	FT (mg kg^{-1})
5.73	65.41	2.04	8.87	20.73	102.011	20.02	107.20

responses. Moreover, good agreement between the actual experimental results and the predicted values generated by the polynomial regression model (Fig. 1) suggests that the quadratic model is able to identify the optimal conditions for hydrolysis of tuna dark flesh to produce FPH.

Degree of hydrolysis: The DH values in the experiments varied from 16.55% to 21.19% (Table 2). This result is in agreement with results reported by Normah *et al.* (2005) they showed 20% DH to be the optimum activity of endogenous enzymes, which is within the acceptable range

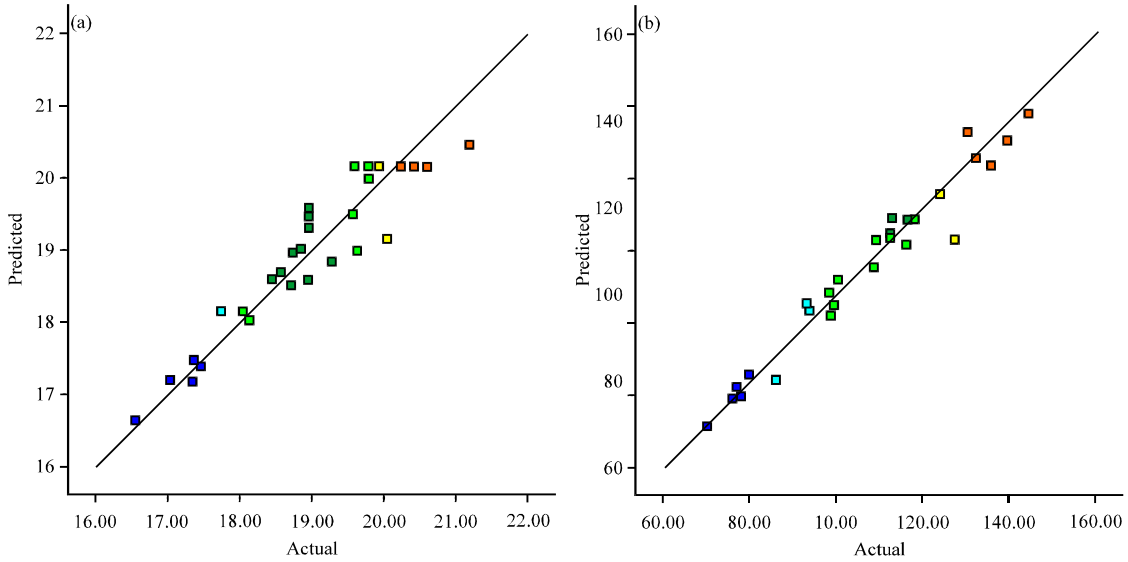

Fig. 1: Comparison between predicted and actual value of degree of (a) Hydrolysis and (b) Free tryptophan

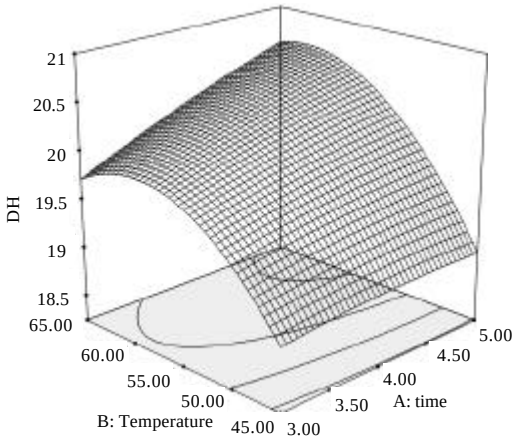

Fig. 2: Response surface of degree of hydrolysis (DH) for the effect of time (h) and temperature at constant Alcalase 2.4 L concentration 2% and pH 8.0

(15-25%) quoted by Novozymes (2001) for Alcalase 2.4 L®. A significant p value ($p<0.05$) for linear effects of time, temperature and pH shows that these variables significantly affected the DH. Increasing the temperature of hydrolysis of dark flesh induced an increase in the DH, although when a certain temperature was reached, the DH value began to decline. The time variables showed a similar trend, although the fluctuation was not pronounced (Fig. 2). The DH value rose with increased of the pH conditions.

According to the Alcalase 2.4 L® application sheet (Novozymes, 2001), the optimum temperature of hydrolysis is 50-60°C at pH 8. However, our experimental results showed that maximum DH was obtained at 55°C and pH 8.00 (Table 2). The differences in raw material used could affect the enzymatic hydrolysis. See *et al.* (2011) found that the optimum conditions that resulted in the

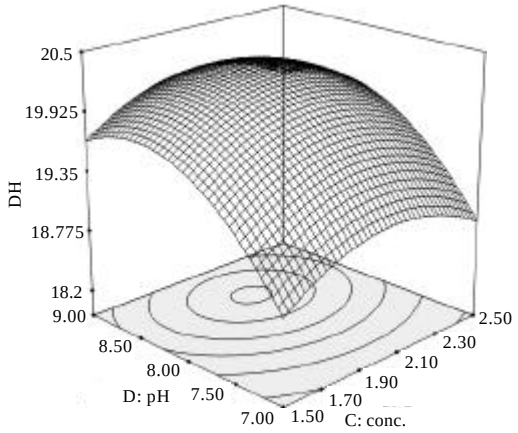

Fig. 3: Response surface of degree of hydrolysis (DH) for the effect of concentration (%) and pH at constant time 4 h and temperature 55°C

highest DH for hydrolysate production from Salmon (*Salmo salar*) using Alcalase were 55.3°C and pH 8.39. Taheri *et al.* (2011) reported a temperature of 45.62°C and a pH of 8.5 to be the optimum conditions for hydrolysis of goldstripe sardine (*Sardinella gibbosa*). Similarly, Ovissipour *et al.* (2009b) found 50°C and pH 8.5 to be the optimum conditions for hydrolysate production from Beluga sturgeon (*Huso huso*) using Alcalase. Other researchers including Benjakul and Morissey (1997), Normah *et al.* (2005) and Chabeaud *et al.* (2009) reported the optimum conditions for hydrolysis of Pacific whiting (*Merluccius productus*) to be 60°C and pH 9.5, that of threadfin to be 60°C and pH 8.5 and Saithe (*Pollachias virens*) to be 60°C and pH 9.0, respectively.

The significant quadratic effect of Alcalase 2.4 L® concentration is shown by the increase in the DH value prior to the stationary phase and decrease inclination (Fig. 3). Ovissipour *et al.* (2009a) observed the same pattern in the hydrolysis of beluga sturgeon (*Huso huso*) when Alcalase 2.4 L® was used. The decreasing pattern that occurred at higher concentration of enzyme has been described previously by Moreno and Cuadrado (1993), who found that this phenomenon led to a decrease of available peptide bonds, leading to a decrease in enzyme activity and product inhibition. This condition in turn contributed to the downward tendency of the hydrolysis curve.

Generally, the DH value slightly increased with increasing hydrolysis time in this experiment. The incubation time, which was 3.00 to 5.00 h, resulted in no significant improvement of DH. This condition is similar with the result found by Normah *et al.* (2005) who reported that a gradual plateauing of the curve occurred when the substrate was further hydrolyzed. O'Meara and Munro (1984) mentioned that rapid cleavage of the susceptible peptide bonds of the substrate during the initial stage of hydrolysis followed by a slower cleavage of the less susceptible bond at the later stage formed the pattern for their hydrolysis curve. In addition, Adler-Nissen (1986) mentioned that the inhibition process was caused by the products. The products compete with the remaining substrates for the binding site of the enzyme during the later stage.

Free tryptophan content: In our study, FT content was affected by linear effect of temperature, Alcalase 2.4 L® concentration and pH variables and the interaction of time and temperature; the interaction of time and pH. The experiment was also affected by the quadratic value of all variables (Table 3).

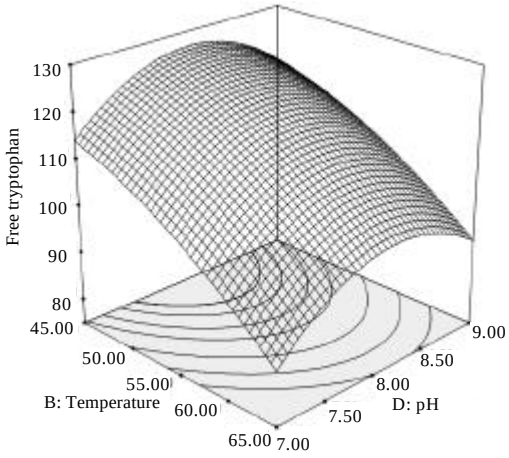

Fig. 4: Response surface of free tryptophan (FT) content for the effect of temperature (°C) and pH at contants Alcalase® concentration 2 % and time 4 h

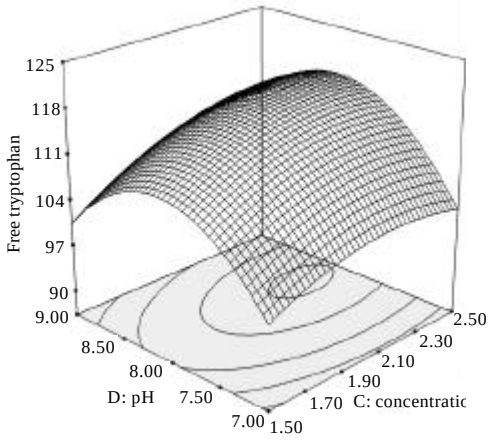

Fig. 5: Response surface of free tryptophan (FT) content for the effect of Alcalase® concentration (%) and pH at constant temperature 55°C and time 4 h

Temperature showed significant linear and quadratic effect on the experiment (Fig. 4). Generally, increasing the temperature resulted in decreasing of the FT content. In addition to the temperature variable, the Alcalase 2.4 L® concentration had a significant linear effect and quadratic effect on the hydrolysis process. Generally, increased enzyme concentration used in conjunction with any of the other factors produced hydrolysate with a higher FT content. The linear and quadratic values of pH also had significant effects on the process. As shown in Fig. 5, the experiment was clearly optimized only at certain range of pH, which shows decrease or increase inclination after the optimum pH.

The time variable showed significant effect on the quadratic value and when it interacts with other variables. Longer hydrolysis time at high pH produced lower FT content while longer hydrolysis time at low pH resulted in higher FT content. Contrarily, the FT content result for the interaction of time and temperature showed the opposite.

Because bitterness is an undesirable component in a food product, bitterness (represented here by the FT content) has to be as low as possible. The FT values in our experiment varied from

70.00 to 138.90 ppm (w/w) hydrolysate (Table 2), thus they were under the tryptophan threshold of bitterness in food products (1021.15 ppm in water, eqv 5 mmol L^{-1}) (Warmke and Belitz, 1993). We found that DH and FT were positively correlated, which means that the FT content increased as the DH increased. This result agreed with that of Nilsang *et al.* (2005), who noted that free tryptophan content continuously increased during enzymatic hydrolysis of fish soluble protein. However, in our optimization study using the response surface methodology, the model predicted the DH and FT responses (maximize DH and minimize FT) to be 20.73% DH and an acceptable value of FT of 102.01 mg kg^{-1}. In the actual experiment (validation), the values were 20.02% DH and 107.02 mg kg^{-1} FT (Table 6).

CONCLUSION

The production of FPH from skipjack tuna dark flesh using Alcalase 2.4 L® enzyme was successfully performed and optimized. The FT produced in all experiments were under the tryptophan threshold of bitterness in food products. The optimized conditions for both FT and DH were 65.41°C, pH 8.87 and 2.04% Alcalase 2.4 L® for 5.73 h and these conditions resulted in experimental values of 20.02% DH and FT content of 107.20 mg kg^{-1}. These values are acceptable for implementation in food industries. RS000M proved to be effective for studying the optimization of enzymatic hydrolysis process of skipjack tuna dark flesh using Alcalase 2.4 L®.

ACKNOWLEDGMENTS

The authors acknowledge with gratitude the support given by Universiti Sains Malaysia (USM) through the Postgraduate Research Grant Scheme 1001/PTEKINDO/843003 and a USM Fellowship given to the first author in the area of fish processing technology. The authors are also grateful to Mr. N. Ariefandie Febrianto for his technical comments on this paper.

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