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Optimization of enzymatic hydrolysis of yellowfin tuna *Thunnus albacares* viscera using Neutrase

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Abstract

Enzymatic hydrolysis of yellowfin tuna visceral protein using Neutrase was studied by response surface methodology using factorial design. Degree of hydrolysis (DH; %) has been estimated as a response surface to the hydrolysis conditions (enzyme activity, reaction temperature, and reaction time). R^2 of 0.91 for the mathematical model indicated that 91% of the variability within the range of values studied could be explained by the model. Lack-of-fit test revealed a non-significant value for the model, indicating that the regression equation was adequate for predicting the degree of hydrolysis under any combination of the variables ($P < 0.05$). Enzyme activity of 39.61 AU/kg protein, temperature of 53° C, and hydrolysis time of 141min were found to be the optimal conditions to reach 30% degree of hydrolysis. The tuna visceral protein hydrolysates had relatively high protein (74.56%), and low lipid (1.86%) content. The chemical score of the hydrolysate indicated that it fulfils adult human nutritional requirements except methionine. Lysine and methionine were the first and the second limiting amino acid in that order. Phenylalanine was predominant amino acid in the hydrolysates with respect to common carp requirement.

Keywords: Fish protein hydrolysate, Tuna visceral protein, Neutrase, Optimization, RSM

Introduction

Recognition of the limited biological resources and increasing environmental pollution have emphasized the need for better and more value-added utilization of the under-utilized fish and the by-products from the fishing industries (Geurard et al. 2002). Commercially viable means for totally utilizing aquatic animals and using them as food have not been successful. More than 100 million tons of fish per year are being harvested (FAO 2006); close to the maximum sustainable yields, of which 29.5% is transformed into fishmeal (Kristinsson and Rasco 2000a).

More than 15% of the harvested biomass is considered processing waste, and is not used for human consumption. Nowadays, industries are no longer permitted to discard their wastes directly to the environment. This results in refining costs for the materials before discarding. Enzyme utilization to hydrolyze food proteins is a

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process of considerable importance that improves the physiochemical, functional, and sensory properties of the intact protein without prejudicing its nutritional value.

Hydrolyzing of proteins can also improve it's intestinal absorption (Kristinsson and Rasco 2000a). It is assumed that peptone from enzymatic hydrolysis of fish wastes is an appropriate nitrogen source for bacterial growth (Gildberg et al. 1989; Safari et al. 2009; Ovissipour et al. 2009a,b,c,d, 2010a,b, 2011). Commercial enzymes provide more controllable reaction and results to produce broad spectrum products with different applications (Kristinsson and Rasco 2000a).

A total world tuna catches are about 3 million metric tons (FAO 2006), canned fish industries generate solid wastes which can be as high as 50- 70% of the original raw material, Preparation of tuna waste hydrolysate would provide means for better utilization of the by-products, traditionally used for animal feed (Guerard et al. 2002).

Many researchers, tried to compare some commercial proteases in order to test the most suitable one for fish protein hydrolysis. The most common commercial proteases, to hydrolysis of fish proteins are both from plant source such as papain (Hoyle and Merritt 1994; Shahidi et al. 1995) or from animal origin such as pepsin (Viera et al. 1995), trypsine, and chymotrypsine (Simpson et al. 1998).

Enzymes from microbial origin have been also applied for this purpose. In comparison to animal or plant-derived enzymes, microbial enzymes offer several advantages including a wide variety of available catalytic activities, and greater pH, or temperature stabilities (Diniz and Martin 1997). Comparative studies of commercial enzymes are complicated by the fact that enzymes have different specific activities, specificities and optimal working conditions (Aspmo et al. 2005). In some cases, proteolytic enzymes have been compared by adjusting the hydrolysis condition to the optimum conditions given by the manufacture for each enzyme, without considering that these optimum conditions also may depends on the substrate used (Kristinsson and Rasco 2000a).

Yellowfin tuna *Thunnus albacares* is one of the most interesting tuna fish with an annually catch of 41,000 tons in south of Iran (IFO 2006). The most caught tuna fish are processed into can in oil one, which produces anegotiable amounts of by-products. This study is focused on enzymatic hydrolysis of yellowfin tuna viscera using Neutrase 0.8 L to produce fish protein hydrolysate (FPH).

Materials and methods

Raw materials

Yellowfin tuna (*T. albacares*) caught in south coast of Iran, Bandar Abbas (spring, 2009), immediately frozen on board at -20 °C, and transferred to north of Iran at -20 °C. Fish viscera were obtained from frozen fish using electrical saw from local processing plant (Shenger Co. Amirabad, Iran), immediately (3 h) transferred to the laboratory at Tarbiat Modares University (Nour, Iran). Once received in the laboratory, fish visceral were minced twice using a domestic mixer (5 mm plate size) at medium speed, divided in to plastic containers. It was frozen again at -20 ºC for further analysis. Compositional analyzing experiments conducted within 2 days after mince freezing. The applied enzyme for hydrolyzing was Neutrase® 0.8 L is a bacterial methaloproteinase from Bacillus amyloliquefaciens, which was provided from Novozymes Co. (Bagsvaerd, Denmark) and stored at 4 °C until use. All the chemicals used for the experiments were of analytical grade.

Preparation of fish protein hydrolysate

The hydrolysate production was carried out according to Ovissipour et al. (2009a,b,c,d,2010a,b,2011). Frozen mince was defrosted overnight at 4-5 °C , then cooked at 85 °C in a water bath (W614-B, Fater Rizpardaz, Tehran, Iran) for 20 min to inactivate endogenous enzymes (Guerard et al. 2002; Ovissipour et al. 2009).

The cooked viscera was mixed with distilled water 1:2 (w:v), and homogenized in a mixer for about 1min. The pH was adjusted to the optimum activity of Neutrase, pH 8 by adding 1N NaOH. All reactions were performed in 250 ml glass vessels containing 30 g substrate, using a in shaking incubator (Ivymen System, Comecta, Spain) with constant agitation (200 rpm). After each sampling, reactions were terminated by heating the solution to 95 °C for 15 min (Guerard et al. 2002; Ovissipour et al. 2009), assuring enzyme inactivation. The hydrolysates were then cooled and centrifuged at 8000 g at 10 °C for 20 min in Hermle labortechnik GmbH z206A (Germany) centrifuge, to collect the supernatant.

Proximate Composition

Moisture content was determined by placing approximately 2 g of sample into a pre-weighted aluminum dish. Samples were then dried in an oven at 105 ± 1 °C overnight or until reach to a constant weight. These were cooled in a desiccator and re-weighed. The moisture content was calculated as the difference in the two weight measurements (AOAC 2002). Protein content in raw materials, and FPH was determined using the Kjeldahl method (N × 6.25) according to AOAC (2002). Total lipid in samples was determined by Soxhlet extraction (AOAC 2002). Ash content was determined by burning 2.0 g sample in pre-weighted porcelain crucible at 600 °C until a white ash was formed (AOAC 2002). Protein in the supernatant was measured, following centrifugation, by the Biuret method according to Layne (1957) using bovine serum albumin as a standard protein. Absorbance was measured at 540 nm in a UV/vis spectrophotometer (Jenway, 6305, UK).

Optimization experiments

The hydrolysis conditions were optimized using response surface methodology (RSM) with a completely randomized factorial design. Three different independent variables, enzyme activity (X_1, °C), and reaction temperature (X_2, minute), and reaction time (X_3, Au/kg protein) were employed at five levels (-α, -1, 0, +1, and +α) for the experiment, based on suggestion of enzyme manufacture (Novozymes, Bagsvaerd, Denmark), and our preliminary experiment (unpublished data), which are presented in Table 1.

Table 1. Values of coded levels used in the optimization experiment

Factor	Levels				
	-α[1]	-1	0	+1	+α
Enzyme activity (AU/kg protein) (X_1)	0.13	0.020	0.030	0.040	0.047
Temperature (°C) (X_2)	32	37	45	53	59
Time (min) (X_3)	50	78	120	162	191

[1] α= 1.68

The experimental design consists of eight factorial points, six axial points and four replicates at central point (Table 2). Degree of hydrolysis (DH%) was determined as the response variable for the combination of the independent variables is given in Table 2.

Experimental runs were randomized to minimize the effect of unexpected variability in the observed response, and the Model was explained by the following equation:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where y is the dependent variables (degree of hydrolysis in real value), $ß_0$ is constant, and $ß_i$, $ß_{ii}$ and $ß_{ij}$ are coefficients estimated by the model. X_i, and X_j are levels of the independent variables which represent the linear, quadratic and cross-product effects of the X_1, X_2 and X_3, on the response. The model evaluated the effect of each independent variable to a response (Bhaskar et al. 2008; Cao et al. 2008; Ovissipour et al. 2010a).

Degree of hydrolysis

Degree of hydrolysis was estimated according to Hoyle and Merritt (1994). To the supernatant, one volume of 20% trichloroacetic acid (TCA) was added, followed by centrifugation to collect the 10% TCA-soluble materials. The degree of hydrolysis (DH) was computed as:

%DH = (10% TCA soluble N in the sample/total N in the sample) × 100

Amino acid composition

Fish protein hydrolysates were dried using a freeze-dryer (Edu-7012, Operon, South Korea). Then, freeze-dried sample was exposed to 6M HCl at 110 ± 1 °C for 24h for hydrolyzing the protein. Derivatisation has been occurred by o-phthaldialdehyde prior to HPLC analysis. The total amino acids were analyzed by the Knauer (Germany)

HPLC using C18 column (Kenauer, Germany) at the flow rate of 1 ml/min using fluorescence detector (RF-530, Knauer, Germany).

Table 2. Coded level combinations for three variables with the observed values for the DH as response variable

	Coded levels of variable			
Run No. #	X1	X2	X3	DH (%)
1	1	1	1	39.76
2	1	1	-1	30.11
3	1	-1	1	26.63
4	1	-1	-1	25.44
5	-1	1	1	18.58
6	-1	1	-1	13.07
7	-1	-1	1	18.48
8	-1	-1	-1	16.87
9	0	1.68	0	34.11
10	0	-1.68	0	20.14
11	0	0	1.68	28.22
12	0	0	-1.68	22.14
13	1.68	0	0	30.72
14	-1.68	0	0	19.45
15	0	0	0	30.42
16	0	0	0	34.55
17	0	0	0	33.28
18	0	0	0	34.24

X_1: enzyme activity, X_2: reaction temperature, X_3: reaction time

Results

Proximate Composition

The composition of fresh yellowfin tuna viscera, and its hydrolysates are given in Table 3. Fresh yellowfin tuna viscera had a protein content of 21.5%, and lipid content of 5.08%. The protein content of the hydrolysate was 74.56% ,based on solid material, which is in agree with other researchers reports (Shahidi et al. 1995; Onodenalore and Shahidi 1996; Kristinsson and Rasco 2000b; Nilsang et al. 2005; Wasswa et al. 2007; Souissi et al. 2007; Bhaskar et al. 2008; Ovissipour et al. 2009).

Lipid content in yellowfin tuna hydrolysate reached to 1.86% in current study. Ovissipour et al. (2009) found that, by using Alcalase, the lipid content of Persian sturgeon viscera hydrolysates after 205 min, at 100 AU/kg protein was 0.18%. The lipid content in FPH was greatly reduced when compared to the raw material, because lipids were most likely excluded with insoluble protein fraction (Kristinsson and Rasco 2000b; Nilsang et al. 2005), by centrifugal separation. Decreasing in lipids content in the protein hydrolysate might significantly contribute to lipid oxidation stability. This may enhance the product stability (Shahidi et al. 1995; Diniz and Martin 1997; Kristinsson and Rasco 2000b; Nilsang et al. 2005).

Optimization of hydrolysis parameters for DH

The influence of the enzyme activity (X_1), temperature (X_2), and time (X_3) on the hydrolysis of Yellowfin tuna viscera by Neutrase 0.8 L was determined using a factorial design. The best explanatory model equation for the DH value obtained from is described in equation 2:

$$y = 33.2 + 5.41x_1 + 2.75x_2 + 2.06x_3 - 3.22x_1^2 + 2.68x_1x_2 - 2.49x_2^2 - 3.18x_3^2 \quad (2)$$

According to the ANOVA (Table 4), in addition to linear and quadratic terms was significant ($P < 0.01$), but cross-product term ($X_1.X_2$) was not significant ($P > 0.05$). Statistical analysis indicated that, within each term all three hydrolysis factors of enzyme activity, reaction temperature and reaction time had significant influence on DH ($P < 0.01$). The same results were observed by other researchers (Diniz and Martin 1996; Bhaskar et al. 2008; Bhaskar and Mahendarkar 2008; Nilsang et al. 2005).

Table 3. Proximate composition (%) of the yellowfin tuna viscera and its protein hydrolysate (FPH)[a]

	Protein	Fat	Moisture	Ash
Fresh viscera	21.5 ± 0.5	5.08 ± 1.53	69.66 ± 2.32	4.46 ± 1.21
FPH	74.56 ± 1.23	1.86 ± 0.27	3.24 ± 0.89	19.38 ± 0.94

[a] Mean ± SD (n=3)

The results model showed that all linear, quadratic and one cross-product terms contributed to the response which is in agree with Diniz and Martin (1996). The adjusted coefficient of determination (r^2) implies that 91% of the behavior variation could be explained by the fitted model. Moreover, lack of fit test, which indicates the fitness of the model was not significant, indicating that the model is sufficiently accurate for predicting the degree of hydrolysis for any combination of experimental independent variables used in this study which is similar to other researcher's results (Diniz and Martin 1996; Bhaskar et al. 2008; Bhaskar and Mahendrakar 2008; Ovissipour et al. 2009).

Table 4. ANOVA table of DH affected by enzyme activity, temperature and time during optimization experiment

Source	df[1]	Sum of square	Mean square	F-value
Regression				
Linear	3	561.57	—	18.03**
Quadratic	3	240.743	—	7.73**
Cross-product	3	78.60	—	2.52
Total	9	880.92	—	9.43**
Residual				
Lack of fit	5	72.45	14.49	4.10
Pure error	3	10.61	3.53	—
Total error	8	83.074	10.38	—
$r^2 = 0.96$				
Factors				
Enzyme activity (AU/kg protein)	4	590.34	147.58	14.21**
Temperature (°C)	4	259.05	64.76	6.24*
Time (minute)	4	207.156	51.78	4.99*

[1] Degree of freedom.
** Significant at 1% level.
* Significant at 5% level.

The regression coefficient of DH in this study ($r^2 = 0.91$) was satisfactory, with a low predicted experimental error (Table 4). High correlations of experimental results with those predicted by RSM models for proteolytic reactions have been reported by several researchers (Diniz and Martin 1996; Nilsang et al. 2005; Bhaskar et al. 2008; Bhaskar and Mahendarkar 2008; Ovissipour et al. 2009; 2010a)

In order to determine the optimum conditions, contour plots and response surface graphs were generated by the predictive model to locate the critical point and the influence of each factor on the surrounding region (Fig. 1). All figures showed that in the hydrolysis of yellowfin tuna protein initially has a direct relationship with enzyme activity, reaction temperature, and reaction time. Beyond these criteria DH shoes a slight decrease. Figure 1A

shows a quadratic effect of reaction time and temperature on DH. The contour plot indicated that DH increase up to 39.61% with an increase in reaction temperature (maximum 53 °C), and hydrolysis time up to 141 min. The influence of time and enzyme activity on DH is, shown in Figure 1B. Quadratic effect of time and enzyme activity is clearly presented. DH increased at longer time, but decreased after 90 min of reaction. Also, the DH at the point of 30% could be reached at 39.61 AU/kg protein using Neutrase. Figure 1C shows a quadratic effect of reaction temperature and enzyme activity on DH value. However, temperatures (more than 141 °C), results in higher DH values more than 30%.

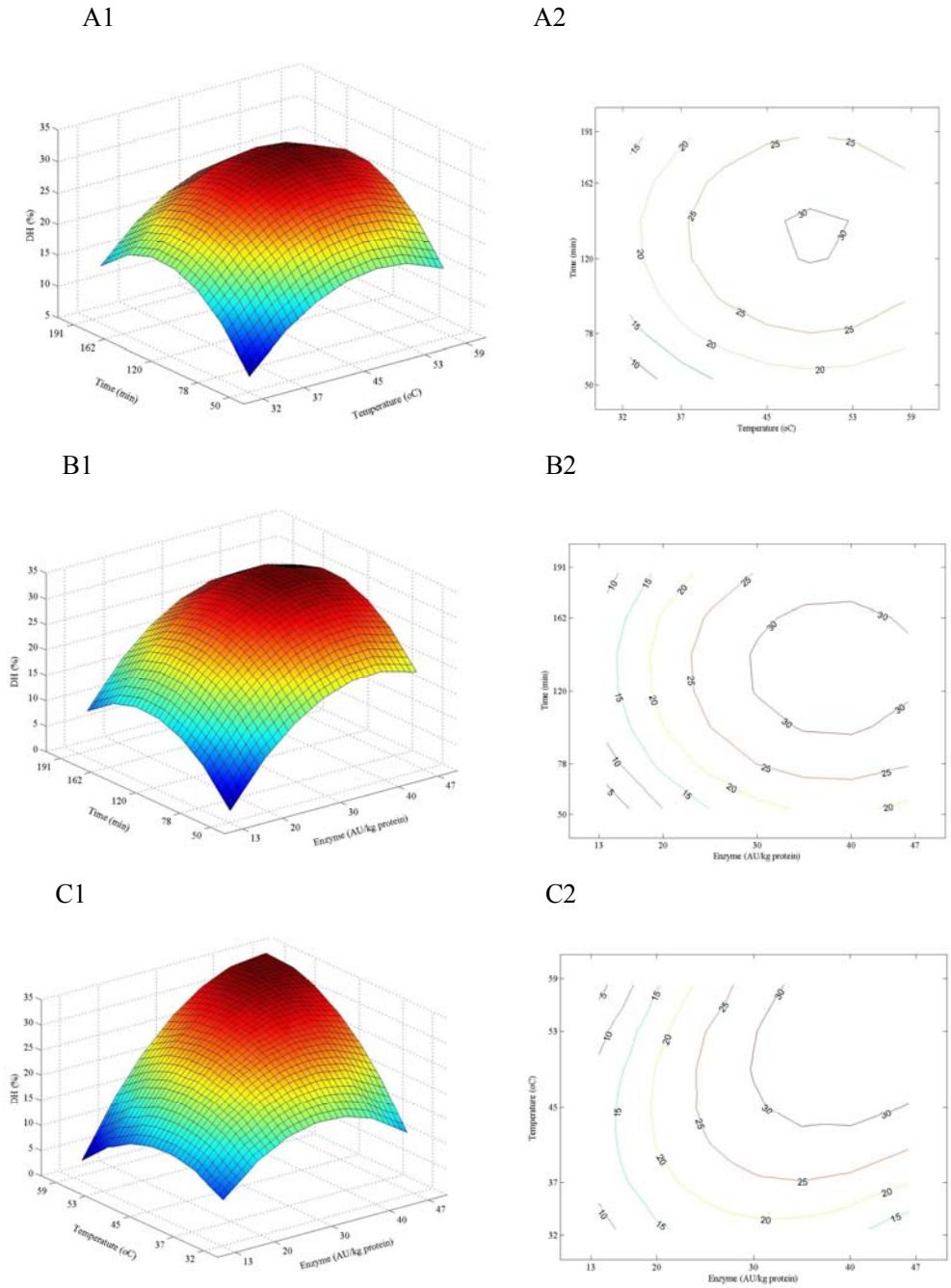

Fig. 1. Response surface graphs and contour plots for the effect of variables on DH as a response of different hydrolyzing conditions: (A) reaction time and temperature, (B) reaction time and enzyme activity (C) reaction temperature and enzyme activity.

The optimum hydrolysis conditions were 53 °C for hydrolyzing temperature, 141 min for hydrolyzing time and 39.61 AU/kg protein for enzyme activity at stationary point. Similar dependence, between enzyme activity, reaction temperature and reaction time has been observed for hydrolytic reactions of food proteins using microbial origin enzymes (Diniz and Martin 1996; Benjakul and Morrisey 1997; Nilsang et al. 2005; Bhaskar et al. 2008; Bhaskar and Mahendrakar 2008).

Ovissiour et al. (2009b) found that the optimum condition for hydrolyzing from visceral of beluga sturgeon (*huso huso*) were 120 min, 50 ^{o}C and Alcalase enzyme activity of 34 AU/kg crude protein. Benjakul and Morrisey (1997) evaluated different combination of reactions for hydrolyzing protease waste materials recovered from processing Pacific whiting (*Merluccius productus*), but to lower levels of hydrolysis.

It is well known, that the peptide chain length and DH, depends upon the extent of hydrolysis, conditions of hydrolysis, enzyme activity and type of the substrate proteins (Kristinsson and Rasco 2000a). Hence, the optimum conditions for hydrolyzing different substrate will be different, and will vary depending upon the substrate used, particularly with the content and reactivity of any endogenous proteases present.

Amino acid composition

The amino acid composition of yellowfin tuna visceral protein hydrolysates, and chemical scores are presented in Table 5.

Table 5. The amino acid composition of yellowfin tuna visceral protein hydrolysate (g/100g) and chemical score in comparison with FAO/WHO reference protein

Amino acid	Quantity (g 100/g)			Chemical score	
	Protein hydrolysate	Reference Protein 1[a]	Reference Protein 2[b]	RP1	RP2
Histidine	7.32	1.6	2.1	4.57	3.48
Isoleucine	6.11	1.3	2.5	4.7	2.4
Leucine	6.54	1.9	3.3	3.44	1.98
Lysine	1.73	1.6	5.7	1.08	0.3
Methionine[c]	1.21	1.7	3.1	0.71	0.39
Phenyl alanine	3.42	_	6.5	_	0.52
Tyrosine	1.62	_	_	_	_
Threonine	4.87	0.9	3.9	5.41	1.24
Tryptophan	_	_	_	_	_
Arginine	7.68	_	1.31	_	5.86
Valine	8.43	1.3	3.6	6.48	2.34
Aspartic acid	10.56	_	_	_	_
Glysine	5.14	_	_	_	_
Alanine	1.85	_	_	_	_
Serine	6.32	_	_	_	_
Glutamic acid	14.68	_	_	_	_

RP1: Chemical score calculated with FAO/WHO reference protein as the base. RP2: Chemical score calculated with amino acid requirements as per NRC (1993).

[a] Suggested profile of essential amino acid requirements for adults (FAO/WHO, 1990).

[b] Essential amino acid requirements of common carp according to NRC (1993).

[c] Methionine + cysteine.

Chemical score provides an estimate of the nutritive value of a protein. This parameter compares levels of essential amino acids between the test, and the standard proteins. In the current study, computed chemical scores are based on the reference protein of FAO/WHO (1990) for adults, and amino acid requirements of juvenile common carp, as listed by NRC (1993).

The amino acid composition in this study and comparison with reference proteins indicate that the amino acid composition of the yellowfin tuna visceral protein hydrolysates were generally higher in essential amino acids, compared with the suggested amino acid recommended by FAO/WHO for adult humans except in terms of

methionine. Similar results are reported by Ovissipour et al. (2009a) for Persian sturgeon viscera hydrolysates. For common carp (*Cyprinus carpio*) the chemical score of the yellowfin tuna visceral protein hydrolysates shows that lysine, methionine and phenylalanine are the most limiting amino acids, in compare to the requirements of juvenile common carp (NRC 1993) (Table 5). Using hydrolysates that have an intermediate chain length and limited amounts of free amino acids would be a valuable ingredient in formulated and nutritionally balanced fish diets (Pigott and Tucker 2002). These results agrees with our previously study on Persian sturgeon hydrolysates chemical score (Ovissipour et al. 2009a).

Conclusion

Yellowfin tuna (*T. albacares*) is one of the most important pelagic fish species with annual catch of 41,000 tons in Iran (IFO 2006). Hydrolysis of yellowfin tuna visceral waste protein using Neutrase resulted in more than 35% DH. The DH is significantly influenced by enzyme activity, reaction temperature and time. Response surface methodology used for optimizing the condition of hydrolysis, resulted in enzyme activity of 37 AU/kg protein, temperature of 50 °C, and time of 60 min. The yellowfin tuna viscera hydrolysate has relatively high protein and low lipid content. Based on tuna hydrolysates amino acid compositions, the hydrolysate prepared from visceral waste has high potential for applications in aquaculture, and animal feeds. It is also an effective nitrogen source (as peptone) for microbial growth media.

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