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Enzymatic hydrolysis of sea rough fish

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Abstract. In the present investigation, sea rough fish were hydrolyzed with alcalase/peptidase combined enzymes (E_A/E_P). Raw material proximate composition was investigated. Rough fish contains potentially valuable fraction with high protein content: protein 15.8 %, lipid 4.5 %, ash 6.1 %, and water 73.6 %. Single-factor experiments were investigated for screening of variables. Response surface methodology (RSM) using Box-Behnken design (BBD) was used for optimization of the E_A/E_P, time, temperature, and enzyme to substrate ratio (E/S) to reach the highest hydrolysis degree (DH). The optimum conditions extracted by the Design Expert Software were E_A/E_P of 2.9, temperature 55.8 ^oC, incubation time of 4.9 h, and substrate concentration at 0.45 %. Under these conditions, a DH value of 56.2 % was obtained. The maximum DH obtained experimentally was 56.6 %. The high hydrolysis efficiency was due to the excellent combination of an endopeptidase and an exopeptidase. Amino acid composition of the product was also evaluated. The results indicated that sea rough fish was successfully converted into hydrolysates that mainly composed of small size peptides and free amino acids. The essential amino acid could be served as a valuable source of nutrition for humans and animals.

Keywords: alcalase/peptidase combined enzymes, sea rough fish.

Classification numbers: 2.5.2, 2.7.1, 2.9.4

1. INTRODUCTION

Fish is considered to be a high protein food sources. Fish protein hydrolysate (FPH) is a mixture of broken protein consisting of smaller peptides and amino acids gained by hydrolyzing of fish protein. FPHs are value food ingredients because of its high nutritional and bioactive properties with complete and good amino acid balance. Amino acid is reported to be a regulator of metabolic pathways and a precursor for synthesis important biological materials [1, 2].

Many investigations have been carried out to evaluate the functional properties of hydrolyzed fish proteins. It was reported that fish protein hydrolysate possess unique bioactive properties such as anti-hypertensive, antimicrobial, antioxidant, and antithrombotic, etc. [3, 4]. Two key factors determining their functionality and application are amino acid composition and DH.

There are three main sources of enzymes aimed at protein hydrolysis including animal sources, plant sources, and microbial sources. Proteolytic enzymes usually cleavage target specific bonds, creating peptides and amino acids of difference in size. Plant enzymes are more specific in their performance compared to microbial sources [4].

Rough fish or by-catch is commonly recognized as small fish species of low economic value. Offshore fishing, each catch has up to 70 % rough fish. In order to increase its value, the fishes should be converted into fish protein hydrolysate, a new product with high functionality. FPH was produced by hydrolysis of fish proteins into shorter peptide chains (from 2-20 amino acid residues) by chemical or enzyme. Quality and composition of the hydrolysate are affected by kind of enzymes, chemical reagent used, pH, temperature, and E/S, etc. [4]. FPHs are hygroscopic, amorphous, containing about 81 - 93 % protein, 3 - 8 % ash, 1 - 8 % moisture, and 3 - 5 % fat [5].

Approach for parameter optimization of fish protein hydrolysis using RSM has been studied. Koray Korkamas et al. determined optimum production conditions of protein hydrolysates from fish wastes by protease, Protamex and Flavourzyme using response surface methodology (RSM) [6]. Shehu Muhammad Auwal *et al.* also reported on hydrolysing stone fish protein by bromelain using response surface optimization. The optimum conditions were targeted for the hydrolysates with maximum antioxidant activities [7]. However, using combined enzymes produced from *Bacillus Licheniformis* (Alcalase E_A) and *Aspergillus oryzae* (Exopeptidase E_p) to hydrolysis sea rough fish using RSM have not received much attention.

The purpose of our study was to optimize and investigate enzymatic hydrolysis conditions of sea rough fish by commercial alcalase/peptidase combined enzymes produced from *Bacillus Licheniformis* (Alcalase E_A) and *Aspergillus oryzae* (Exopeptidase E_p). The combined enzymes exhibit both endoprotease and exoprotease activities. The characteristic of the hydrolysates were investigated. Such studies have rarely been reported before in Viet Nam.

2. MATERIALS AND METHODS

2.1. Materials

Protease enzyme for hydrolysis was purchased from Novoenzymes Company. Rough fish were from the market in LaGi District, Binh Thuan Province, Viet Nam. All reagent used were of analytical grade**.**

2.2. Methods

Single-factor experiment: The specimens were washed, crushed by grinder to prepare substrate for enzymatic hydrolysis. For each hydrolysis reaction, 100 g sample in 100 ml distilled was used in an Erlenmeyer flask. The hydrolysis reactions were performed at E_A/E_P ranged from 1/0 to 4/1, temperature was set in range 45 ÷ 65 ^oC by using a thermostatic shaker, time from 2 ÷ 10 h, without any control of the pH. Hydrolysis was initiated by adding the mixture of E_A/E_P. To stop the reaction, the mixture was heated to 90 ^oC for 15 min. After that, a centrifugation at 3,000 rpm for 30 min was performed, five layers including oil/fat, light lipoprotein, soluble protein, fine particles, and coarse particles were formed. Soluble protein layers were carefully collected, freeze-dried using Alpha 1,4 LD freeze dryer for further characterization.

DH was calculated from the ratio of the number of α-amino nitrogen H and the total number of peptide bonds per mass unit (HLNT) as following [8, 9]:

$$DH = \frac{H}{HLN_T} \times 100.$$

Experimental design: The reaction parameters were optimized by RSM. Table 1 showed the range and central point values of the four independent variables. Average values were calculated from triplicate experiments and the response (Y) was DH.

Table 1. Independent variables and their levels.

Independent variables	Level		
	-1	0	+1
X_1: E_A/E_P	1	3	4
X_2: Temperature	45	55	65
X_3: E/S	0.2	0.4	0.6
X_4: Time (hours)	4	5	6

The following quadratic equation illustrated the behavior of the system:

$$Y = b0 + \sum_{i=1}^{4} biXi + \sum_{i=1}^{4} biiXi^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} bijXiYj \qquad (1)$$

where Y is the dependent variable; b_0, b_i, b_{ii} and b_{ij} are coefficients, X_i, X_j are coded to be levels of the independent variables. In order to optimize the level of each variable for maximum DH value, the point optimization method is used. The combination of different optimization variables which yield the maximum response is resolved.

2.3. Measurements

Moisture content of sea rough fish and the product from hydrolysis was calculated after drying at 105 °C (TCVN 3700-90). Ash content was determined by burning the samples at 600 °C, Kjeldahl method (AOAC/ 3705-90) was applied for total of nitrogen content (HLN_T) and TCVN 3703-2009 for lipid content.

α-amino nitrogen content (%) was determined by titration method [9 - 11]. The amino acid composition was estimated by high-performance liquid chromatography. The experimental design was implemented by Design Expert Software (version 11.0).

3. RESULTS AND DISCUSSION

3.1. Single-factor experiments

Proximate composition of rough fish: Rough fish contains a great quantity of: water 73.6 %, protein 15.8 %, lipid 4.5 %, ash 6.1 % (the results based on triplicates). It indicates that rough fish contain potentially valuable fraction with high protein content so that appropriate enzymes need to be chosen for high protein recovery yields.

Effect of E_A/E_P on the DH and HLN_T: The changes in DH obtained for different E_A/E_P are shown in the Fig. 1. The DH is highest at the ratio E_A/E_P of 3/1. It is in agreement with the results of above proximate composition of rough fish.

Because rough fish contains high lipid content, so that at the ratio E_A/E_P: 3/1, the HLN_T is the highest (Fig. 2). This means that both enzymes participated in the hydrolysis process leading to the best hydrolysis efficiency.

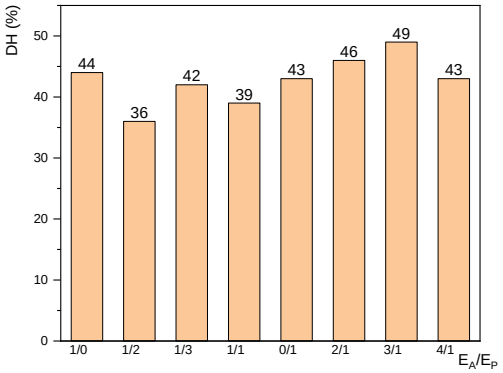

Figure 1. Effect of E_A/E_P on the DH at temperature of 55°, time of 5 hours.

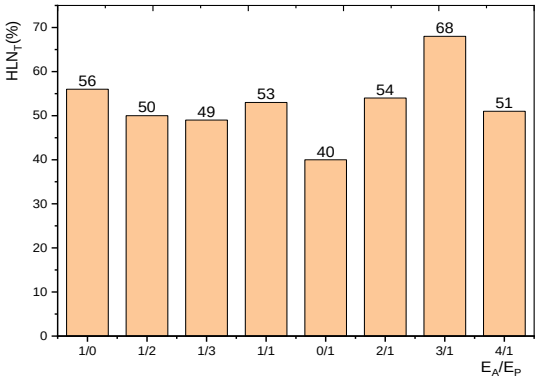

Figure 2. Effect of E_A/E_P on the HLN_T at temperature of 55°, time of 5 hours.

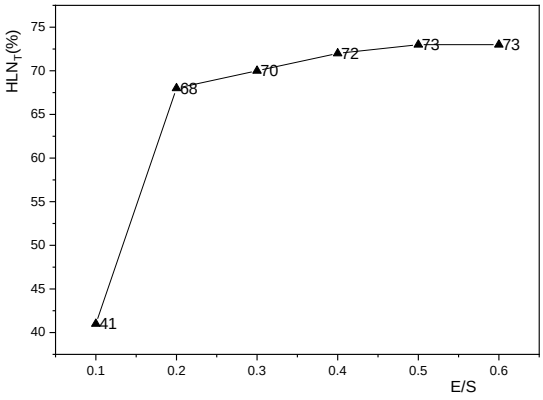

Figure 3. Effect of E/S on the HLN_T at temperature of 55°, time of 5 hours.

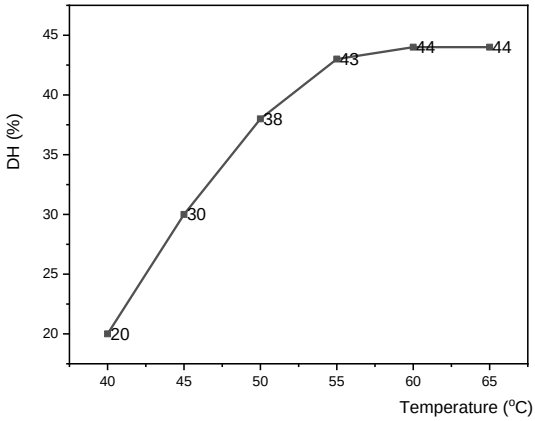

Figure 4. Effect of temperature on the DH at E/S 0.5, E_A/E_P 0.3, time of 5 hours.

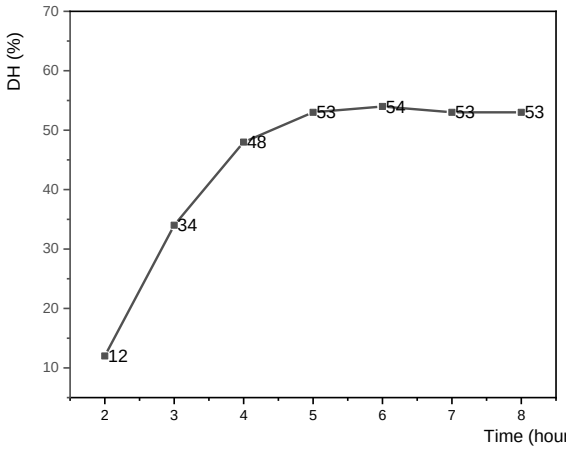

Figure 5. Effect of time on DH (%) at temperature of 55°, E/S of 0.5, E_A/E_P 0.3.

Effect of E/S on the DH: Effect of different ratio E/S (w/w) are shown in the Fig. 3. E/S was set at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 while E_A/E_P: 3/1 and time was set 4 hours. The increase of E/S leading to an increase in DH. The results are due to better hydrolysis of rough fish with increasing enzyme concentration. As results in Fig. 3, the E/S was chosen to be 0.5.

Effect of temperatures on the DH: Effect of different temperatures on the DH is shown in the Fig. 4. The temperature was carried out at 40 ^oC, 45 ^oC, 50 ^oC, 55 ^oC, 60 ^oC, 65 ^oC while the E/S of 0.5, E_A/E_P of 3/1, time of 5 h. The results showed an increase in enzymatic reaction rate as increasing temperature but the DH plateaued at 55^oC. When elevating the temperature higher than 65^oC the enzymes might lose their activities.

Effect of different reaction time on the DH: The reaction time was varied from 2 h to 8.0 h as indicated in the Fig. 5. The results indicated that the DH increased within the first 5 h then plateaued until 8 h. Longer time did not cause significant increase in the DH. So 5 h was selected.

Table 2. BBD with the independent variables.

Run No	X_1	X2	X_3	X_4	DH(%)
1	-1	-1	0	0	35
2	+1	-1	0	0	38
3	-1	+1	0	0	46
4	+1	+1	0	0	49
5	0	0	-1	-1	40
6	0	0	+1	-1	48
7	0	0	-1	+1	48
8	0	0	+1	+1	49
9	-1	0	0	-1	45
10	+1	0	0	-1	46
11	-1	0	0	+1	49
12	+1	0	0	+1	50
13	0	-1	-1	0	33
14	0	+1	-1	0	44
15	0	-1	+1	0	38
16	0	+1	+1	0	49
17	-1	0	-1	0	43
18	+1	0	-1	0	45
19	-1	0	+1	0	48
20	+1	0	+1	0	50
21	0	-1	0	-1	35
22	0	+1	0	-1	39
23	0	-1	0	+1	36
24	0	+1	0	+1	52
25	0	0	0	0	56
26	0	0	0	0	55
27	0	0	0	0	56

3.2. Experimental design

Box-Behnken design (BBD) with 27 experiments, including three replicates at the center point, were conducted for four factors (EA/EP, Temperature, E/S and time) at three levels. The results were presented in the Table 2.

The quadratic model explains the experimental data as followings:

$$Y = 55.66 + 1.10X_1 + 5.34X_2 + 2.42X_3 + 2.58\ X_4 + 3.0\ X2X4 - 1.75\ X_3X_4 - 3.79\ X_1^2 - 10.0\ X_2^2 - 4.91\ X_3^2 - 4.66\ X_2^4 \quad (2)$$

Table 3. ANOVA for quadratic model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1109.94	14	79.28	84.57	< 0.0001	significant
X_1-Time	12.00	1	12.00	12.80	0.0038	
X_2-Temperature	341.33	1	341.33	364.09	< 0.0001	
X_3-pH	70.08	1	70.08	74.76	< 0.0001	
X_4-E/S	80.08	1	80.08	85.42	< 0.0001	
X_1X_2	0.0000	1	0.0000	0.0000	1.0000	
X_1X_3	0.0000	1	0.0000	0.0000	1.0000	
X_1X_4	0.0000	1	0.0000	0.0000	1.0000	
X_2X_3	0.0000	1	0.0000	0.0000	1.0000	
X_2X_4	36.00	1	36.00	38.40	< 0.0001	
X_3X_4	12.25	1	12.25	13.07	0.0035	
X_1^2	76.68	1	76.68	81.79	< 0.0001	
X_2^2	537.79	1	537.79	573.64	< 0.0001	
X_3^2	128.93	1	128.93	137.52	< 0.0001	
X_4^2	116.15	1	116.15	123.89	< 0.0001	
Residual	11.25	12	0.9375			
Lack of Fit	10.58	10	1.06	3.18	0.2632	not significant
Pure Error	0.6667	2	0.3333			
Cor Total	1121.19	26				
Adjusted R^2				**0.98**		
Predicted R^2				**0.95**		

The coefficient R^2 was 0.98 indicated that the models are well adapted to the responses. The Predicted R^2 was 0.95 which was in accordance with the Adjusted R^2 of 0.98.

The Model F-value of 84.57 suggested that the model is significant. P-values < 0.0500 demonstrated that model terms were significant. In this model, E_A/E_P, temperature, E/S, time, temperature*time, E/S*time, E_A/E_P * E_A/E_P, temperature* temperature, E/S* E/S, time* time are significant model terms. The Lack of Fit F-value of 3.18 is not significant relative to the pure error.

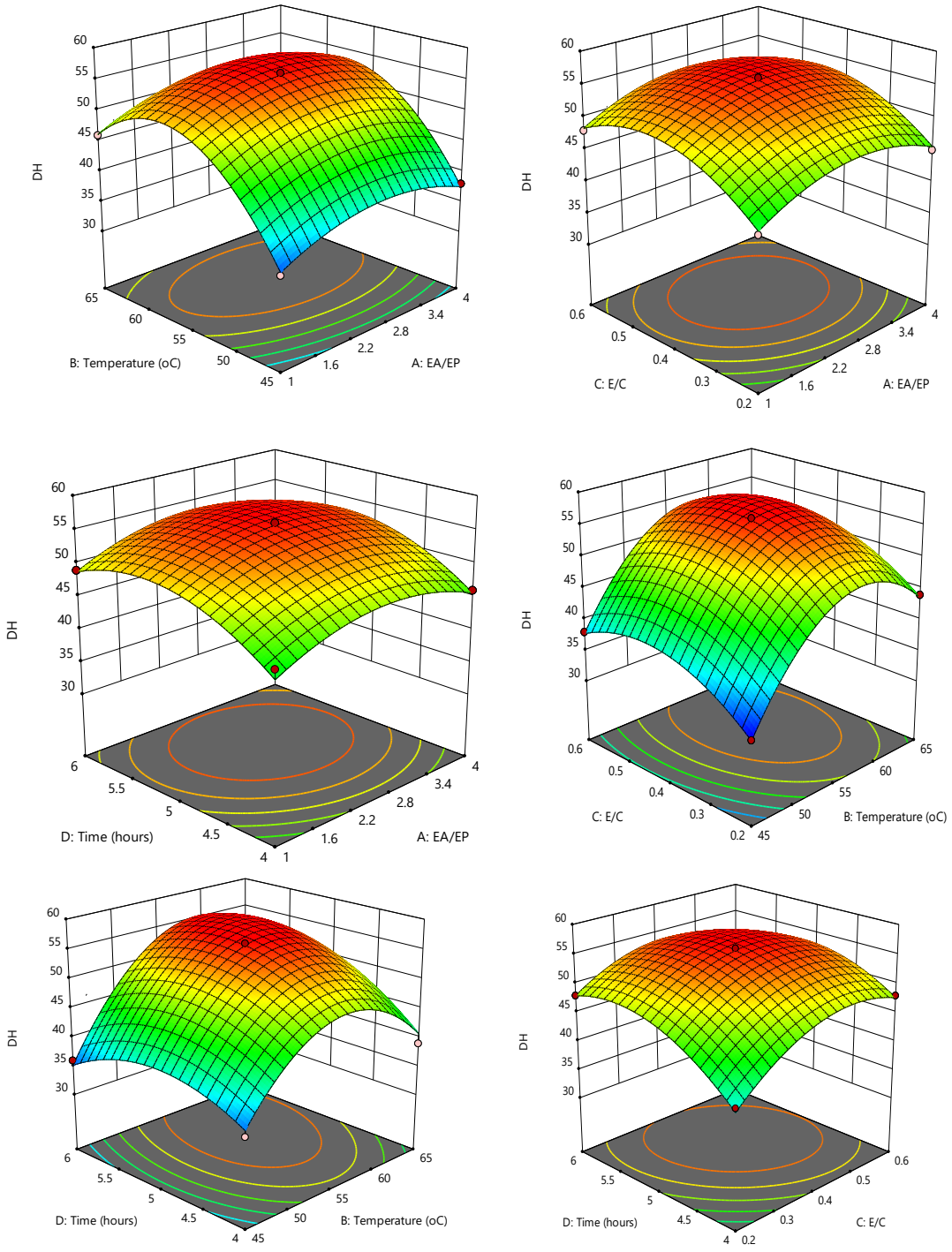

Figure 6. The effect of time, temperature, pH and E/S on the response Y.

The 3D response surfaces of the response using Eq. (2) are shown in Fig. 6. The optimal conditions obtained from the Software for the highest value of DH were E_A/E_p at 2.9, temperature at 55.8 ^oC, incubation time at 4.9 h, and substrate concentration at 0.45 %. Under

these conditions, value Y of 56.2 % was obtained. The maximum Y obtained experimentally was found to be 56.6 %. This is really in accordance with the model prediction.

The results of single factors experimental and Box-Behnken design (BBD) indicated that maximum DH value could be obtained by choosing optimal condition for hydrolysis of sea rough fish by EA/EP. The high hydrolysis efficiency was due to the excellent combination of an endopeptidase that break peptide bonds of nonterminal amino acids (Alcalase from *Bacillus licheniformis*) and an exopeptidase (*Aspergillus oryzae*) that catalyzes the cleavage of the terminal (or the penultimate) peptide bond.

3.3. Amino acid composition of the hydrolysis

The results indicated that the obtained hydrolysate mainly composed of small size peptides and free amino acids. Chromatogram, composition and content of amino acid of the hydrolysate were shown in the Fig. 7 and Table 4. The data suggested that rough fish hydrolysate contained essential amino acids to serve as a valuable source of nutrition for humans and animals. Our results was in agreement with references [5, 12].

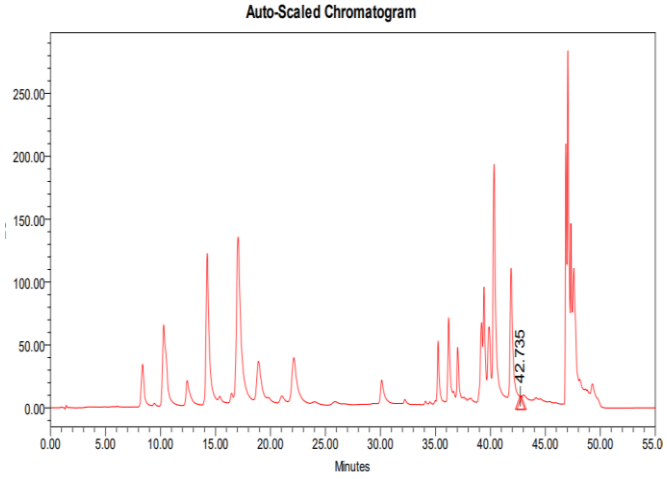

Figure 7. HPLC profile of amino acids of the hydrolysate.

Table 4. Amino acid profile of the hydrolysate.

TT	Amino acids	µg/mg	TT	Amino acids	µg/mg
1	Aspartic	9,16	10	Cystein	0,30
2	Serine	10,01	11	Tyrosine	3,25
3	Glutamic acid	13,77	12	Valine	4,28
4	Glycine	6,47	13	Methionine	0,81
5	Histidine	2,28	14	Lysine	7,79
6	Arginite	7,90	15	Isoleucine	4,02
7	Threonine	6,08	16	Leucine	7,54
8	Alanine	7,12	17	Phenylalanine	4,08
9	Proline	5,12	18	Tryptophan	0,02

4. CONCLUSIONS

In conclusion, alcalase/peptidase combined enzymes showed an efficient hydrolysis toward sea rough fish. The optimum parameters optimized using response surface methodology with Box-Behnken design models were E_A/E_P of 2.9, temperature 55.8^oC, incubation time of 4.9 h, and substrate concentration at 0.45 %. Under these conditions, DH value of 56.2 % was obtained. The hydrolysate contained high protein content with essential amino acids and could be used as food for animal which could greatly improve the sea rough-fish value. Further studies need to be investigated on hydrolysis technology of marine rough fish for practical application.

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Author contributions: Nhi Tran Thi Y, Ha Le Thi Thanh: Formal analysis, Software, Methodology, Investigation, Conceptualization, Thien Do Truong: Writing-Review & Editing Investigation, Conceptualization, Supervision.

Conflict statements: The authors declare that they have no conflict of interest.

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