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OPTIMIZATION OF ENZYMATIC HYDROLYSIS PROCESS FROM SHRIMP BY-PRODUCT FOR SHRIMP SAUCE PRODUCTION

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Abstract. Shrimp by-product from shrimp processing industry was hydrolyzed by alcalase and flavourzyme and the process was optimized by response surface methodology. Shrimp by-product was ground and treated with fixed alcalase 0.2 % (4.8A U/kg protein) and flavourzyme of different loadings (0.1 - 0.4 %), pH (6.0 - 9.0), temperature (45 - 65 ^{o}C) and hydrolysis time (5 - 1 3 h). At optimal conditions of pH of 7.5, temperature of 59 ^{o}C, flavourzyme loadings of 0.4 % (100 LAPU/g protein), alcalase of 0.2 %, and hydrolysis time of 8.2 h, hydrolysis degree was 36.76 % when compared to control sample (hydrolysis by HCl 6N at 100 ^{o}C for 24 h). Shrimp hydrolysis solution was mixed with 25 % of NaCl before fermentation. After 10 days of fermentation, shrimp sauce had total nitrogen of 13.2 g/l, amino nitrogen of 9.625 g/l, NH_3 of 2.13 g/l. These properties and sensory quality were equivalent to control sample (2.5 months of fermentation by traditional process).

Keywords: shrimp by-product, protein hydrolysis, degree of hydrolysis, alcalase, flavourzyme.

Classification numbers: 1.5.1, 1.3.1.

1. INTRODUCTION

Shrimp industry plays an important part in Vietnam fishery export during the last 2 decades. Annually, shrimp sector contributes around 40–45 % of the total export value, equivalent to 3.5–4 billion USD per year with total processing capacity of 1 million tons per year. Frozen products make up about 90 % of shrimp quantity with many different types of products (Whole, head on shell on shrimp, peeled and deveined shrimp, peeled undeveined shrimp, peeled tail-on shrimp, etc.). About 35–45 % by weight of shrimp raw material is discarded as waste depending on the species and processing method applied [1]. Taking into account the considerable generation of shrimp by-products and intense market competition for the seasoning products, development of value-added products from the shrimp waste to maintain the economic viability of the industry as well as reducing environmental pollution has been an urgent need. Shrimp heads and shells generally contain good percentage of protein with balanced amino acid profile, implying the feasibility of the recovery of protein fraction from the shrimp by-products to produce a traditional seasoning that is similar to fish sauce.

Fish sauce is a clear amber liquid containing free amino acids and oligopeptides with specific aroma and flavour. Indian anchovy (*Stolephorus indicus*) is widely used as raw material for fish sauce manufacturing in Southeast Asia. Typically, fish is mixed with 20–30 % solar salt and then left in a concrete tank at ambient temperature for 8–12 months [2]. The long fermentation time is considered the major limitation in fish sauce processing.

Fish endogenous proteinases and microbial proteinases could play an important role in protein hydrolysis during fish sauce fermentation. During fermentation, fish proteins are hydrolyzed under the action of proteases, the endogenic ones (mostly from the digestive tract) and those produced by halophilic bacteria [3]. Different solutions have been proposed to shorten the very long time of processing in fish sauce production, most notably the liquefaction of fish by addition of proteolytic enzymes or use of selected bacteria as starter culture [4].

The recovery of protein fraction from the shrimp waste by enzymatic hydrolysis has been widely studied to be used in feed animal [5], showing certain advantages since accelerated hydrolysis allows for control of hydrolysis and thus minimizes undesirable reactions. Protein digesting enzymes breakdown protein into smaller peptides, making hydrolysates very rich source of amino acids for protein biosynthesis [6]. Enzymes from microbial sources operating at alkaline pH, such as Alcalase, Neutrase, Protamex, Flavourzyme, are efficient in the hydrolysis of shellfish proteins. Shrimp by-product hydrolysates produced under controlled conditions yield desirable functional properties, high nutritive value and reduced bitterness [**Error! Reference source not found.**]. However the results were varied widely and the most researchers did not specifically mention about the organoleptic properties of these products and the application in shrimp sauce has been not reported yet.

The objective of the present investigation was to optimize extraction procedure of protein hydrolysates from shrimp waste mainly using commercial proteases and study the influence of physical parameters viz. pH, temperature, substrate concentration and time on the protein hydrolysis reaction in order to apply for shrimp sauce production.

2. MATERIALS AND METHODS

2.1. Materials

Shrimp by-product was purchased from the market and kept in ice during transportation time to the laboratory.

Enzyme: Alcalase 2.4 L (Optimal conditions: 30 - 65 °C, pH 7 - 9) and Flavorzyme 500 LAPU/g (Optimal conditions: around 50 °C, pH 5 - 7.) were purchased from Novozyme.

2.2. Methods

- Total nitrogen was determined according to AOAC 940.2. Protein content was determined with nitrogen factor equal to 6.25.
- Amino acid nitrogen was identified by determining formaldehyde nitrogen according to AOAC 2.066 and subtracting by ammoniacal nitrogen according to AOAC 2.065.
- Moisture content was determined by oven – drying method using an overnight drying period at 105 ^{o}C until reaching a constant weight according to AOAC 972.20.
- Sensory evaluation: Samples taken for sensory examination shall be assessed by persons trained in such examination and in accordance with Annex A and the Guidelines for the Sensory Evaluation of Fish and Shellfish in Laboratories (CAC/GL 31 - 1999).

- *Degree of hydrolysis (DH%)*

Degree of hydrolysis (DH) is defined as the ratio between number of peptide bonds cleaved during hydrolysis reaction and total peptide bonds in raw material. DH of protein hydrolysates was determined by measuring the amount of α amino acid according to the Nyhydrin method. Ninhydrin 2 % and Pyridine 20 % were added to the protein hydrolysates. The solution was kept in dark chamber at 70 - 75 ^{o}C for 7 - 10 min and cooled to room temperature. The purple color formed by the reaction of α amino acid with ninhydrin and absorbance was measured at 570 nm. Free α amino acid was obtained using the standard curve of Tyrosine and the DH was calculated using equation

$$DH = \frac{Lt - Lo}{Lmax - Lo} \times 100\ \%$$

where: Lt: is the amount of α- amino acid of shrimp by-product protein hydrolysates hydrolyzed for t hours; Lo: is the amount of α- amino acid of raw material shrimp by-product; Lmax: is the amount of α- amino acid of shrimp by-product protein hydrolysates completely hydrolyzed by HCl 6 N at 100 ^{o}C for 24 hours.

- *Preparation of protein hydrolysis of shrimp by-product with combining alcalase and flavourzyme*

The highest DH (21.3 %) was obtained with 0.4 % alcalase concentration, at temperature of 60 ^{o}C, pH of 8 and 6 hours of hydrolysis time and DH of 22 % with 0.4 % flavourzyme concentration at 50 ^{o}C, pH of 7 and 13 hours of hydrolysis time (Unpublished data). Alcalase is an endo-protease of the serine type. It has abroad substrate specificity and can hydrolyze most peptide bonds within a protein molecule. Flavourzyme is a peptidase preparation that liberates amino acids by hydrolysis of the N-terminal peptide bond. In order to achieve higher degree of hydrolysis than using alcalase or flavourzyme alone, the combination of endopeptidase action of the alcalase with exopeptidase capability of flavourzyme could induce positive effect.

Shirmp by-products (heads and shells) met food safety were minced and hydrolyzed by fixed 0.2 % alcalase and flavourzyme. The hydrolysis process was done in water bath with agitation of 100 rpm. Protein hydrolysis process was optimized with four factors and experiments were layout in Table 1

Table 1. Experiment design by Box-Behnken matrix with four factors.

Factors	coded	Unit	Low level (-1)	Central (0)	High level (+1)
Time	A	h	5	9	13
Temperature	B	^{o}C	45	55	65
pH	C	pH	6	7.5	9
Flavourzyme concentration	D	%	0.1	0.25	0.4

In total, 27 experiments with three central points were carried out. The experiment was performed in three replicates. The statistical significance of the regression coefficients was evaluated using ANOVA. The fitted values predicted by the response regression equation were compared with the experimental values for validation of the model. Three-dimensional response surface plots were drawn using Minitab software version 16.0 to figure out the relationship between levels of the process variables and the outcome of response.

3. RESULT AND DISCUSSION

3.1. The proximate composition of raw material and degree hydrolysis

Table 2. Proximate composition and sensory quality of shrimp by-product.

Moisture (%)	72.1	Color	Grayish, 25 % dark in the head
Protein (N×6.25) (%)	15.91	Flavour	Fresh, no strange odor
pH	7.52	NH3 (mg N%)	0.02

As shown in Table 2, the shrimp by-product used in this study was composed of 15.91 % of protein, pH 7.52, and 0.02 mg N% of NH3. Raw material has fresh flavor, no strange odor and grayish color. Based on NH_3 content and sensory quality, shrimp by–product met food safety standard.

Table 3. Degree of hydrolysis of protein of shrimp by-product with 0.2 % fixed alcalase at different experiments.

Run order	**Independent variables**				**Actual DH (%)**
	Time (h)	**Temperature (^oC)**	**pH**	**Flavourzyme (%)**	
1	5	45	7.5	0.25	16.39
2	13	45	7.5	0.25	22.42
3	5	65	7.5	0.25	20.39
4	13	65	7.5	0.25	36.4
5	9	55	6	0.1	5.38
6	9	55	9	0.1	11.5
7	9	55	6	0.4	18.2
8	9	55	9	0.4	29.21
9	5	55	7.5	0.1	16.69
10	13	55	7.5	0.1	33.67
11	5	55	7.5	0.4	32.67
12	13	55	7.5	0.4	36.25
13	9	45	6	0.25	9.98
14	9	65	6	0.25	13.78
15	9	45	9	0.25	13.64
16	9	65	9	0.25	15.98
17	5	55	6	0.25	4.21
18	13	55	6	0.25	14.9
19	5	55	9	0.25	14.15
20	13	55	9	0.25	19.32
21	9	45	7.5	0.1	23.29
22	9	65	7.5	0.1	23.41
23	9	45	7.5	0.4	28.2
24	9	65	7.5	0.4	36.6
25	9	55	7.5	0.25	28.34
26	9	55	7.5	0.25	31.39
27	9	55	7.5	0.25	30.34

Degree of hydrolysis (DH), which indicated the percentage of cleaved peptide bonds [7], is one of the basic parameters that describe the properties of the hydrolysates, but also serves as indicator of protease activity and efficiency. Using Box-Bennhken matrix, 27 experiments (including 3 center points) were carried out. Shrimp by-product protein hydrolysis were produced under these conditions and DH is shown in Table 3.

A probability test of 0.05 was used to estimate the statistical significance of variation in the observed responses using ANOVA. Other statistical parameters including coefficient of determination R^2 (R-sqd), adjusted coefficient of determination R^2-adjusted (R^2-adj), F-test probability, and lack of fit values are also given in Table 4.

Table 4. Regression coefficients, R^2, and F-test probability for DH.

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	2235	8	279.38	34.47	< .0001	significant
A-Time	284.8	1	284.8	35.14	< .0001	
B-Temperature	8.78	1	88.78	10.95	0.0039	
C-pH	116.25	1	116.25	14.34	0.0013	
D- E/S ratio	376.21	1	376.21	46.42	< .0001	
AD	44.89	1	44.89	5.54	0.0302	
A^2	42.62	1	42.62	5.26	0.0341	
B^2	75.09	1	75.09	9.27	0.007	
C^2	1317.15	1	1317.15	162.53	< .0001	
R^2 (R-sqd)				0.9387		
R^2 (R-adj)				0.9115		
Residual	145.87	18	8.1			
Lack of Fit	141.07	16	8.82	3.67	0.2349	not significant

The large coefficient of determination (R^2) and nonsignificant lack of fit values ($p > 0.05$) of Y responses demonstrated the fitness of the experimental values to the theoretical values predicted by the model's regression equation. The adjusted coefficient of determination (R^2-adj) showed that the observed data variation of 91.15% for DH occurred due to the effects of the process conditions. The Fisher test (F-test) revealed high F-values and low p values of $p < 0.05$, which further validated the suitability of the models to the experimental data. From the model, the final equation in terms of coded factors was as follows:

$$Y = 31.07 + 4.87A + 2.72B + 3.11C + 5.6D - 3.35AD - 2.67A^2 - 3.54B^2 - 14.82C^2$$

The equation showed that the most influential factor for shrimp by-product hydrolysis to obtain maximum hydrolysis degree were initial pH, followed by temperature, hydrolysis time and enzyme concentration. This equation could be used to predict and control shrimp by-product hydrolysis using Alcalase and Flavourzyme.

3.2. Response surface plots and the effects of factors for DH response

Three-dimensional response surface graphs were presented to illustrate the interactive effects of the independent variables on DH and to determine the optimum level of each variable for maximum response. Figure 1 shows the response surface plot with interaction between temperature and time of hydrolysis, temperature and pH; time of hydrolysis and pH; E/S ratio and pH.

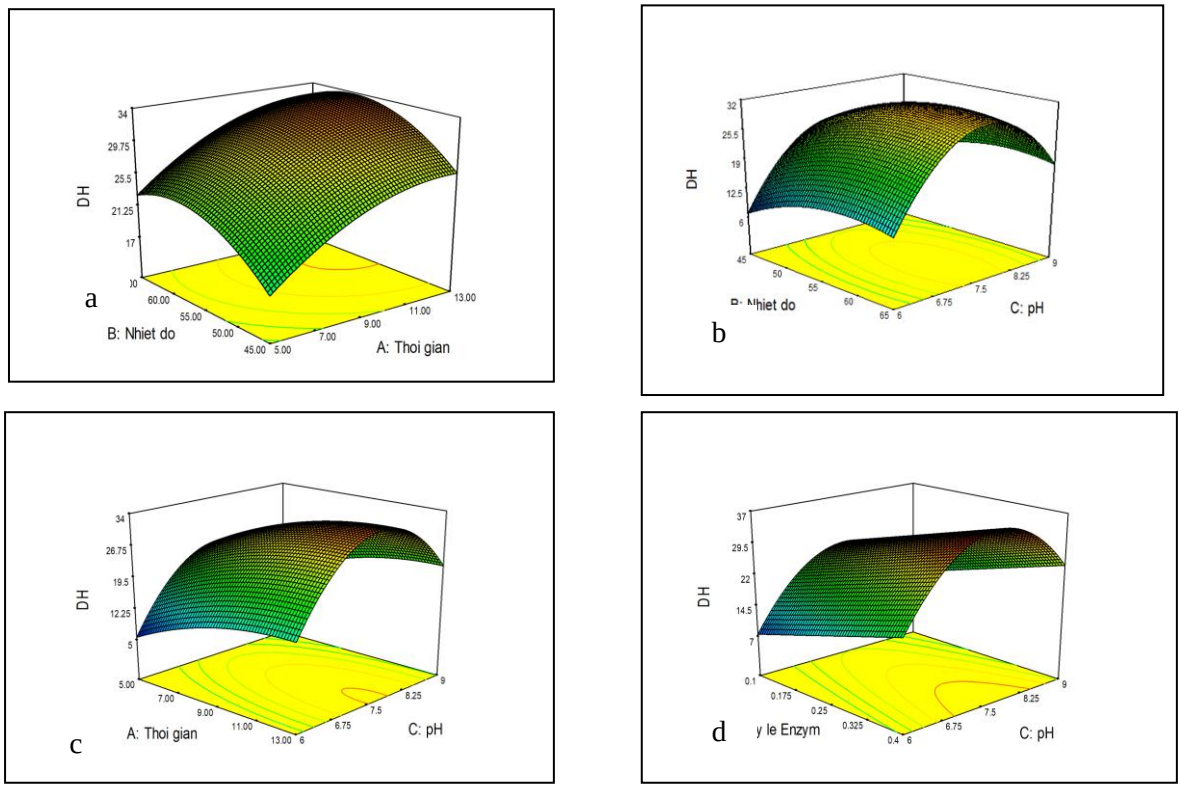


Figure 1. 3D plots for degree of hydrolysis: (a) temperature and time; (b): temperature and pH; (c): time and pH; (d) E/S ratio and pH.

The result demonstrated that the response surface curve had a plateau-shaped graph with a maximum point in the moderate range of independent variables, for both temperature and time of hydrolysis, temperature and pH; time of hydrolysis and pH.

The optimum pH range for Alcalase was from 6.5 to 8.5 and from 5.0 to 7.0 for flavourzyme. pH could affect both the substrate and enzyme by changing the distribution and confirmation of the molecules at very acidic or alkaline condition. However, at high pH value, the enzymes tend to undergo irreversible denaturation and loss of stability [9]. After optimum condition was reached, the DH dropped. According to Nelson [9], the protein structure of enzymes might denature and influence the proteolytic activity if the hydrolysis is kept at higher temperature. Longer hydrolysis time and higher temperature were necessary to produce peptides to increase DH (Fig 1a, 1b, 1c).

Based on Figure 1d, the optimum level was observed at the highest enzyme concentration and moderate pH. Flavourzyme concentration gave linear effect to DH. After reaching the optimum level, the DH will decrease gradually with increasing in pH.

3.3. Optimization of degree of hydrolysis

The degree of importance differs between independent variables and dependent response variable where a greater number implied the high importance of the variable. The degrees of importance for independent variables were set to 3, while that of the response variable was 5. Following the setting, RSM suggested several optimum conditions to hydrolysis shrimp by-product protein producing maximum DH. The suggested conditions were at temperature of 59 ^{o}C, pH of 7.5, 492 minutes of hydrolysis time and 0.4 % flavourzyme concentration and alcalase concentration at 0.2. The DH was 36.67 % with the desirability of 1.0.

In order to validate the suggested mathematical model, shrimp by-product protein hydrolysis was conducted under the optimum conditions. The degree of hydrolysis of shrimp by-product protein is 35.16 %. This result is similar to that of Satya S. D. & Krushna C. D. [10] when shrimp by-product protein was hydrolyzed at conditions: temperature of 59.37 ^{o}C, pH of 8.25, alcalase concentration of 1.84 and hydrolysis time of 84.42 min, corresponding to maximum DH of 33.13 %.

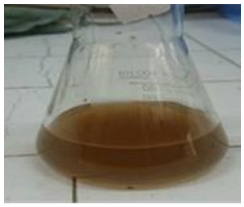
3.4. Application on shrimp sauce production

Sample 1: Shrimp by-product, after being hydrolyzed by alcalase and flavourzyme at previous optimum conditions, was mixed with 25 % NaCl, put into lidded pottery jars and stored at ambient temperature for 10 days.

Sample 2: Shrimp by-product was mixed with 25 % NaCl and put into lidded pottery jars and stored at ambient temperature for 75 days (traditional fish sauce processing method).

The results of quality comparison between two samples were presented in Table 5.

Table 5. The quality of hydrolysate solution after fermentation by traditional method and enzymatic method.

Target	Sample 1	Sample 2
Color	Red brown	Red brown
Flavor	Shrimp flavour, absent of rotten and rancid odour	Shrimp flavour, absent of rotten and rancid odour
Total Nitogen (g/l)	13.29	11.86
NH_3 (g/l)	2.13	3.269
amino Nitrogen (g/l)	9.625	8.5
Image		

Shrimp by-product protein hydrolyzed by alcalase and flavourzyme resulted in liquefied suspension with higher amino nitrogen content and lower NH_3 content than control sample (2.5

months of fermentation by traditional process). Shrimp solution had red brown color and shrimp flavor, not rotten and rancid odour. By this method, it is suggested that manufacture of shrimp sauce via protein hydrolysis could shorten the traditional fermentation by 60 days without significantly compromising quality and sensory characteristics of the product.

4. CONCLUSIONS

This study confirmed that the addition of commercial proteases may significantly contribute to the liquefaction of shrimp by-product in shrimp sauce production compared to classical autolysis. Indeed, after 492 minutes of hydrolysis using 0.2 % alcalase and 0.4 % flavourzyme at 59 ^{o}C, shrimp hydrolysates could be mixed with 25 % of NaCl, yielding a shrimp solution that had total nitrogen of 13.2 g/l, amino nitrogen of 9.625 g/l, and NH_3 of 2.13 g/l after 10 days. These properties and sensory quality were equivalent to control sample (2.5 months of fermentation by traditional process).The protein hydrolysis by commercial protease could result in an fermentation period that is 60 day shorter than that of traditional method in shrimp sauce production.

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