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Optimization of protein hydrolysis conditions from shrimp head meat (*Litopenaeus vannamei*) using commercial alcalase and flavourzyme enzymes

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ABSTRACT

The aim of this research was to study the hydrolysis capacity of proteins in white shrimp head meat by using commercial alcalase and flavourzyme enzyme. A response surface methodology with two concentration factors, alcalase enzyme (10÷30 UI/g) and flavourzyme enzyme (20÷30 UI/g), including 11 experiments was utilized to optimize the hydrolysis process. In addition, such experimental conditions of pH (6.9 - 7.2), temperature (53 - 58°C), and hydrolysis time (2 – 4 hours) were also investigated by using the response surface methodology with 17 experimental units. As a result, the hydrolysis solution was achieved with a high hydrolysis efficiency (90.19%) and good antioxidant activity (86.16%) by using the following optimal conditions of pH (7.01, temperature (54.94°C), hydrolysis time (2.96 hours), alcalase enzyme (19.42 U/g), and a flavourzyme (32.09 U/g).

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1 INTRODUCTION

Shrimp processing is a major industry in the fisheries sector. Accordingly, there are so many by-products of this industry such as shrimp heads and shrimp shells. Depending on species, by-products generated in the shrimp processing ranges from 48 to 56%, which bases on the total body weight. The major components in these by-products are protein (30-50%), chitin (15-25%), minerals (10-15%) and astaxanthin (Gunasekaran *el al.*, 2015). In addition, shrimp heads which account for 35-45% (Zhao *el al.*, 2011) are the important sources in shrimp processing industries. Until now, there are few reports about producing of protein enriched products which can be supplemented into other food processing. Shrimp head was mainly used to produce chitin, chitosan, fertilizers, feeds for poultry and livestocks, and shrimp powder. Several researchers have suggested that by-product can be used to produce functional protein hydrolysates (He *el al.*, 2006). For instance, protease enzyme was used to create peptide and amino acid from hydrolyzed processes, which can enhance valuable nutrients and bioactive components of the by-products. The use of hydrolysates of these materials has become common practice in the food industry (Murueta *el al.*, 2007) due to the bioactive peptides related to their amino acid composition, senquence and molecular weight. For example, antioxidant peptides are inactive within the sequences of parent proteins (Najafian and Babji, 2012) that must be released and activated by pro-

cessing through different techniques. Basically, antioxidant peptides can be generated from precursor proteins by microbial fermentation and enzymatic hydrolysis (Korhonen and Pihlanto, 2006). Enzymatic hydrolysis is the most commonly applied method of producing such antioxidant peptides. Hydrolysis leads to the production of tailor-made bioactive peptides with desired functional and biological properties, which can improve the technofunctional properties of proteins such as solubility, emulsification, gelation, and water-holding capacity (Wang *el al.*, 2013). It has also been proposed as a way to reduce allergenicity of food proteins, in particular betalactoglobulin and ovalbumin (Moure *el al*., 2005). However, the study of the antioxidant capacity of protein hydrolysates from shrimp heads has not been sufficiently investigated. Researchers are mainly focusing on the preparation and antioxidant activity of the crude shrimp hydrolysates (Zeng *el al.*, 2013). For the present, many proteases which derived from microorganisms such as Alcalase, Flavourzyme, Protamex, etc. have been produced in order to be make better use of protein of hydrolysates products from shrimp by-products, a peptide source with biological activities with significant potentials for use in pharmaceuticals (Gildberg and Stenberg, 2001; He *el al.*, 2006; Zhao *el al.*, 2011; Dey and Dora, 2014). However, one problem during the production of protein hydrolysates is that bitterness can develop, limiting the use of these products in human food and animal feed. The bitter taste can be ascribed to hydrophobic peptides and results from the degradation of the protein substrate (Adler-Nissen, 2008). Previous recently researches showed that applying Flavourzyme with its combination of exopeptidase and endopeptidase activity minimises the risk of bitterness (NOVONORDISK, 1999). The main objective of this study was to investigate the hydrolyzing of shrimp heads in combination with commercial enzymes (Flavourzyme and Alcalase) to produce protein hydrolysates with high degree of hydrolysis and antioxidative activity, which can gradually improve their commercial values.

2 MATERIALS AND METHODS

2.1 Materials

White shrimp head was collected directly from the frozen shrimp production line at Hoa Trung seafood Corporation (Luong The Tran Ward, Cai Nuoc District, Ca Mau Province). The outer carapace, shell, legs, beard, etc. were removed and washed in tap water and the meat separated. After collection, the shrimp head meat was immediately packed in polyamide (PA) plastic bags (2 kg per bag) and transported to the laboratory (Can Tho Univeristy (Ninh Kieu District, Can Tho city) within 5 hours. In the laboratory, they were divided into smaller samples (200 g per bag), packed in PA bag and stored at −20 °C until use.

Enzyme Alcalase® AF 2.4 L and Flavourzyme® 500 MG were provided by Novozyme (Denmark). All other chemicals were of analytical grade.

2.2 Protein hydrolysis

Frozen shrimp head meat was ground by grinder (level 2) for 3 minutes before hydrolysis. During the grinding process, temperature was maintained under 5°C. After grinding, shrimp head meat was mixed with water (ratio 1:1 w/v). Alcalase was firstly added to achieve a predigestion and incubated for 40 minutes before adding flavourzyme (Table 1). In the control process (Table 2), hydrolysis process was enhanced by using the magnetic stirrer (speed: 200 rpm). After that, the sample was centrifugated for 20 minutes at 6.000 rpm. The supernatant (hydrolysis solution) was collected. Finally, the degree of protein hydrolysis and antioxidant activity were determined. Protein hydrolysates from shrimp head meat were diluted 20 times before measuring DPPH (1,1-diphenyl-2-picrylhydrazyl) values (%).

2.3 Experimental design

2.3.1 Determination of proximate composition of shrimp head meat

Shrimp head meat was sampled randomly (200 g/sample, n = 3). Samples were ground to obtain uniformity and used to analyze the proximate compositions, consist of pH, moisture content and total protein. All analysis were determined with three replicates.

2.3.2 Experiment 1: The interaction effect of flavourzyme and alcalase on protein hydrolysis from shrimp head

Hydrolysis experiment was proceeded with 2 major factors, acalase and flavourzyme concentrations in 2 hours by orthogonal method which was designed to optimize the enzyme concentration using for hydrolysis process. Reaction parameters were optimized by using the response surface methodology (RSM). Moreover, Acalase enzyme (UI/g), flavourzyme (UI/g) were optimized using the central composite design of RSM. The range and central point values of 2 independent variables are shown in Table 1. The experiment was conducted with 2 factors X_1-Alcalase enzyme concentrations (5 levels of 5.86, 10, 20, 30, and 34.14UI/g) and X_2 flavourzyme enzyme concentrations (5 levels of: 15.86, 20, 30, 40, and 44.14 UI/g). The 2^2 + star central composite design (CCD) was used with the optimal number of treatments of 11 (Table 1), in which three experiments in the central project to check the coefficient

significance of regression equation. Hydrolysis was performed by 11 experimental units as shown in Table 1. Corresponding to each experiment, filtration, centrifugation, hydrolysate collection conditions and hydrolysis productivity were determined. Based on the average activity of the obtained protease, which corresponded to 11 experimental units, the Statgraphics Centrution 16.1 program was used to evaluate the data and calculate the regression equation, where the objective function Y was DH (degree of hydrolysis) (%) and DPPH (%).

Table 1: Design matrix of the optimization of enzyme factor for the experiment for DH (%)

Design points	Independent variable levels		Coded levels	
	X_1	X_2	Acalase concentration (UI/g)	Flavourzyme concentration (UI/g)
1	-1	+1	10.00	40.00
2	0	-1.41	20.00	15.86
3	-1	-1	10.00	20.00
4	+1	+1	30.00	40.00
5	+1	-1	30.00	20.00
6	0	+1.41	20.00	44.14
7	0	0	20.00	30.00
8	+1.41	0	34.14	30.00
9	-1.41	0	5.86	30.00
10	0	0	20.00	30.00
11	0	0	20.00	30.00

2.3.3 Experiment 2: Optimization reaction conditions of protein hydrolysis from shrimp head meat

To standardize hydrolysis procedure, reaction parameters were optimized using response surface methodology (RSM). The central composite design (CCD) was employed in this regard. The range and center point values of three independent variables presented in Table 2 were based on the results of preliminary experiments. Reaction temperature (X_3), pH (X_4), and hydrolysis time (X_5) were chosen for independent variables. Corresponding to each experimental condition, centrifugal hydrolysates and sustaintial protein preparations were used to determine hydrolysis efficiency and DPPH (%).

– X_3: temperature with 5 survey levels including the lowest level (-1.68) corresponds to 51.3°C, -1 corresponds to 53°C, center (0) is 55.5°C, and two high temperature levels are 58° C (+1) and 59.3°C (+1.68).

– X_4: pH at 5 levels: the lowest level (-1.68) is pH 6.80; -1 is pH 6.9; level 0 is pH 7.05 and two high pH values are 7.3(+1) and 7.3(+1.68).

– X_5: time (hours) with 5 survey levels: (-1.68) is 1.32 hours, (-1) is 2 hours, centre (0) is 3 hours, and two time levels are 4 hours (+1) and 4.68 hours (+1.68).

Table 2: Design matrix for the optimization of reaction conditions on degree of hydrolysis (%DH) and antioxidant activity (%DPPH) for shrimp head meat hydrolysis

Design point	Independent variable levels			Coded levels		
	X_3	X_4	X_5	Temperature (°C)	pH	Time (hour)
1	0	-1.68	0	55.50	6.80	3.00
2	0	0	0	55.50	7.05	3.00
3	+1.68	0	0	59.70	7.05	3.00
4	0	0	-1.68	55.50	7.05	1.32
5	0	+1.68	0	55.50	7.30	3.00
6	+1	+1	+1	58.00	7.20	4.00
7	-1.68	0	0	51.30	7.05	3.00
8	-1	+1	-1	53.00	7.20	2.00
9	+1	+1	-1	58.00	7.20	2.00
10	-1	-1	-1	53.00	6.90	2.00
11	+1	-1	+1	58.50	6.90	4.00
12	0	0	+1.68	55.50	7.05	4.68
13	-1	-1	+1	53.00	6.90	4.00
14	+1	-1	-1	58.00	6.90	2.00
15	-1	+1	+1	53.00	7.20	4.00
16	0	0	0	55.50	7.05	3.00
17	0	0	0	55.50	7.05	3.00

2.4 Analysis method and criterial measured

– Proximate analysis: All proximate analysis of raw samples was determined according to AOAC (2005). Moisture content was determined by drying samples overnight at 105°C until constant weight was achieved. Crude protein content was determined using the Kjeldahl method. pH value was measured by pH meter.

– Degree of Hydrolysis (DH) determination: The degree of hydrolysis (DH) is defined as the percentage of cleaved peptide bond: DH (%) = $(h/h_{tot})\times 100$. Where, h is the number of hydrolysed bonds and h_{tot} is the total number of peptide bonds per equivalent of the protein studied.

The DH was determined by the o-phthaldialdehyde (OPA) method (o-phthalaldehyde) (Nielsen *el al.*, 2001), based on amine groups. The principle is that acid amine or peptide reacted to Ortho-phthaldialdehyde with –SH group in dithiothreitol or –mercaptoethanol to create the compound which has ability to absorb at wavelength 340 nm.

– Determination of antioxidant activity: The antioxidant capacity of the hydrolysates was determined by DPPH (1,1- diphenyl-2-picrylhydrazyl). DPPH radical scavenging activity (DSA) is measured by the method of Wu *el al.* (2003) and Zhao *el al.* (2011). The analyses were conducted by using on the concentration of hydrolysis solution. The hydrolysis solution (0,375ml) was added to the test tubes containing 2 mL 1, 1- diphenyl-2-picrylhydrazyl (DPPH), 0,1 mmol L-1 DPPH prepared in methanol solution. The mixture was shaken and placed in a dark room for 30 minutes before being measured by spectroscopy method at the wavelength of 517 nm. Solution of DPPH prepared in methanol solvent was used as blank sample. Antioxidant activity (DPPH%) was calculated by the following formula:

$$DPPH\%(DSA\%) = 1 - \frac{(A_S - Ao)}{A_{DPPH}} * 100$$

Where: A_s; A_0 and A_{DPPH} abbreviates for the absorbance of real sample, blank sample and control sample, respectively; DSA: DPPH radical scavenging activity

2.5 Statistical analysis

All tests were done in triplicate, and data were averaged. Standard deviation was also calculated.

Analysis of the experimental design and calculation of predicted data were carried out using Statgraphics Centurion Software (version 16.1, USA) to estimate the response of the independent variables. Statistical testing of the regression model has been done by the Fisher's statistical test for ANOVA (analysis of variance) for quadratic model. F value, R^2 value, P value, Residual error, Pure error and Lack of fit were calculated for the model. Thus a model equation was proposed from the outcome of the study, for optimizing hydrolysis condition which will produce maximum degree of hydrolysis (%DH) and maximum antioxidant activity (%DPPH) for obtaining shrimp meat protein hydrolysate.

3 RESULTS AND DISCUSSION

3.1 The composition of shrimp head materials

Chemical composition of shrimp heads is shown in Table 3.

Table 3: The composition of shrimp heads

Components	Content
Moisture (%)	83.24±0.68
pH	7.78±0.02
Total protein (%)	12.80±0.25

**Average result of 5 sampling, 3 replications/ samples*

The result showed that the total protein in shrimp heads was quite high (average 12.80%). This proves that the shrimp head meat is a good source of protein bioactive peptides. However, the high total protein content and the slightly alkaline pH (7.78) are good conditions for growing proteolytic microorganisms, which gives a rise to severely altered proteins and biological inactivity reduction. Hydrolysis with external protease enzyme is the most effective way to recover protein hydrolysates.

3.2 The interaction effect of of alcalase and flavourzyme enzyme on shrimp head protein hydrolysis

In all commercial enzymes, alcalase is a well-known non-specific endoprotease used widely in the protein hydrolysis research (Synowiecki and Al-Khateeb, 2000; Gildberg and Stenberg, 2001). The protein recovery efficiency was higher than Flavourzyme, Neutrase, Protamex, and Flavourzyme in the case of black tiger shrimp (Shahidi *el al*., 1995). However, an exo-enzyme mixture and endopeptidases were commonly used in order to increase the hydrolysis efficiency (Vioque *el al*., 1999), a unique enzyme can not provide an extensive hydrolysis in a reasonable period of time (Lahl and Braun, 1994)

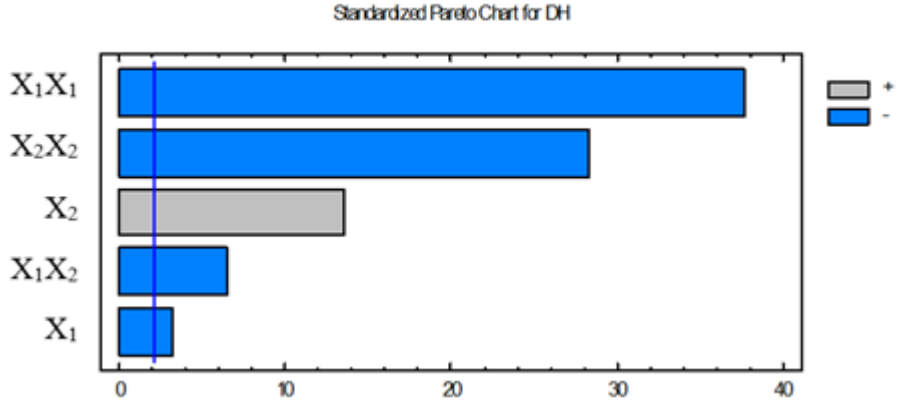

Fig. 1: Interaction of alcalase and flavourzyme on the degree of hydrolysis DH (%)

According to Fig. 1, the first-order regression coefficients of X_1, X_2 as well as the interaction coefficient of alcalase and flavourzyme ($X_1.X_2$) and the quadratic terms (X_1^2, X_2^2) had a significant effect ($P < 0.05$) at the maximum DH. This contributes to the influence of each independent variable as well as the significant interactions in the hydrolysis process. The stimulation hydrolysis productivity (Y_1 theoretical) was determined by replacing the variables with real values into the following equation:

Y_1 (DH%) = -52.6632 + 4.07231X_1 + 4.6687X_2 – 0.0895902X_1^2 – 0.0184128X_1X_2 – 0.0671562X_2^2 (1), R^2 = 0.9881 (Fig. 2).

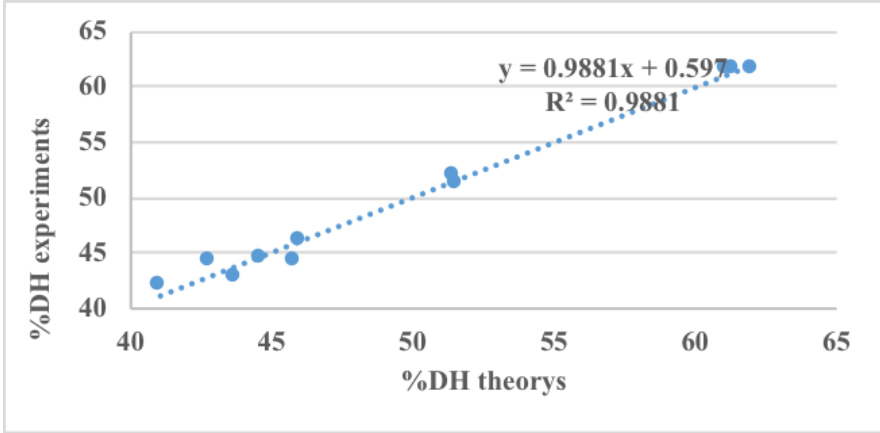

Fig. 2: Correlation graph between experimental hydrolysis productivity and calculated by the regression equation

The Fig.2 desmontrated that the regression equation describes correctly the experimental results. The correlation coefficient indicates that 98.81% of variation in hydrolysis productivity is due to the effect of independent variables X_1 and X_2, respectively, and only 1.19% of variation is caused by unspecified factors

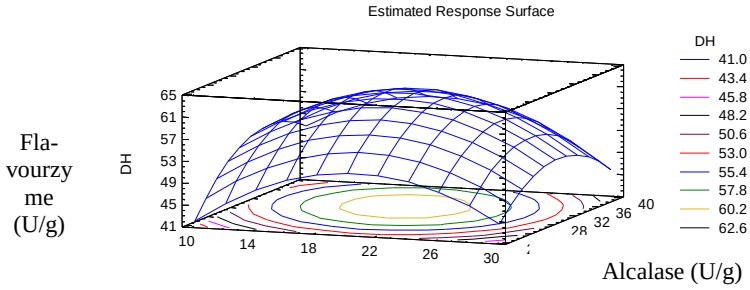

Fig. 3: Response surfaces graph depicting effects of Alcalase and Flavourzyme concentrations on DH% during hydrolysis of shrimp head meat

The optimal concentration values of two commercial enzymes using for shrimp head hydrolysis processes were obtained from the regression equation (1). According to the results shown in Fig. 2 and 3, the appropriate conditions to hydrolyze shrimp heads for subsequent experiments correspond to X_1 = 19.43U/g and X_2 = 32.09 U/g, which significantly improves protein hydrolysis efficiency in shrimp heads. For example, it is 61.82% higher than the optimal alcalase hydrolysis efficiency described by Gunasekaran *el al.* (2015). The results were compatible with the study of Vioque *el al.* (1999), using alcalase enzyme and flavourzyme for protein hydrolysis in rapeseed seeds with a hydrolysis yield of 60%.

3.3 The interactive effect of temperature, pH and hydrolysis time on protein hydrolysis efficiency of shrimp heads

Based on the optimal conditions of intrinsic protease and commercial enzyme, three factors of pH, temperature and hydrolysis time have a significant influence on protease activity. This may be the main reason for changing effective shrimp heads protein hydrolysis. The effect of factors on the regression equation was based on DH values (%) (Fig. 4) and DPPH (%) antioxidant capacity (%) (Fig. 5).

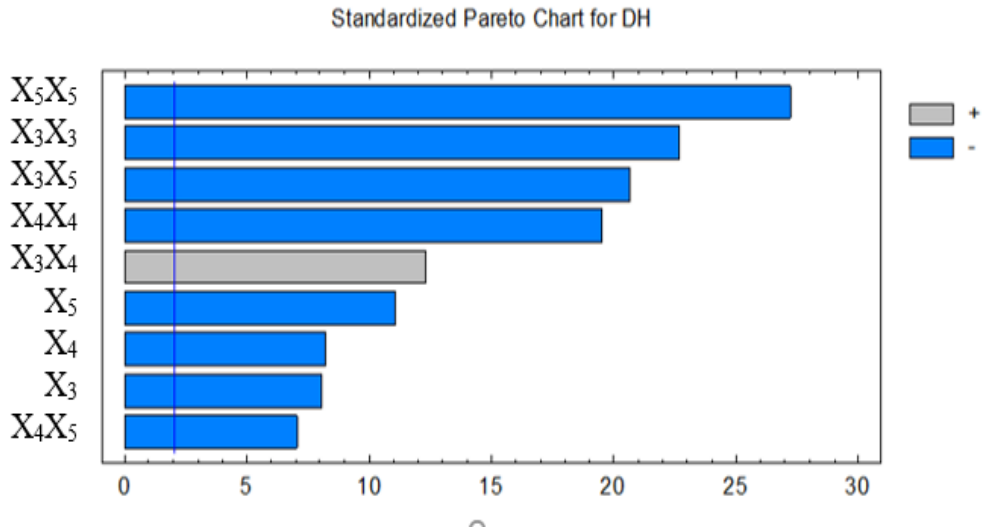

Fig. 4: Effect of temperature, pH and hydrolysis time on degree hydrolysis

The Pareto graph (Fig. 4) was used to reveal the main effect as well as interaction effects of all variables on protein hydrolates production. From Fig.4, all coefficients, consist of first-order regression coefficients of temperature (X_3), pH (X_4) and hydrolysis time (X_5), the interaction coefficients as well as the quadractic terms were a significant effect with statistically different at 95% confidence intervals.

Analysis of variance from RSM yielded the following final regression equation (2) in terms of hydrolytic efficiency (Y_2, DH%) as a function of reaction temperature (X_3), initial pH (X_4) and hydrolysis time (X_5):

$$Y_2\ (DH\%) = -15161.4 + 56.7271X_3 + 37.3768X_4 + 400.736X_5 - 1.33152X_3^2 + 14.2967X_3X_4 - 3.59857X_3X_5 - 318.284X_4^2 - 20.5364X_4X_5 - 9.98803X_5^2 \quad (2)$$

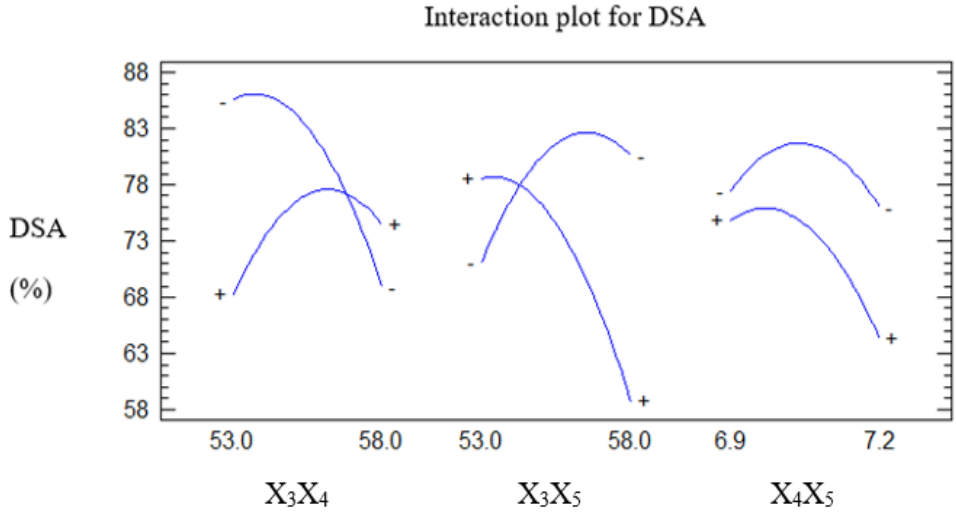

Fig. 5: Effect of temperature, pH and hydrolysis time on on antioxidant activities

The optimal conditions obtained for hydrolysis efficiency are shown in the recent studies. Shrimp (*Metapenaeus dobsoni)* head meat was used as the raw material; the DH was from 45.32% to 91.62%, and optimum value of DH was 90.20% at temperature of 54.94 ◦C, pH of 7.01 for 2.96 hours. It has been previously reported that DH was 42.44% when alcalase enzyme was used to hydrolyze shrimp (*Metapenaeus dobsoni*) head waste at pH = 8.2, temperature of 45.4◦C, and E/S at 1.8% (Gunasekaran *el al., 2015)*. Quaglia and Orban (1990) reported that a DH

of 70–75% was achieved when hydrolysis of defatted meat of sardine using alcalase was conducted for 140 minutes. Moreover, the DH for *Acetes chinensis* hydrolysis using optimized conditions was found to be around 26.32% (Cao *el al*., 2009). Consequently, the DH will be varied depending upon the hydrolysis conditions and using enzymes types.

The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability (Siddhuraju and Becker, 2007). In this study, it is indicated that DH can greatly affect the antioxidant properties of peptides. Smaller peptides have a higher level of radical scavenging than larger peptides; this finding is consistent with that of Li *el al*. (2007) and Dong *el al*. (2008).

Y_3 (DPPH%) = -8325.72 + 3.26072X_3 + 2258.86X_4 + 307.971X_5 – 0.933347X_3^2 – 15.3321X_3X_4 - 2.9289X_3X_5 – 218.724X_4^2 – 15.2187 X_4X_5 – 6.95327X_5^2 (3)

Optimal value (DPPH) 86.92% obtained at temperature of 53.24°C, pH of 6.91 and 3.37-hour-hydrolysis time. Many antioxidant peptides seem to share some common structural characteristics, which include a relatively short peptide residue length and often present hydrophobic amino acid residues in the sequence (Wang *el al.*, 2013). The DPPH is changed depending upon the release of this compound, which is directly the function of the degree of hydrolysis. In fact, Kim *el al.* (2007) reported that the lower molecular-weight fraction (1–3 kDa) was found to be the most effective inhibitor of lipid peroxidation, compared to four molecular-weight fractions (5–10, 3–5, 1–3 and <1 kDa) of the peptic hydrolysate of hoki-frame proteins. It was also mentioned that the lowest molecular-weight fraction (<1 kDa) from the tryptic hydrolysate of conger eel-muscle protein possessed the strongest antioxidant potency (Ranathunga *el al*., 2006).

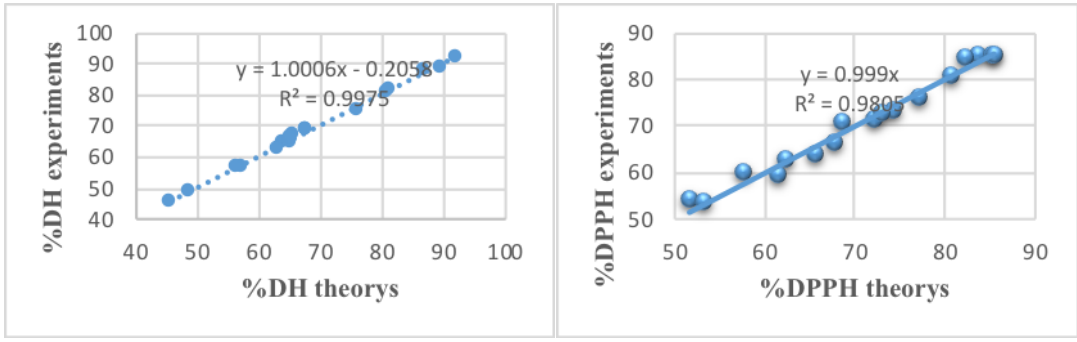

Fig. 6: The correlation between empirical hydrolysis efficiency and computed regression based on degree of hydrolysis and the antioxidant index

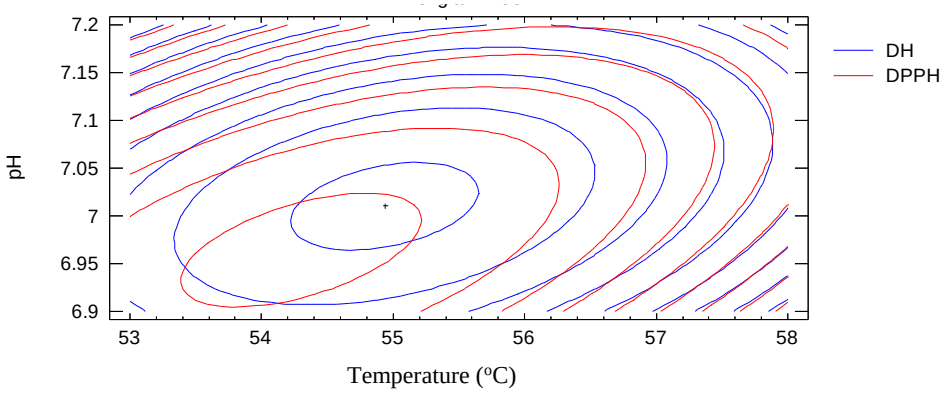

Fig. 7: The interaction of pH and temperature on protein hydrolysis efficiency (DH), antioxidant activity (DPPH) of hydrolysates

Thus, it can be concluded that the regression equation described correctly the experimental results. The coefficient of determinations (R^2) was 0.9775 of the variation in protein hydrolysis and and 0.9805 of the change in antioxidant activity (Fig. 6) which ensured a satisfactory adjustment of the quadratic model to the experimental data. It also desmonstrated that statistical model could explain 99.75% of variability in the degree of hydrolysis (response) or 98.05% of variability in the % DPPH value and only 0.25% (in case of %DH) or 1.95% (%DPPH)

of the total variations were not explained by the model. The interactions between the two functions are shown in Fig. 7 in the hydrolysis process that hydrolysis yields with high hydrolysis efficiency and good antioxidant activity.

As can be seen from Fig. 7, the optimal parameters were determined as pH of 7.01, temperature at 54.94 ^{o}C and hydrolysis time for 2.96 hours for obtaining high hydrolysis efficiency (90.19%) and good antioxidant activity (86.16%).

4 CONCLUSIONS

The results of this study showed the prominent feasibility of combining alcalase and flavourzyme, which results in a high hydrolysis efficiency (%DH) of 90.19% and a good antioxidant activity with 86.16% DPPH. The hydrolysis conditions were optimized by the RSM, which consequently gives the following optimal conditions: pH = 7.01, temperature (54.94^{0}C), 2.96-hour-hydrolysis time with concentrations of 19.42 U/g and 32.09 U/g for alcalase and flavourzyme enzyme, respectively.

REFERENCES

Cao, W., Zhang, C., Hong, P., and Ji, H., 2009. Optimising the free radical scavenging activity of shrimp protein hydrolysate produced with Alcalase using response surface methodology. *Int. J. Food. Sci. Technol*. 44(8): 1602–1608.

Murueta, J.H.C. and Carreño, F.G., 2007. Concentrates of fish protein from bycatch species produced by various drying processes. *Food Chemistry*, 100(2): 705-711.

Dey, S.S., and Dora, K.C., 2014. Optimization of the production of shrimp waste protein hydrolysate using microbial proteases adopting response surface methodology. *Journal of* Food Science and Technology. 51(1): 1-9.

Dong, S., Zeng, M., Wang, D., Liu, Z., Zhao, Y., and Yang, H., 2008. Antioxidant and biochemical properties of protein hydrolysates prepared from silver carp (*Hypophthalmichthys molitrix*). Food Chemistry. 107(4): 1485–1493.

Gildberg, A. and Stenberg, E., 2001. A new process for advanced utilisation of shrimp waste. Process Biochemistry, 36(8-9): 809-812.

Gunasekaran, J., Kannuchamy. N., Kannaiyan, S., Chakraborti, R., and Gudipati, V., 2015. Protein hydrolysates from Shrimp (*Metapenaeus dobsoni*) head waste: Optimization of extraction conditions by response surface methodology. Journal of Aqutic Food products Technology, 24: 429-442.

He, H., Chen, X., Sun, C., Zhang, Y., and Gao, P., 2006. Preparation and functional evaluation of oligopeptide-enriched hydrolysate from shrimp (*Acetes chinensis*) treated with crude protease from *Bacillus* sp. SM98011. Bioresource Technology, 97: 385-390.

Lahl, W. J. and Braun, S. D., 1994. Enzymatic production of protein hydrolysates for food use. Food Technol. *48: 68–71.*

Li, B., Chen, F., Wang, X., Ji, B., and Wu, Y., 2007. Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. Food Chem. 102: 1135–1143.

Kim, S. Y., Je, J. Y., and Kim, S. K., 2007. Purification and characterization of antioxidant peptide from hoki (Johnius belengerii) frame protein by gastrointestinal digestion. Journal of Nutritional Biochemistry. 18: 31–38.

Korhonen, H., and Pihlanto, A., 2006. Bioactive peptides: production and functionality. International Dairy Journal. 16: 945–960.

Moure, A., Dom´ınguez, H., and Parajo, J. C., 2005. Fractionation and enzymatic hydrolysis of soluble protein present in waste liquors from soy processing. J Agric Food Chem. 53: 7600–7608.

Najafian, L., and Babji, A. S. 2012. A review of fish-derived antioxidant and antimicrobial peptides: their production, assessment, and applications. Peptides. 33: 178–185.

Nielsen, P. M., Petersen, D. and Dambmann, C., 2001. Improved Method for Determining Food Protein Degree of Hydrolysis. Journal of food science. 66 (5): 642-646

Quaglia, G. B., and Orban, E., 1990. Influence of enzymatic hydrolysis on structure and emulsifying properties of sardine (*Sardina pilchardus*) protein hydrolysates. J. Food Sci. 55(6): 1571–1573.

Ranathunga, S., Rajapakse, N., and Kim, S. K., 2006. Purification and characterization of antioxidative peptide derived from muscle of conger eel (Conger myriaster). European Food Research and Technology. 222: 310–315.

Shahidi, F., Han, X.Q., Synowiecki, J., 1995. Production and characteristics of protein hydrolysates from capelin (Mallotus villosus). Food Chem. 53: 285–293.

Siddhuraju, P. and Becker, K., 2007. The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* L. Walp.) seed extracts. Food Chem. 101: 10–19.

Synowiecki, J. and Al-Khateeb, N.A.A.Q., 2000. The recovery of protein hydrolysate during enzymatic isolation of chitin from shrimp Crangon crangonprocessing discards. *Food Chemistry*, 68: 147-152.

Vioque, J., Vioque, R.S., Clemente, A., Pedroche, J., Bautista, J., and Millan, F., 1999. Production and characterization of an extensive Rapeseed protein hydrolysate. Journal of the American Oil Chemists' Society. 76: 819-822.

Wu, H. C., Chen, H. M., and Shiau, C. Y., 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austriasicus). Food Res. Int. 36: 949-957.

Wang, T., Zhao, Q., and Wang, Q., 2013. Production and Antioxxidant Properties of marine-derived bioactive peptides. In: Se-Kwon Kim, eds. Marine protein and peptides: Biological activities and applications (pp. 385–406). Ames: Wiley-Blackwell.

Zeng, M., Dong, S., Zhao, Y. and Liu, Z., 2013. Anti-oxxidant activitive of marine peptide from Fish and Shrimp. In: Se-Kwon Kim, eds. Marine protein and peptides: Biological activities and applications (pp. 449–466). Ames: Wiley-Blackwell.

Zhao, J., Huang, G.R., Zhang, M.N., Chen, W.W., and Jiang, J.X., 2011. Acid amin composition, molecular weight distribution and antioxidant stability of shrimp processing byproduct hydrolysate. American Journal of Food technology, 1-10.