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Optimization of Enzymatic Hydrolysis for the Production of Antioxidative Peptide from *Nannochloropsis gaditana* using Response Surface Methodology

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ABSTRACT

In the present research, microalgae protein hydrolysate of *Nannochloropsis gaditana* (MPH) was extracted via enzymatic hydrolysis using alcalase enzyme. Hydrolysis conditions like (pH, temperature, enzyme concentration and substrate concentration) were optimized by Response Surface Methodology (RSM) using Central Composite Design (CCD). Four range of independent variables namely; pH (7-9), temperature (45-55°C), substrate concentration (2-6 g/L) and enzyme concentration (0.2-0.4 g/L) were used to study the influence of these parameters on the degree of hydrolysis. The CCD consisted of twenty-four experimental points and six replicates of central points with the optimum conditions obtained from this experiment were at pH 8.14, a temperature 51.4°C, a substrate concentration 5.48 g/L and an enzyme concentration 0.26 g/L with maximum degree hydrolysis of 55.76%. All experiments were fixed at 24 hours reaction time. The degree of hydrolysis of MPH was analysed using O-phthaldehyde (OPA) method to quantify the cleavage of peptide bond. The optimized sample was evaluated for its antioxidant activity using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay with 52.19% and 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid (ABTS) assay with 14.13%. The bioactive peptides contained in *Nannochloropsis gaditana* have the ability to scavenge free radicals and act as good antioxidants

Keywords: Antioxidant, bioactive peptide, degree hydrolysis, microalgae, optimization

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INTRODUCTION

Microalgae are known as unicellular photosynthetic microorganisms and live in freshwater or saline environment. They utilize water, carbon dioxide, and sunlight to convert into algal biomass. For many years, microalgae have been considered as important potential feedstock in the biofuels and chemical area due to its valuable bioactive compounds (Markou & Nerantzis, 2013; Nobre et al., 2013). Lipids from microalgae have been utilized as biofuel feedstock and the sugars are extracted from microalgae as substrate for bioethanol production has been studied widely around the world (Chen et al., 2012; Chng, 2016; Chu, 2017; Geun Goo et al., 2013; Harun et al., 2010; Lee et al., 2013; Patnaik & Mallick, 2015; Shuping et al., 2010; Subhadra, 2010; Watanabe et al., 2014). However, the study on microalgae protein especially peptides is not as much as lipid and carbohydrate.

Peptides derived from protein can be found in all living beings of plant and animal origin and are classified as one of major food component. Peptides usually can be obtained through technological processes such as fermentation and enzyme hydrolysis (Martínez-maqueda et al., 2013). The process involving the breakage of protein molecules into various size of small peptides and eventually amino acids is called enzymatic protein hydrolysis (Sbroggio et al., 2016). These peptides typically have 3–20 amino acid residues, and their activities rely upon their sequences and amino acid composition (Pihlanto-leppa, 2001). Principally, the characteristics of an enzyme used must be at least a food grade and non-pathogenic if the enzyme is microbial origin (Bhaskar, 2008). However, enzymatic protein hydrolysis using commercial food grade enzymes is more desirable and consistent due to the better control in term of hydrolysates properties (Slizyte et al., 2005).The applications of protein peptide have been studied for antioxidant, anticoagulant, antimicrobial and antihypertensive which could be utilized as a component in food or pharmaceutical healthcare products (Kim et al., 2001; Mendis et al., 2005; Suetsuna et al., 2004).

The chemical composition of bioactive compounds in microalgae varies depending on species, cultivation, growth condition and type of extraction (Mirsiaghi & Reardon, 2015). One of microalgae species, *Nannochloropsis* is potentially known as source of lipids for production of biodiesel (Gouveia & Oliveira, 2009; Moazami et al., 2012). However, it is also considered as a source of protein, carbohydrates, carotenoid, and phenolic compounds (Lubián et al., 2000).The study on protein peptide from *Nannochloropsis gaditana (N.gaditana) sp* is very few and not much of literature can be found as most of researchers are focusing on the biofuel production. Research done by Sheih et al., (2009) reported that algae residual protein from *Chlorella vulgaris* sp possess antioxidative activity which had significant protective effects on DNA and could prevent cellular damage. Neverthless, depth study on the algae protein in terms of optimization and selectivity of bioactive peptide still has not been explored so far. *N.gaditana* is well known among researchers as one of the best candidate for biofuel productions (Andrich et al., 2005; Gerde et al., 2013; Nobre et

al., 2013; Pan et al., 2010). However, the extraction of lipids for biofuel usually produces microalgal residual which consists of protein and carbohydrate. It is normally discarded as a waste. The microalga residues of *N.gaditana* ~~is~~ mainly consist of protein and carbohydrates which have value-added. Therefore, in this research, a microalga *N.gaditana* was chosen as a model to produce bioactive peptide for antioxidant activity. Antioxidant activity is mainly depending on the type of peptide produced during enzymatic hydrolysis process. Enzymatic hydrolysis is a crucial step in the bioactive peptide production. A suitable enzyme and parameter during the hydrolysis process should be chosen wisely. Ngo et al., (2013) reported that enzymes derived from microorganisms and animal digestive system was best to be used in obtaining bioactive peptide. Alcalase, neutrase, flavourzyme, protamex and kojizyme from microorganism and trypsin, papain and α-chymotrypsin from animals are commercial enzymes that could be found in the market. Among these enzymes, alcalase was used in this study because the alcalase hydrolysate exhibited good bioactivity (Wijesekara, Qian, Ryu, Ngo & Kim 2011), good functional properties (Amiza, Nurul Ashikin, & Faazaz, 2011) and most suitable for hydrolysis of protein especially in transpeptidation according to the manufacturer. Thus, a suitable approach in bioactive peptide production is needed for scaling up or commercialization in the future.

Optimization during enzymatic hydrolysis process and statistical analysis is required in maximizing protein peptide extraction and determine the independent and interaction effects of various process parameters on the extraction yields. Response Surface Methodology (RSM) is a known statistical method for planning an experiment, evaluating the impact factors, building a model, and optimizing the selected parameters (Kalil et al., 2000; Parimi et al., 2015). It is a statistically designed experimental protocol in which several factors are simultaneously varied (Rao et al., 2000) unlike the traditional method. The main objective of this research is to apply statistical method in optimizing the enzymatic hydrolysis parameters of protein peptide from microalgae *N.gaditana sp*. In this paper, four main parameters that influence the performance of enzymatic hydrolysis namely; pH, temperature, substrate concentration and enzyme concentration were optimized by RSM to determine the optimal condition for peptide production. The Central Composite Design (CCD) which required five levels of each factor was chosen to carry out experiments and estimate the coefficients of a quadratic model.

MATERIALS AND METHODS

Materials

Microalgae biomass of *Nannochloropsis gaditana* was purchased from Pure Bulk Inc. (USA), and delivered in a green powdered form. Liquid Alcalase® 2.4 L,(≥2.4 U/g) proteinase Subtilisin A from *Bacillus licheniformis* was purchased from Sigma-Aldrich.

All the chemicals used in different analysis were of analytical grade and purchased from Sigma-Aldrich and R&M Chemical.

Methods

Optimization of Enzymatic Hydrolysis Conditions of MPH by Response Surface Methodology (RSM). Optimization of enzymatic hydrolysis of *N.gaditana* was analysed using Response Surface Methodology (RSM) in Design Expert Software Version 10. RSM with randomized factorial design which is Central Composite Design (CCD) was employed to analyse the results. CCD is based on two-level factorial designs, augmented with center and axial points to fit quadratic models. A total number of 30 run with 24 non-center points and 6 center points was designed xfor the experiment. The center points were replicated to provide good predictive capabilities near the center of the factor space. Four different factors; pH(X_1), temperature (X_2), substrate concentration (X_3) and enzyme concentration (X_4) were employed over response variable; degree hydrolysis ,DH (Y). Enzymatic hydrolysis process value range for *N.gaditana* is shown in Table 1. Each run was performed with 100 mL of sodium phosphate buffer (PBS) containing microalgae biomass at specific pH, temperature, substrate concentration and enzyme concentration with a pre-determined reaction time. After 24 hours, enzymatic hydrolysis reaction was stopped by heating the hydrolysate in water bath at 95°C for 10 minutes. Then, the protein hydrolysate was centrifuged at 4000 × *g* using centrifuge model KUBOTA for 20 minutes followed by filtration to remove the solid residual (Agrawal et al., 2017). The supernatant was collected for the degree hydrolysis analysis.

Table 1
The variable levels of CCD for enzymatic hydrolysis of antioxidative peptide from Nannochloropsis gaditana

Factor level	pH, X_1	Temperature, X_2 (°C)	Substrate concentration, X_3 (g/L)	Enzyme concentration, X_4 (g/L)
-1	7	45	2	0.2
0	8	50	4	0.3
1	9	55	6	0.4

Determination of Degree Hydrolysis. Degree of hydrolysis (DH) plays an important role in protein peptide hydrolysis reaction. DH is defined as the percentage of cleaved peptide bond and can be calculated as per equation (1):

$$DH = \frac{h}{h_{tot}} \times 100 \quad (1)$$

where, h is the number of hydrolyzed bonds and h_{tot} is the total number of peptide bonds in the substrate. DH was measured by O-phthaldehyde (OPA) method according to (Nielsen et al., 2001) with slightly modification. Firstly, OPA reagent was prepared by dissolving 3.81 g sodium tetraborate decahydrate and 100 mg of sodium dodecyl sulphate with deionized water. 80 mg of O-Phthaldehyde 98% in 2 ml of ethanol 95% was mixed with the sodium solution. Then, 200 µl of β-mercaptoethanol was added and deionized water was top up to 100 ml.

Determination of Antioxidant Activity. The microalgae protein hydrolysate at optimum condition were analysed for antioxidant activity.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Assay. The DPPH assay was measured according to the method of (Huang & Mau, 2006). A 1 mL of protein hydrolysates was mixed with 1 mL of methanol solution containing 1 mM DPPH radicals. The mixture was allowed to stand for 40 min in the dark, and the absorbance was monitored at 517 nm using UV-Vis spectrophotometer model GENESYS. Distilled water was used instead of hydrolysates as a blank. Scavenging DPPH activity was calculated according to the equation (2):

$$\text{Antioxidant activity} = \frac{A_0 - A_1}{A_0} \times 100 \qquad (2)$$

where A_1 is sample absorbance and A_0 is blank absorbance.

2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid (ABTS)) Assay. The radical cation was prepared by mixing 2.45 mM potassium persulfate with 7 mM ABTS stock solution with 1:1 ratio. The mixture was left for 4-16 h until the reaction was complete and the absorbance was stable. The $ABTS^+$ solution was diluted with ethanol 95% to an absorbance of 0.700 ± 0.05 at 734 nm for measurements. The ABTS assay was measured by mixing 0.9 mL of $ABTS^+$ solution with 0.1 mL of tested samples and mixed for 45 seconds. The mixture was measured immediately using UV-Vis spectrophotometer model GENESYS at 734 nm after 15 minutes. The antioxidant activity of microalgae protein hydrolysate was measured according to equation (2).

RESULTS AND DISCUSSION

Optimization of Hydrolysis Conditions by Response Surface Methodology (RSM)

The optimal enzymatic hydrolysis conditions was analysed using Central Composite Design (CCD) in RSM based on the degree of hydrolysis (DH) at four independent variables as

shown in Table 2. The DH value of *N.gaditana* was obtained from 18.12% to 73.03% with different hydrolysis conditions. This maximum DH value from *N.gaditana* was quite high if compared to algae source ;*Spirulina* sp.LEB-18, 60% (Pereira et al.,2016) and fish source; fish soluble concentrate, 68% (Nilsang et al., 2005); *Nemipterus japonicus*, 42.15% and *Exocoetus volitans,*43.21% (Naqash & Nazeer, 2012). The DH value of *N.gaditana* was higher compared to other source probably due to the different source of protein, different type of enzymes used and different range of parameters used.

Table 2
Enzymatic hydrolysis experimental design of independent variables along with the observed values for the response variable, degree of hydrolysis for N. gaditana (Y_1)

Run	pH (X_1)	Temperature,°C (X_2)	Substrate Concentration,g/L (X_3)	Enzyme Concentration,g/L (X_4)	Degree Hydrolysis,% (Y)
1	9	45	2	0.2	53.08
2	8	40	4	0.3	56.98
3	8	50	4	0.3	70.76
4	9	55	6	0.4	39.22
5	9	55	6	0.2	36.04
6	9	45	6	0.4	35.4
7	7	45	6	0.4	32.3
8	6	50	4	0.3	38.03
9	8	50	4	0.5	73.03
10	9	45	2	0.4	46.15
11	8	60	4	0.3	39.78
12	7	45	6	0.2	32.37
13	7	55	2	0.2	18.12
14	8	50	4	0.3	69.35
15	10	50	4	0.3	58.98
16	7	45	2	0.2	32.89
17	7	55	6	0.4	36.16
18	9	55	2	0.2	32.49
19	9	55	2	0.4	45.64
20	8	50	4	0.3	67.14
21	8	50	4	0.3	70.12
22	9	45	6	0.2	35.32
23	8	50	4	0.3	72.46
24	7	45	2	0.4	31.86
25	8	50	4	0.1	54.91
26	7	55	6	0.2	37.03
27	7	55	2	0.4	30.46
28	8	50	4	0.3	65.89
29	8	50	0	0.3	4.12
30	8	50	8	0.3	53.52

Data was analyzed for its analysis of variance (ANOVA), coefficient variation, diagnostic case statistics and response surface plots and effect of factors. The prediction model for the response variable could be fitted into linear, two-factor interaction (2FI), quadratic or cubic (Mat Amin et al., 2016). Table 3 shows the suggested model for the degree hydrolysis of *N.gaditana*. It can be seen that cubic model has the highest value of R-Squared but due to it was an aliased model, the model could not be appropriate and useful model. Thus, quadratic model was selected since the desirability value was close to 1 and the model fitted the experimental data with an acceptable determination coefficient $R^2 = 0.9708$ showing that the model had 97.08% of the total deviation of experimental data. While for the adjusted R-Squared, it is preferable to have the value close to 1. The adjusted R-squared that is closed to 1 and not aliased is quadratic model with 0.9394. In fact, the predicted R-Squared of 0.7976 is in reasonable agreement with the adjusted R-Squared which the difference is less than 0.2. Overall, quadratic model is suggested as the most suitable conditions to obtain the optimum DH with the predicted value is 55.76%.

Table 3
Model Summary statistics

Sources	Standard deviation	R-Squared	Adjusted R-Squared	Predicted R-Squared	
Linear	16.41	0.1169	-0.0367	-0.2025	
2FI	18.37	0.1815	-0.3000	-1.4064	
Quadratic	3.97	0.9708	0.9394	0.7976	Suggested
Cubic	2.22	0.9958	0.9810		Aliased

In Table 4 shows that pH, temperature and enzyme concentration had a linear effect, while pH, temperature and substrate concentration had a quadratic effect on DH value. Interaction for DH value could be seen from the interaction of pH and substrate concentration ($p<0.01$), temperature and substrate concentration ($p<0.01$), temperature and enzyme concentration ($p<0.5$). The highest positive value of esteemed regression coefficient was observed for the pH (X_1=4.75) indicating that it was the most influential parameter of DH while the least influence was substrate concentration with highest negative value (X_3^2= -21.63).

The regression equation for the degree of hydrolysis for *N.gaditana* with respect to four different independent variables; pH(X_1), temperature(X_2), substrate concentration(X_3) and enzyme concentration(X_4) were derived as follows:

$$Y = + 69.29 + 4.75\ X_1 - 2.44X_2 - 0.43X_3 + 2.34X_4 - 0.56X_1X_2 - 3.49X_1X_3 - 0.056\ X_1X_4 + 3.15X_2X_3 + 2.23X_2X_4 - 0.95X_3X_4 - 5.20X_1^2 - 5.23X_2^2 - 21.63X_3^2 - 1.33X_4^2$$

The results for analysis of variance (ANOVA) for degree of hydrolysis of *N.gaditana* by alcalase enzyme was observed that the model was highly significant at 99% confidence level ($p<0.0001$) as shown in Table 4. The suggested optimum hydrolysis conditions for enzymatic hydrolysis of *N.gaditana* were pH 8.14, 51.4°C, 5.48 g/L substrate concentration and 0.26 g/L enzyme concentration.

Table 4
Results of ANOVA on degree of hydrolysis (DH) for N. gaditana

Factors	Sum of square	df	Mean square	F value	P value
Model	6804.53	14	486.04	30.87	< 0.0001
X_1-*pH*	541.98	1	541.98	34.43	< 0.0001
X_2-*Temperature*	143.13	1	143.13	9.09	0.0099
X_3-*Substrate Concentration*	2.93	1	2.93	0.19	0.6731
X_4-*Enzyme Concentration*	131.09	1	131.09	8.33	0.0128
X_1X_2	4.96	1	4.96	0.32	0.5841
X_1X_3	195.37	1	195.37	12.41	0.0037
X_1X_4	0.050	1	0.050	3.145E-003	0.9561
X_2X_3	158.32	1	158.32	10.06	0.0074
X_2X_4	79.88	1	79.88	5.07	0.0422
X_3X_4	14.46	1	14.46	0.92	0.3554
X_1^2	647.82	1	647.82	41.15	< 0.0001
X_2^2	655.63	1	655.63	41.64	< 0.0001
X_3^2	2806.44	1	2806.44	178.26	< 0.0001
X_4^2	42.40	1	42.40	2.69	0.1247
Residual	204.67	13	15.74		
Lack of Fit	175.58	8	21.95	3.77	0.0800
Pure Error	29.08	5	5.82		
Cor Total	7009.20	27			

P =level of significance

Response Surface Analysis. The effect of four main parameters in enzymatic hydrolysis of *N.gaditana* (pH, temperature, substrate concentration and enzyme concentration) was manifested in 3D response surface contour plot as shown in Figure 1.

As we can see from Figure 1(a), the DH value increased as the substrate concentration increased along the pH. The interaction between substrate concentration and pH had strong effect on the curvature of the response in according to high negative value of interaction coefficient ($X_1X_3 = -3.49$).This is because at pH 8 to 8.5, the enzyme is highly energetic and provide more active site to lock the substrate. While in Figure 1(b), the degree of hydrolysis of *N.gaditana* was indirectly proportional to the substrate and enzyme concentration. The degree of hydrolysis at high substrate concentration, 6 g/L was much lower if compared

to low substrate concentration. During the hydrolysis reaction, the enzyme activity of the enzyme can be inhibited by the product formed. Competition between original substrate and the peptide toward the active sites of enzymes was expected. After hydrolysis of a

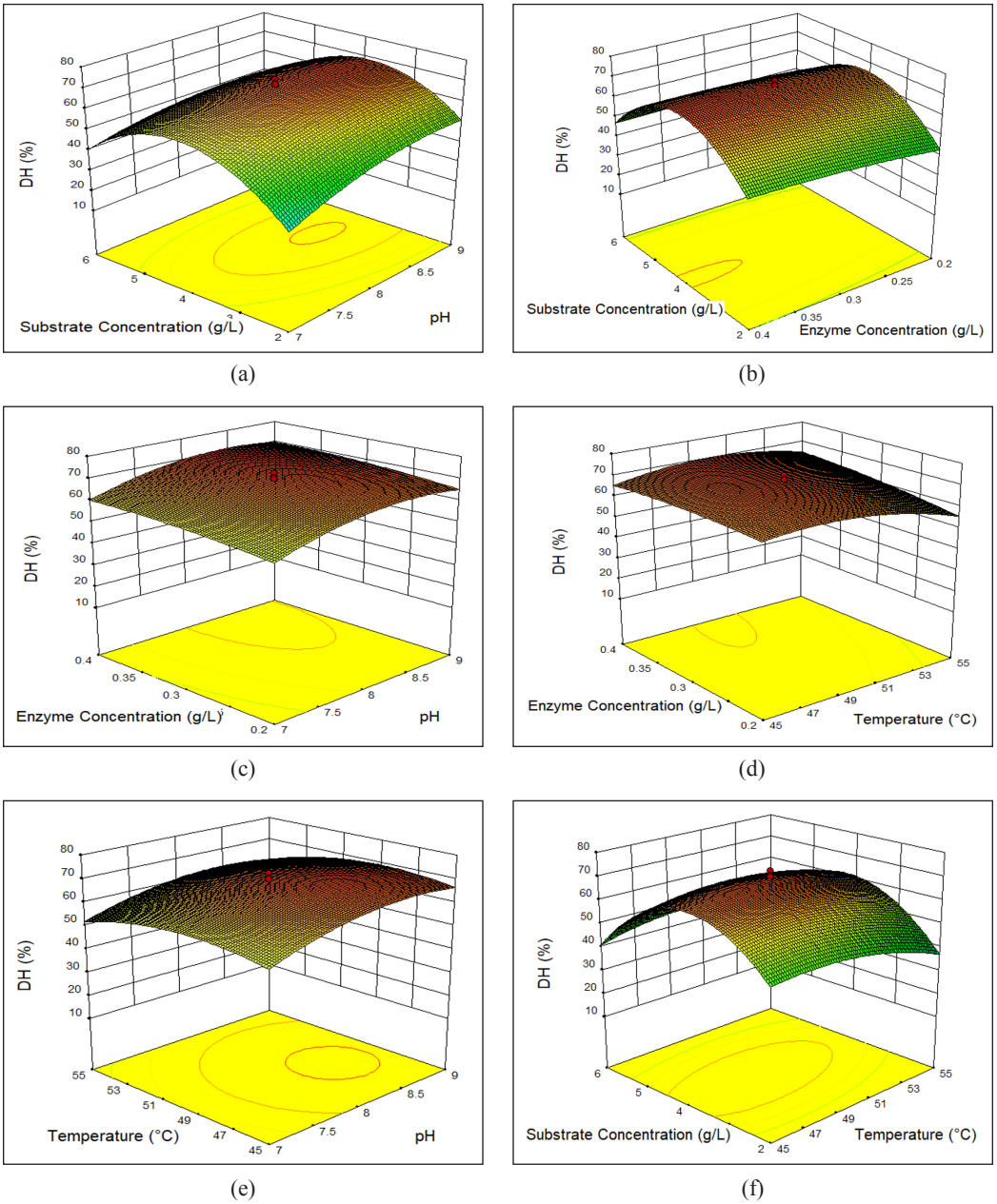

Figure 1. The three-dimensional response surface plot for the effect of (a) substrate concentration and pH (b) substrate concentration and enzyme concentration (c) enzyme concentration and pH (d) enzyme concentration and temperature (e) temperature and pH and (f) substrate concentration and temperature on DH of *N.gaditana*

peptide bond, terminal acyl is produced and could act as inhibitor, which can bind to the active site of the enzyme, forming an acyl-enzyme complex (Lisboa et al., 2014) .Thus,at high substrate concentration, the enzyme has high chances to bind with other inactive protein region, thus preventing the formation of enzyme-substrate complex and reduce the formation of products (Pereira et al., 2016). At low substrate concentration, hydrolysis rate was decreased could be due to low concentration of peptides available to be bonded with enzyme. By having moderate substrate concentration at low usage of enzyme as obtained in this study is preferable and economic for scaling up.

In Figure 1(c), we can see that the DH increased as the pH value increased. The same finding was reported by (Montilha et al., 2017). This was because the pH was strongly influenced the stability of enzyme, which could cause irreversible denaturation of its conformational structure and loss of enzyme activity (Whitaker, 1994). The optimum pH in this study was found to be at pH 8 which was in the range of pH 7 to 9 as recommended by manufacturer. Other studies found that alcalase worked best at pH 7.7 for Angel Wing Clam (*Pholas orientalis*) meat (Mat Amin et al., 2016), pH 7 for soybean (Nguyen, 2015), pH 8.5 for shrimp waste (Dey & Dora, 2014), pH 8.0 for goat milk casein (Shu et al., 2016) and pH 8 for Viscera of Tuna (*Euthynnus affinis*) (Salwanee et al., 2013). It showed that the optimal pH might vary according to the system used in such reaction (i.e: substrate, enzyme concentration) (Salwanee et al., 2013). In Figure 1 (d) the three-dimensional response surface graph demonstrated that the interaction between temperature and enzyme concentration greatly influenced the DH value with low effect on the response surface curvature according to interaction coefficient (X_2X_4= 2.23). This is because; any changes in temperature might change the shape of the enzyme and make it less complement to lock the substrate (Salwanee et al., 2013). In fact, the optimum temperature range of alcalase enzyme is between 45 to 60°C as per stated by the manufacturer.

Validation of Model

Based on the statistical results, the optimum conditions for the production of bioactive peptide from *N.gaditana* are as follows: pH of 8.14, temperature of 51.4°C, substrate concentration of 5.48 g/L and an enzyme concentration of 0.26 g/L with maximum predicted degree hydrolysis of 55.76%. The conditions are modified to make it easy for controlling and measure the parameters. Thus, a new experiment with modified optimal conditions of pH 8, temperature 51°C, substrate concentration 5 g/L and enzyme concentration 0.3 g/L were performed for verification the optimization. The degree hydrolysis at modified at optimal conditions is 57.59% which is 3.28% more than the predicted value. The value is slightly different might be due to the modification in the parameters of enzymatic hydrolysis. These results showed and confirmed the model is appropriate and robust to estimate the value of experiments.

Antioxidant Evaluation Assay

The best condition of enzymatic hydrolysis was further analysed for its antioxidant properties. Table 5 shows the antioxidant activity of the same sample but was evaluated with different free radical scavenging activity. DPPH assay with 52.19% gave high antioxidant activity compared to ABTS assay. This showed that microalgae protein hyrolysate had high ability to inhibit DPPH free radical at the same conditions. Similar result was found by Shalaby and Shanab (2013) where the protein using water extraction exhibit high antioxidant activity with DPPH instead of ABTS. It could be due to the increasing number of electron and hydrogen donating properties of the active peptides (Sbroggio et al., 2016). These active peptides have high possibility to react with free radicals in producing more stable products, and hence the radical chain reaction could be terminated (Pazinatto et al., 2013). DPPH is a stable free radical with a maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance, the radical is scavenged and the absorbance is reduced (Cian et al., 2013).

Table 5
Antioxidant assay of microalgae protein hydrolysate

Antioxidant Assay	DPPH	ABTS
Microalgae Protein Hydrolysate at optimum condtions	52.19%	14.13%

CONCLUSION

In this study, we found that the optimum conditions for enzymatic hydrolysis of *N.gaditana* is at pH 8.14, 51.4°C , 5.48 g/L substrate concentration and 0.26 g/L enzyme concentration with predicted degree hydrolysis of 55.76%. Antioxidant activity of microalgae protein hydrolysate varied depending on the free radical scavenger used. In this evaluation, DPPH assay revealed high antioxidant activity compared to ABTS assay. In conclusion, bioactive peptide derived from *N.gaditana* can be a valuable source for antioxidant. However, further characterization and purification of microalgae protein hydrolysate are needed to determine the properties of protein peptide.

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REFERENCES

Agrawal, H., Joshi, R., & Gupta, M. (2017). Isolation and characterisation of enzymatic hydrolysed peptides of green tender sorghum and their antioxidant activities. *LWT - Food Science and Technology*, *84*, 608-616.

Andrich, G., Nesti, U., Venturi, F., Zinnai, A., & Fiorentini, R. (2005). Supercritical fluid extraction of bioactive lipids from the microalga Nannochloropsis sp. *European Journal of Lipid Science and Technology*, *107*(6), 381–386.

Bhaskar, N., Benila, T., Radha, C. & Lalitha R. G. (2008). Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catla catla*) for preparing protein hydrolysate using a commercial protease. *Bioresource Technology*, *99(2)*, 335–343.

Chen, R., Yue, Z., Deitz, L., Liu, Y., Mulbry, W., & Liao, W. (2012). Use of an algal hydrolysate to improve enzymatic hydrolysis of lignocellulose. *Bioresource Technology*, *108*, 149–154.

Chng, L. M., Chan D. J. C. & Lee, K. T. (2016). Sustainable production of bioethanol using lipid-extracted microalgae from *Scenedesmus dimorphus*. *Journal of Cleaner Production*, *130*, 68-73

Chu, W. L. (2017). Strategies to enhance production of microalgal biomass and lipids for biofuel feedstock. *European Journal of Phycology*, *52*(4), 419–437.

Cian, R. E., Alaiz, M., Vioque, J., & Drago, S. R. (2013). Enzyme proteolysis enhanced extraction of ACE inhibitory and antioxidant compounds (peptides and polyphenols) from *Porphyra columbina* residual cake. *Journal Applied Phycology*, *25*(4), 1197–1206.

Dey, S. S., & Dora, K. C. (2014). Antioxidative activity of protein hydrolysate produced by alcalase hydrolysis from shrimp waste (*Penaeus monodon* and *Penaeus indicus*). *Journal of Food Science and Technology*, *51*(3), 449–457.

Gerde, J. A., Wang, T., Yao, L., Jung, S., Johnson, L. A., & Lamsal, B. (2013). Optimizing protein isolation from defatted and non-defatted Nannochloropsis microalgae biomass. *Algal Research*, *2*(2), 145–153.

Geun Goo, B., Baek, G., Jin Choi, D., ll Park, Y., Synytsya, A., Bleha, R., Ho Seong, D., Gyun Lee, C., Kweon Park, J. (2013). Characterization of a renewable extracellular polysaccharide from defatted microalgae *Dunaliella tertiolecta. Bioresource Technology*, *129*, 343–350.

Gouveia, L., & Oliveira, A. C. (2009). Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology & Biotechnology*, *36*(2), 269–274.

Harun, R., Danquah, M. K., & Forde, G. M. (2010). Microalgal biomass as a fermentation feedstock for bioethanol production. *Journal of Chemical Technology and Biotechnology*, *85*(2), 199–203.

Huang, S., & Mau, J. L. (2006). Antioxidant properties of methanolic extracts from *Agaricus blazei* with various doses of g -irradiation. *LWT - Food Science and Technology*, *39*(7), 707–716.

Kalil, S. J., Maugeri, F., & Rodrigues, M. I. (2000). Response surface analysis and simulation as a tool for bioprocess design and Optimization. *Process Biochemistry*, *35*(6), 539–550.

Kim, S., Kim, Y.-T., Byun, H., Park, P., & Ito, H. (2001). Purification and characterization of antioxdative peptide from Bovine Skin. *Journal of Biochemistry and Molecular Biology*, *34*(3), 219–224.

Lee, O. K., Kim, A. L., Seong, D. H., Lee, C. G., Jung, Y. T., Lee, J. W., & Lee, E. Y. (2013). Chemo-enzymatic saccharification and bioethanol fermentation of lipid-extracted residual biomass of the microalga, *Dunaliella tertiolecta*. *Bioresource Technology*, *132*, 197–201.

Lisboa, C. R., Pereira, A. M., Ferreira, S. P., & Costa, J. A. (2014). Utilisation of Spirulinasp .and Chlorellapyrenoidosa biomass for the productionof enzymatic protein hydrolysates. *Journal of Engineering Research and Applications*, *4*(5), 29–38.

Lubián, L. M., Montero, O., Moreno-garrido, I., Huertas, E., Sobrino, C., González-del Valle, M., & Parés, G. (2000). Nannochloropsis (Eustigmatophyceae) as source of commercially valuable pigments. *Journal of Applied Phycology*, *12*(3-5), 249–255.

Markou, G., & Nerantzis, E. (2013). Microalgae for high-value compounds and biofuels production: A review with focus on cultivation under stress conditions. *Biotechnology Advances*, *31*(8), 1532–1542.

Martínez-maqueda, D., Hernández-ledesma, B., Amigo, L., Miralles, B., & Gómez-ruiz, J. Á. (2013). *Extraction / Fractionation Techniques for Proteins and Peptides and Protein Digestion*. Boston: Springer.

Mat Amin, A., H.A, L., & H, Z. (2016). Madridge Journal of Food Technology Optimization of enzymatic protein hydrolysis conditions to obtain maximum angiotensin-i- converting enzyme (ACE) inhibitory activity from Angel Wing Clam (*Pholas orientalis*) meat. *Madridge Journal of Food Technology*, *2*(1), 65–73.

Mendis, E., Rajapakse, N., & Kim, S. (2005). Antioxidant Properties of a Radical-Scavenging Peptide Purified from Enzymatically Prepared Fish Skin Gelatin Hydrolysate. *Journal of Agricultural and Food Chemistry*, *53*(3), 581–587.

Mirsiaghi, M., & Reardon, K. F. (2015). Conversion of lipid-extracted Nannochloropsis salina biomass into fermentable sugars. *Algal Research*, *8*, 145–152.

Moazami, N., Ashori, A., Ranjbar, R., Tangestani, M., Eghtesadi, R., & Nejad, A. S. (2012). Large-scale biodiesel production using microalgae biomass of Nannochloropsis. *Biomass and Bioenergy*, *39*, 449–453.

Montilha, M. S., Sbroggio, M. F., Figueiredo, G. De, Ida, E. ., & Kurozawa, L. E. (2017). Optimization of enzymatic protein hydrolysis conditions of okara with endopeptidase Alcalase. *International Food Research Journal*, *24*(3), 1067–1074.

Nguyen, P. M. (2015). Alcalase and Protamex Hydrolysis of Bioactive Peptides from Soybean. *Bulletin of Environment, Pharmacology and Life Sciences*, *4*(7), 132–143.

Nielsen, P. ., Petersen, D., & Dambmann.C. (2001). Improved Method for Determining. *Food Chemistry and Toxicology*, *66*(5), 642–646.

Nilsang, S., Lertsiri, S., Suphantharika, M., & Assavanig, A. (2005). Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. *Journal of Food Engineering 70*(4), 571–578.

Nobre, B. P., Villalobos, F., Barragan, B. E., Oliveira, A. C., Batista, A. P., Marques, P. A. S. S., Mendes, R. L., Sovova, H., Palavra A. F. & Gouveia, L. (2013). A biorefinery from Nannochloropsis sp. microalga - Extraction of oils and pigments. Production of biohydrogen from the leftover biomass. *Bioresource Technology*, *135*, 128–136.

Pan, P., Hu, C., Yang, W., Li, Y., Dong, L., Zhu, L., Tong, S., Qing, R. & Fan, Y. (2010). The direct pyrolysis and catalytic pyrolysis of Nannochloropsis sp. residue for renewable bio-oils. *Bioresource Technology*, *101*(12), 4593–4599.

Parimi, N. S., Singh, M., Kastner, J. R., Das, K. C., Forsberg, L. S., & Azadi, P. (2015). Optimization of protein extraction from *Spirulina platensis* to generate a potential co-product and a biofuel feedstock with reduced nitrogen. *Frontiers in Energy Research*, *3*, 1–9.

Patnaik, R., & Mallick, N. (2015). Utilization of S*cenedesmus obliquus* biomass as feedstock for biodiesel and other industrially important co-products: An integrated paradigm for microalgal biorefinery. *Algal Research*, *12*, 328–336.

Pereira, A. M., Lisboa, C. R., Pereira, A. M., Alberto, J., & Costa, V. (2016). Biopeptides with antioxidant activity extracted from the biomass of Spirulina sp . LEB 18. *African Journal of Microbiology Research*, *10*(3), 79–86.

Pihlanto-leppa, A. (2001). Bioactive peptides derived from bovine whey proteins : opioid and ace- inhibitory peptides. *Trends in Food Science & Technology*, *11*(9-10), 347–356.

Rao, K. J., Kim, C. H., & Rhee, S. K. (2000). Statistical optimization of medium for the production of recombinant hirudin from *Saccharomyces cerevisiae* using response surface methodology. *Process Biochemistry*, *35*(7), 639–647.

Salwanee, S., Aida, W. M. W., Mamot, S., Maskat, M. Y. & Ibrahim,. S. (2013). Effects of Enzyme Concentration , Temperature , pH and Time on the Degree of Hydrolysis of Protein Extract from Viscera of Tuna (*Euthynnus affinis*) by Using Alcalase. *Sains Malaysiana*, *42*(3), 279–287.

Sbroggio, M. F., Montilha, M. S., Ribeiro, V., Figueiredo, G. De, Georgetti, S. R., & Kurozawa, L. E. (2016). Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates. *Food Science and Technology*, *36*(2), 375–381.

Shalaby, E. A., & Shanab, S. M. M. (2013). Comparison of DPPH and ABTS assays for determining antioxidant potential of water and methanol extracts of *Spirulina platensis*. *Indian Journal of Geo-Marine Sciences*, *42*(5), 556–564.

Sheih, I., Fang, T. J., & Wu, T. (2009). Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste. *Food Chemistry*, *115*(1), 279–284.

Shu, G., Zhang, B., Zhang, Q., Wan, H., & Li, H. (2016). Effect of temperature, pH, enzyme to substrate ratio, substrate concentration and time on the antioxidative activity of hydrolysate from goat milk casein by alcalase. *Food Technology*, *20*(2), 29–38.

Shuping, Z., Yulong, W., Mingde, Y., Kaleem, I., Chun, L., & Tong, J. (2010). Production and characterization of bio-oil from hydrothermal liquefaction of microalgae *Dunaliella tertiolecta* cake. *Energy*, *35*(12), 5406–5411.

Slizyte, R., Falch, E., Rustad, T., Dauks, E., & Storro, I. (2005). Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (*Gadus morhua*) by-products. *Process Biochemistry*, *40*(3-4), 1415–1424.

Subhadra, B. G. (2010). Sustainability of algal biofuel production using integrated renewable energy park (IREP) and algal biorefinery approach. *Energy Policy*, *38*(10), 5892–5901.

Suetsuna, K., Maekawa, K., & Chen, J. R. (2004). Antihypertensive effects of *Undaria pinnatifida* (wakame) peptide on blood pressure in spontaneously hypertensive rats. *Journal of Nutritional Biochemistry*, *15*(5), 267–272.

Watanabe, H., Li, D., Nakagawa, Y., Tomishige, K., Kaya, K., & Watanabe, M. M. (2014). Characterization of oil-extracted residue biomass of *Botryococcus braunii* as a biofuel feedstock and its pyrolytic behavior. *Applied Energy*, *132*, 475–484.

Naqash, S. Y., & Nazeer, R. A. (2012). Optimization of enzymatic hydrolysis conditions for the production of antioxidant peptides from muscles of *Nemipterus japonicus* and *Exocoetus volitans* using response surface methodology. *Amino Acids, 43*(1), 337–345.