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Original Article

Optimization of enzymatic hydrolysis condition of edible bird's nest using Protamex to obtain maximum degree of hydrolysis

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Abstract

This study reported on the optimization of enzymatic hydrolysis condition of edible bird's nest (EBN) using Protamex® to obtain maximum degree of hydrolysis degree (DH). Besides, the proximate analyses of soaked cleaned raw EBN and its lyophilized hydrolysate powder prepared under optimum condition (suggested in this study) were also compared. Response surface methodology (RSM) was employed using a three-level face-centered Central Composite Design (CCD) at four different parameters, namely temperature (40-60°C), concentration of Protamex® (0.5-1.5%), pH (5.5-9.5) and hydrolysis time (2-6 hr). It was found that a quadratic fit could explain the effect of these four variables on the DH of EBN. The optimum condition was obtained at temperature 59.9°C, pH of 6.3, Protamex® concentration of 2% and hydrolysis time of 5.4 hr. The DH achieved under this optimum condition (33.88%) was close to the predicted DH (34.11%). It was found that the lyophilized EBN hydrolysate powder prepared under the optimum condition gave similar protein and carbohydrate content, but lower fat content and higher ash content as compared to cleaned raw EBN.

Keywords: Degree of hydrolysis, Edible bird's nest, Enzymatic hydrolysis, Protamex

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Introduction

Edible bird's nest (EBN) refers to the nests produced by the interwoven strands in saliva secreted by the swiftlet's sublingual salivary glands (Guo, 2006; Tung et al., 2008). Swiftlets were distributed throughout the South Pacific and South East Asia region (Stimpson, 2013). In Malaysia, most EBN are from the white-nest swiftlet species, *Aerodromus fuciphagus* (Norhayati et al., 2010). Edible bird's nest is well known as "Caviar of the East" which brings a symbol of wealth, prestige and status. It has been used in traditional Chinese medicine as a treatment for malnutrition, a boost to the immune system as well as enhancement to the metabolism and skin complexion (Ma and Liu, 2012; Hamzah, 2013). Currently, EBN products are mainly limited to whole EBN, EBN pieces, bird nest drink or soup (Wu et al., 2010) and cosmetic products (Zainab et al., 2013).

Previous study on edible bird's nest has been reported on its nutritional composition (Marcone 2005) and the structure of monosialyl oligosaccharides as a result of hydrolysis of edible bird's nest by chemical and enzymatic methods (Wieruszeski et al., 1987).

Marcone (2005) reported that glycoprotein is the main component in edible bird's nest. Ma and Liu (2012) reported that bioactivities of EBN were only exhibited in EBN hydrolysates. Most of the scientific research on enzymatic hydrolysis of edible's bird nest is based on medicinal benefits. Until now, several studies have been reported on protein hydrolysis of EBN to improve antioxidative activity, digestibility and ACEI activity (Yida et al., 2014; Amiza et al., 2014; Muhammad et al., 2015).

Enzymatic protein hydrolysis improves the physiochemical, functional, and sensory properties of the intact protein without prejudicing its nutritional value (Kristinsson and Rasco, 2000). Enzymatic protein hydrolysis using commercial food-grade enzymes is more favourable than endogenous enzymes due to better control and reproducibility in terms of properties of the hydrolysates such as the length of the peptide, resulting in desirable and consistent products (Slizyte et al., 2005). Therefore, the enzymatic protein hydrolysis always been selected for the purpose of producing high quality, toxic free degradation product and high essential amino acid product (Garcia et al., 2012). Many studies have reported the comparison between the proximate composition of a protein with its resulting hydrolysate following enzymatic protein hydrolysis and drying process (Oon, 2014; Beak and Cadwallader, 1995; Ovissipour et al., 2009; Nilsang et al., 2005; Amiza and Masitah, 2012; Severin and Xia, 2006).

Several factors need to be considered before, during and after the enzymatic protein hydrolysis process such as hydrolysis time, hydrolysis temperature, hydrolysis pH and concentration of enzyme. All these factors would affect the outcome of enzymatic hydrolysis process (Bhaskar and Mahendrakar, 2008). The extent of protein hydrolysis can be measured using degree of hydrolysis (DH). DH is defined as the percentage ratio between the number of peptide bonds cleaved and the total number of peptide bonds in the substrate studied (Adler-Nissen, 1986). DH is the principal variable to be used in optimization to achieve the desired protein hydrolysate, since DH is positively correlated with the solubility of the hydrolysates and digestibility of the protein (Huong, 2013).

Optimization of protein enzymatic hydrolysis can be tailored to achieve certain targets such as to obtain high DH, high bioactivities, desirable properties etc. It is important to get the optimal conditions for production of food protein hydrolysate to reduce cost and time. Response Surface Methodology (RSM) is commonly used for optimization study. It is a collection of mathematical and statistical techniques successfully applied in optimization of food processing operations, including enzymatic hydrolysis processes (Thompson, 1982; Bezerra et al., 2008) in order to solve multivariate problem. The results are graphically represented as response surface (Raissi and Farsani, 2009). Based on the experimental data, RSM could suggest the optimum conditions to obtain the desired responses and the mathematical model in explaining the relationship between the independent variables and its response (Bezerra et al., 2008; Gan et al., 2010).

To date, limited optimization studies has been reported on enzymatic protein hydrolysis of EBN. Oon (2014) reported that enzymatic protein hydrolysis of EBN using Alcalase® to obtain maximum DH can be predicted by using a two factor interaction (2FI) model while Amiza et al. (2014) found that enzymatic protein hydrolysis of EBN using Alcalase® to obtain maximum angiotensin-converting enzyme (ACE) inhibitory activity can be predicted using quadratic model. Thus, in this study, optimization of EBN hydrolysis using food-grade enzyme, Protamex® to obtain maximum DH was employed using RSM. Protamex® has been chosen due to its ability to produce bitterless hydrolysates compared to other proteinase (Liaset *et al*., 2003).

Material and Methods

Raw materials and chemicals

One kilogram of unprocessed edible bird's nest (EBN) was purchased from a swiftlet house operator in Gong Badak, Kuala Terengganu in July 2014. The unprocessed EBN was stored in a plastic container at room temperature until further use. Prior to analysis, the raw unprocessed bird's nests were cleaned by soaking them in tap water in order to ensure maximum water absorption until the nests' cement is softened and expanded. After that, they were rinsed as needed with tap water to get rid of contaminants (contaminant, guano, cracked eggs, dust) and swiftlet's feathers. The fine feathers and impurities were the plucked and removed manually by using a fine tips tweezers and with the aid of illuminated magnifying glass. This step was repeated until all the small feathers and impurities were removed. Finally, the cleaned EBN was kept in a plastic container and stored in freezer (-20°C) until further use.

Protamex® (1.5 AU/g), which is a light brown, free flowing granulate, was purchased from Novozymes Malaysia Sdn. Bhd. It was stored at 4°C until further use. All other chemical reagents used in this study were of analytical grades from Sigma Aldrich.

Determination of proximate analysis of edible bird's nest sample

Proximate analysis of edible bird's nest sample including moisture, crude protein, ash, crude fat and carbohydrate content were analysed according to AOAC method (AOAC, 2000).

Optimization of EBN hydrolysis using response surface methodology (RSM)

The effect of four variables of enzymatic hydrolysis of EBN towards degree of hydrolysis (DH) was studied using response surface methodology (RSM). A three-level face-centered central composite design (CCD) was used. The independent experimental variables were temperature (A: 40, 50, 60°C), enzyme to substrate concentration (B: 0.5, 1.5, 2.5 E/S, %w/w), hydrolysis time (C: 2, 4, 6 hr) and pH (D: 5.5, 7.5, 9.5), which were employed at three equidistant level (-1, 0 and +1), with DH as the response variable. In this study, a total of 30 runs of EBN enzymatic hydrolysis were employed as suggested by Design Expert software (Stat-Ease, Inc.). The independent variables were chosen based on recommended conditions by Protamex manufacturer (Novozyme Malaysia Sdn. Bhd.) and previous studies (Oon, 2014; Huong, 2013). Experimental runs were randomised to minimise the effects of unexpected variability in the observed response.

Enzymatic hydrolysis of edible bird's nest (EBN)

The EBN hydrolysis was performed according to the procedure of Bhaskar and Mahendrakar (2007) with slight modifications.

Determination of degree of hydrolysis (DH)

DH of lyophilized EBN hydrolysate powder was determined using trichloroacetic acid (TCA) method as described by Hoyle and Merritt (1994) with a slight modification. Total nitrogen content was determined by analyzing 0.5 g of freeze dried EBN powder using Kjeldahl method. As for determination of 10% TCA soluble nitrogen, 0.5 g of freeze dried EBN powder was added to 10 ml of distilled water. Then, 10 ml of 20% (w/v) TCA was mixed with the sample. The sample was left to stand for 30 min for precipitation. Later, the sample was centrifuged (High speed centrifuges model 1580R, Gyrozen Co., Ltd, Korea) at 4000 rpm for 15 min. The resulting supernatant was filtered and analyzed using Kjeldahl method (AOAC, 2000).DH is defined as the percentage ratio between the number of peptide bonds cleaved and the total number of peptide bonds in the sample (Adler-Nissen, 1979). DH was computed as follows:

$$\% \text{ DH} = \frac{\text{10\% TCA soluble N in the sample}}{\text{Total N in the sample}} \text{ x } 100$$

Verification of model

Four replications of EBN hydrolysis prepared at the predicted optimum condition were carried out to validate the model. The resulting supernatant from the hydrolysates were then freeze dried prior to determination of DH. Experimental values of DH were then compared with the predicted value obtained from RSM, using one-sample t-test. The predicted DH given by Response Surface Methodology (RSM) was then compared with the experimental DH obtained.

Statistical analysis

Optimization data were subjected to analysis of variance (ANOVA) using Design Expert 9.0.3 software (StatEase Inc.) at 95% confidence level ($p < 0.05$).

Results and Discussion

Optimization of enzymatic protein hydrolysis of EBN using Protamex®

From the experimental data, it was found that the DH values of lyophilized EBN hydrolysates ranged from 13.79% to 33.26%**.** The highest DH (33.26%) was obtained at temperature of 60°C, pH of 5.5, hydrolysis time of 6 hr and enzyme concentration of 2.5%. Meanwhile, the lowest DH (13.79%) was obtained at temperature of 60°C, pH of 9.5, hydrolysis time of 2 hr and enzyme concentration of 0.5%.

The range of DH values obtained in this study are slightly lower than EBN hydrolysed using Alcalase® (14.27%-37.89%) (Oon, 2014). Comparing this study with other protein hydrolysis catalyzed using Protamex® shows that this DH range is similar with those of Yellowfin tuna (*Thunnus albacares*) by-products using Protamex®(16.8%-32.3%) (Nguyen et al., 2011). The differences in DH obtained in these studies could be due to the differences in protein source used as well as the different range of

parameters such as time, pH, enzyme concentration and temperature applied during enzymatic hydrolysis.

Analysis on response of optimization condition

Analysis of variance (ANOVA) for degree of hydrolysis (DH)

If the experimental data can be fitted to a model, there are 3 possible models which will be suggested by the Design Expert software version 9.0.3 (Stat-Ease, Inc.), i.e. linear, quadratic, cubic or two-factor interaction (2FI). In this study, the quadratic model was suggested for EBN hydrolysis. This indicated that the quadratic model fitted well to represent the real relationships among the chosen hydrolysis variables with response. Model reduction was carried out to further improve the model. After model reduction was performed (i.e. by excluding the insignificant model terms (p-value > 0.05), the ANOVA table of Response Surface Reduced Quadratic model for DH is shown in Table 1.

It is important to examine the fitted model to ensure adequate approximation is provided to the true system (Myers and Montgomery, 2002). Table 1 shows the model "F-value" of 19.74, which implies that the model was significant. There was only a 0.01% chance that a 'Model F-Value' this large could occur due to noise in the experiments. Meanwhile, the values of "Prob> F" which less than 0.05 indicated that A, B, C, D, AB, AC, A^2, B^2, and D^2 all were significant model terms for the response of DH. Based on Table 1, the "p value" for "Lack of Fit" of 3.97 implied that the model was more fitted to the data after model reduction. A good fit means that the generated models adequately explained the data variation and significantly represented the actual relationships between the reaction parameters.

Table 2 shows the summary statistics of model for DH after model reduction.

The goodness of the model can also checked by determination of coefficient that is "R-Squared". In this case, the value for "R-Squared" of the model was 0.8988, which indicated that the model was suitable for adequately representing the real relationships among the selected reaction variables.

Based on the sequential model sum of squares, the appropriate models were selected based on the highest-order polynomial where the additional terms where significant. The application of RSM offers, on the basis of parameter estimate, an empirical relationship between the response variable and the test variable under consideration (Rastogi and Rashmi, 1999). By applying multiple regressions analysis on the experimental data, the response variable and the test variables was generated by the following model equation for DH of EBN.

The model equation for DH of Protamex®-catalyzed enzymatic hydrolysis using in terms of coded factors was as follows:

Degree of hydrolysis (DH) = + 24.60 + 0.92 *A + 1.96 *B + 2.06 *C – 2.58 *D + 2.03 *AB + 1.67 *AC + 5.30 *A^2 – 3.33 *B^2 -5.27 *D^2

Where, A = Temperature, B = Enzyme Concentration, C = Hydrolysis time, D = pH.

Table. 1. ANOVA table for response surface reduced quadratic model for degree of hydrolysis

Source	Sum of Squares	Df	Mean Square	F value	p-value Prob>F	
Model	581.54	9	64.62	19.74	< 0.0001	significant
A-Temperature	15.35	1	15.35	4.69	0.0426	
B-Enzyme concentration	69.50	1	69.50	21.23	0.0002	
C-Hydrolysis time	76.14	1	76.14	23.26	0.0001	
D-pH	119.71	1	119.71	36.57	< 0.0001	
AB	65.81	1	65.81	20.11	0.0002	
AC	44.66	1	44.66	13.64	0.0014	
A^2	79.84	1	79.84	24.39	< 0.0001	
B^2	31.51	1	31.51	9.63	0.0056	
D^2	78.74	1	78.74	24.05	< 0.0001	
Residual	65.47	20	3.27			
Lack of Fit	60.40	15	4.03	3.97	0.0676	Not significant
Pure Error	5.07	5	1.01			
Cor total	647.01	29				

The final equation in terms of actual factors given by Design Expert software was:

Degree of Hydrolysis = + 105.54210 – 5.84942 *A + 1.81915 *B – 3.14823 *C + 18.46027 *D + 0.20281 *AB + 0.083531 *AC + 0.053034 *A^2 – 3.33159 *B^2 – 1.31665 *D^2

Table. 2. Summary statistics of model for degree of hydrolysis after model reduction

Std. Dev	1.81	R-Squared	0.8988
Mean	22.63	Adj R-Squared	0.8533
C.V.	8.00	Pred R-Squared	0.7739
PRESS	146.27	Adeq Precision	19.720

The equation in terms of coded terms is useful for identifying the relative impact of the factors by comparing the factor coefficients (Stat-Ease, Inc., 2002). The equations shows that the most significant factors were given by pH, followed by temperature, then hydrolysis time and finally enzyme concentration. The resulting equation shows that the significant interaction effect obtained found in this study was between temperature and enzyme concentration (AB) and temperature and hydrolysis time (AC).

The suggested quadratic model in this study was similar to a previous study on optimization of DH from fish soluble concentrate (Nilsang et al., 2005), visceral waste protein of beluga sturgeon *Husohuso* (Ovissipour et al., 2009), visceral waste of Catla (Bhaskar and Mahendrakar, 2008) and blood cockle (Amiza and Masitah, 2012). However, the quadratic model is not consistent with the study reported by Oon (2014) whereby she reported 2-factor interaction model for Alcalase®-catalyzed hydrolysis of EBN. This difference in model equation could be due to the differences in the enzymes used in hydrolysis as well as in the ranges of parameters used.

Response surface plots for interaction effect

Figure 1 shows the response surface plot of DH as affected by temperature and enzyme concentration.

The figure shows a synergistic effect towards DH is maximum at high temperature and high enzyme concentration. The antagonistic effect towards DH is observed at intermediate temperature and extreme enzyme concentration. This is in agreement with Silva et al. (2010) whom stated that more active sites are available on the enzyme at higher enzyme concentration, thus resulting in great cleavage of the peptide bonds, consequently, resulting in high DH. DH decreased with an increasing temperature (40°C to 50°C), then exhibited an increasing trend from the temperature of about 50°C to 60°C. For most enzymatic hydrolysis reactions, rate of hydrolysis increased when temperature increases. In this study, the lower percentage of DH at 40°C to 50°C may due to the insufficient energy provided for the Protamex® to react with the substrate as reported by for tuna viscera hydrolysis (Salwanee et al., 2013). This is also supported by Zheng et al. (2013) whom reported on blood cells hydrolysis whereby they stated that the theory of the Arrhenius activation energy was the key reason for the increased enzymatic reaction. On the contrary, Silva et al. (2010) found that DH is not significantly affected by temperature from 46°C to 64°C for Protamex®.

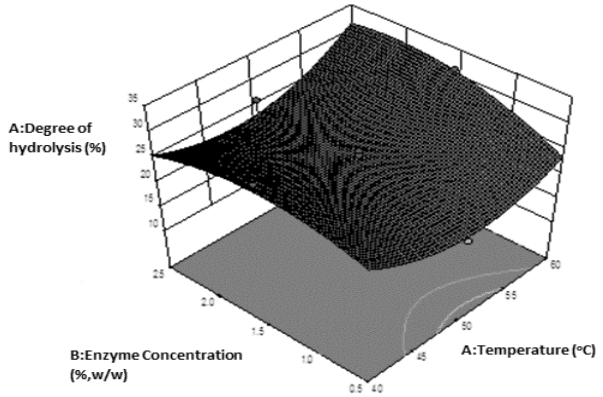

Fig. 1. Response surface plot for enzyme concentration and hydrolysis time on degree of hydrolysis of EBN

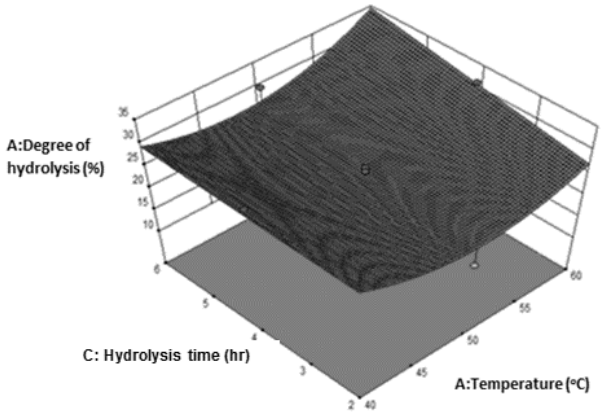

Fig. 2. Response surface plot for temperature and hydrolysis time on degree of hydrolysis of EBN

Figure 2 shows a synergistic effect towards DH is maximum at high temperature and high hydrolysis time.

The antagonistic effect towards DH is observed at intermediate temperature throughout the hydrolysis time used in this study. This is in agreement with Ovissipour et al. (2010) whom stated that an increase in DH is achieved by increase in reaction time.

The results obtained from Figure 1 and 2 showed that DH was higher at higher temperature (°C), enzyme concentration (%, w/w) and hydrolysis time (hr). This result was in agreement with the study done by Molla and Hovannisyan (2011) in the hydrolysis of beluga protein whereby increase in DH is achieved by increasing temperature, time, and enzyme concentration up to certain levels. Similar dependence between enzyme activity, temperature, and reaction time has been observed for hydrolytic reactions of food proteins using enzymes of microbial origin (Bhaskar et al., 2008; Diniz and Martin, 1997; Shahidi et al., 1995).

Optimization of degree of hydrolysis (DH)

Conditions for optimum response

The optimum condition suggested by Design Expert software was at temperature of 59.9°C, enzyme concentration of 2.0%, hydrolysis time of 5.4 hr and pH of 6.3. The maximum DH under this optimal condition was 34.11%. In general, for all the four independent factors, the optimum conditions generated by software were all in the range except temperature. However, the optimum condition (59.9°C) for temperature is still in the range recommended by the manufactures (Novozymes A/S). Bhd (2001) for Protamex® (35°C to 60°C).

To confirm the validity of the model, the protein hydrolysis was conducted under the predicted optimal conditions. Four parallel experiments were performed, and the mean DH obtained was 33.88%. A one-sample t-test shows that there was no significant difference between experimental DH value (33.88%) and the predicted DH value (34.11%), which shows that the model was suitable for estimation of the experimental value. Nilsang et al. (2005) and Cao et al. (2008) also reported that RSM has been successfully used to optimize the parameters affecting DH of protein.

In terms of optimum protein hydrolysis conditions, Oon (2014) found that the optimum condition were temperature of 64.99°C, enzyme concentration of 2%, pH of 9.46 and hydrolysis time of 2.99 hr. Only optimum enzyme concentration is similar for EBN hydrolysis using Alcalase® and Protamex®. However, Alcalase® gave maximum DH at higher temperature and pH and lower hydrolysis time compared to that of Protamex®.

Comparison of proximate composition of soaked cleaned raw edible bird's nest and its lyophilized protein hydrolysate powder

To study the effect of EBN hydrolysis by Protamex®, proximate analysis was carried out on the soaked cleaned raw EBN and its lyophilized hydrolysate powder. The EBN hydrolysate was prepared using the optimum hydrolysis conditions suggested in this study (temperature of 59.9°C, pH of 6.3, Protamex® concentration of 2% and hydrolysis time of 5.4 hr). The proximate composition of the soaked cleaned EBN and lyophilized EBN hydrolysate powder are presented in Table 3 (stated as wet and dry basis).

Table. 3. Proximate composition of the soaked cleaned edible bird's nest (EBN) and lyophilized EBN hydrolysate powder prepared under optimum condition (stated as wet and dry basis)

Material	Moisture (%)	Protein (%)	Carbohydrate (%)	Fat (%)	Ash (%)
Soaked cleaned EBN (wet basis)	90.91±0.14	5.39±0.04	2.54±0.24	0.90±0.10	0.25±0.05
Soaked cleaned EBN (dry basis)	-	59.35±0.49	27.99±2.67	9.93±0.11	2.74±0.50
Lyophilized EBN hydrolysate powder (wet basis)	8.04±0.65	60.84±0.33	25.76±0.42	1.21±0.03	4.36±0.07
Lyophilized EBN hydrolysate powder (dry basis)	-	66.16±0.36	28.01±0.46	1.31±0.03	4.74±0.08

The lyophilized EBN hydrolysate has lower moisture content (8.04%) as compared to the moisture contained in soaked cleaned raw EBN (90.91%). The low moisture content lyophilized EBN hydrolysateis due to freeze drying process. Protein content of lyophilized EBN hydrolysate samples (in wet basis) is 60.84%. This value is slightly lower than that of lyophilized EBN hydrolysate prepared under Alcalase®-catalyzed protein hydrolysis (65.03% (wet basis)) (Oon, 2014). The difference in protein content between both EBN hydrolysate samples could be due to the different proteinase used in the hydrolysis process. Beak and Cadwallader (1995) also reported that Alcalase® gave higher protein recovery compared to Protamex® in crayfish processing by-product hydrolysis. Based on dry basis, the protein content of soaked cleaned EBN was 59.35%, while the protein content of the lyophilized EBN hydrolysate powder was 66.16%. This result shows that there is a slight difference in protein content between the raw materials (soaked cleaned EBN) and its hydrolysate. According to Ma and Liu (2012) and Marcone (2005), the protein content of dry raw EBN (in wet basis) was in the range of 42% to 63%. This shows that the protein content of EBN samples in this study were slightly higher compared to previous study by Ma and Liu (2012) and Marcone (2005). The difference in protein content could be due to differences in the source of EBN, cleaning extent and moisture content between the samples. The protein content of EBN hydrolysate in this study was higher than that of spray-dried Tilapia flesh hydrolysate (37.7- 49.6%) (Azizah et al., 2001) and Catla viscera hydrolysate (14.25%) (Bhaskar et al., 2008) but lower than those of sardine, mackerel and white croacker hydrolysate (82.7–85.1%) (Arvanitoyannis and Kassaveti, 2008).

In dry basis, it was found that the ash content in the soaked cleaned EBN was higher than EBN hydrolysates which were 2.74% and 4.74%, respectively. The increase in ash content may due to addition of NaOH to adjust the pH to alkaline range prior to enzymatic hydrolysis. Amiza and Masitah (2012) and Severin and Xia (2006) have reported that the addition of NaOH during the adjustment of pH before enzymatic hydrolysis caused an increased in ash content. The ash content in EBN hydrolysates (4.36%; wet basis) was higher compared to ash content in EBN hydrolysate catalysed by Alcalase® (Oon, 2014) (2.63%; wet basis).

Meanwhile, the carbohydrate content of soaked cleaned EBN (in dry basis) 27.99% was quite similar to EBN hydrolysate (28.01%). Since the carbohydrate content was calculated by difference method, it may directly affected by the other proximate component of EBN. The carbohydrate content in this study (25.76%; wet basis) is consistent with values reported by Ma and Liu (2012) and Marcone (2005). Thus, this study shows that lyophilized EBN hydrolysate powder prepared using Protamex® gave higher ash content and lower fat content, while protein and carbohydrate content did not change much as compared to raw EBN. The fat content (dry basis) in soaked cleaned EBN (9.93%) was higher compared to EBN hydrolysate (1.31%). The loss of fat from EBN could occur during soaking, storage, heat treatment, hydrolysis and freeze-drying process (Oon, 2014). Low fat content is desirable in protein hydrolysate because it will increase the stability of the hydrolysate toward lipid oxidation and enhance the hydrolysate stability (Ovissipour et al., 2009; Nilsang et al., 2005). The fat content in EBN hydrolysate (1.21%; wet basis) was higher compared to fat content reported by Oon (2014) (0.21%; wet basis). Previous study on EBN has reported that the fat content in EBN was in the range of 0.14% to 1.28% (Ma and Liu, 2012; Marcone, 2005).

Conclusion

The relationship between four variables of enzymatic hydrolysis of EBN using Protamex® can be predicted using quadratic model. Optimum condition to obtain maximum DH was found at temperature of 59.9°C, pH of 6.3, Protamex® concentration of 2% and hydrolysis time of 5.4 hr. It was found that the lyophilized EBN hydrolysate powder prepared under the optimum condition gave similar protein and carbohydrate content, but lower fat content and higher ash content as compared to cleaned raw EBN.

Contribution of Authors

Amiza MA: Author of the manuscript and advised on experimental design and technical aspect.
Khuzma D: Author of the manuscript and advised on technical aspect.
Kee CH: Author of the manuscript and conducted laboratory work.

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