OPTIMIZATION OF PROTEIN EXTRACTION FROM FREEZE DRIED FISH WASTE USING RESPONSE SURFACE METHODOLOGY (RSM)

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ABSTRACT

The present study embarked on the objective of optimizing protein extraction from freeze dried fish waste (FD-FW), sardine (Sardina pilchardus). Introduction of freeze drying prior to extraction was aimed at reducing the risk of protein loses in fish waste (FW) during frozen storage before the extraction process. Response surface methodology (RSM) was used to study the effect of independent variables, namely time (30-60 minutes), pH (7-11), rotation speed (100-300 rpm), and NaOH: substrate ratio (1-3) on protein extraction from FD-FW. From RSM-generated model, the optimum conditions for extraction of protein from FD-FW were identified to be at pH 10.56 in 48.61 minutes reaction time, with rotation speed of 104.77 rpm and NaOH: substrate ratio of 1.54. Predicted protein yield was 85.02 mg/ml while an experimental protein yield was 83.51 mg/ml as revealed by confirmatory studies.

***Keywords**: Sardine pilchardus; freeze drying; freeze dried fish waste; Response surface methodology.*

INTRODUCTION

Nowadays, fish processing industry, wet market and fish loading are looked upon as producers of worthless garbage by discarding a huge number of wastes which are parts of fish body. The fish waste (FW) is discarded without attempt for recovery. Without proper utilization, these wastes may cause environmental problems and for now, most of the FW is dumped as garbage or directly used as feedstuff. FW consists of fish head that constitute approximately 20% of the fresh water fish biomass, and are a rich source of protein and polyunsaturated lipids [2]. This solid waste has approximately the same protein content as fish flesh [3]. Although some amount of FW is being utilized today as feedstuff, a huge amount is still being discarded. The reason for introducing FW as feedstuff is due to good source of protein content in the FW [7,8,9]. However, using FW directly as feedstuff might be harmful for animal due to microbiological factor contributed by the composition of undesirable substances in the FW [6].

Before FW was being extracted in extraction process, FW was stored in freezer in order to accumulate all the FW from several FW producers. Storage of FW in freezer will affect the composition of the sample [10]. Noticeable composition (in dry matter) that showed reduction after undergo frozen storage were ash and protein [11]. In order to overcome this matter, freeze drying was introduced prior to the extraction process. The main reason on introducing freeze drying was to reduce the risk of protein denaturing. In freeze drying process, FW would be converted into dry form which in turn would enable it to be kept at room temperature rather than in freezer. Previous research on effect of different drying method onto antioxidant properties (AOP) showed that freeze drying had three main advantages which are, least decline in AOP composition but at the same time might show an enhancement of AOP after drying and remain stable for storage at room temperature [12].

In our present study, freeze dried fish waste (FD-FW) was being extracted in order to get protein by using alkaline method, which has been reported to be the most commonly used procedure for protein extraction. Another method that has been primarily used to extract protein is by enzymatic method. Studies have claimed that from both methods, a good protein yield from FW obtained only at more alkaline conditions [1]. In the alkaline method, the most common alkaline solutions used were sodium hydroxide and calcium

hydroxide. From previous studies, sodium hydroxide has shown higher percentage of total nitrogen in solution compared to calcium hydroxide. Besides that, extraction using sodium hydroxide would not affect the amount of extracted protein if any changes in temperature occurred [3].

Above all, several factors in the extraction process, such as time, pH, rotation speed and level of sodium hydroxide to substrate, may affect final properties of the extracted protein from FD-FW. When more than a few factors affect the desired responses in a certain process designs, response surface methodology (RSM) becomes an effectiveness tool for optimizing the process. The advantages of using RSM have been reported to include reduction in the number of experimental trials needed to evaluate multiple parameters, and the ability of the statistical tool to identify interactions. In addition to analyzing the effects of the independent variables, the experimental methodology also generates a mathematical model that describes the overall process [3].

With respect to the background described above, freeze drying was undertaken to reduce the amount of protein content loses in FW due to frozen storage before the extraction process, and the specific objective of the study was to optimize the parameters (time, pH, rotation speed and level of sodium hydroxide to sample) in extraction of protein from FD-FW.

MATERIALS AND METHODS

Fish waste

FW was supplied by Protigam Food Industries Sdn. Bhd., which contained fish head. FW was then minced using a standard electrical blender, Panasonic.

Pre-treatment process

Before undergoing freeze drying, the FW was treated with petroleum ether for the purpose of fat removal. De-fatted FW were placed in glass container specifically designed for the freeze dryer.

Freeze drying process

The FW was freeze dried for about 24 hours using Labconco-freeze drying system. Afterwards, FD-FW was ground into powder form and placed in a closed container at room temperature.

Proximate composition of protein

Proximate composition of protein was carried out by Kjehdahl method using Kjeltec protein analyser.

Protein extraction process

FD-FW was mixed with distilled water with a ratio of 1:10 (established after several preliminary experiments, data not shown) before the addition of sodium hydroxide [5]. Amount of sodium hydroxide added and the condition of extraction were based on different combinations, as shown in Table 1. 29 individual points were employed in the extraction process. The independent variables and their levels were selected based on previous studies. Extraction was done using a mixer (Wise Stire, Model HS30D). Following the extraction process, FD-FW solution was centrifuged (Sigma, Model 3K18) at 13000 rpm and 4^{o}C. The supernatant containing soluble protein was collected.

Protein determination in supernatant

Soluble protein in supernatant was determined using Bradford method.

Experimental design

Response Surface Methodology (RSM) was used in this study to determine the optimum conditions for the extraction of protein from FD-FW samples. The experimental design and statistical analysis were performed using Design Expert Software. The experiments were based on a box-behnken design with a quadratic model in order to study the combined effects of four independent variables (time, pH, rotation speed and level of sodium hydroxide to sample). These four independent variables were represented by X1, X2, X3 and X4, respectively. Each independent variable had 3 levels which were -1, 0 and +1, as shown in Table 2. The dependent variable was known as response function.

Table 1: Actual level of independent variables along with the observed values for the response variable, extracted protein (Y)

Run	Independent variables				Dependent variables
	X1	X2	X3	X4	Y
1	2.00	200.00	30.00	7.00	71
2	1.00	200.00	60.00	9.00	74.85
3	2.00	200.00	30.00	11.00	73.7
4	3.00	300.00	45.00	9.00	73.33
5	2.00	100.00	45.00	11.00	70.4
6	2.00	200.00	45.00	9.00	73.1
7	2.00	200.00	45.00	9.00	73.1
8	1.00	100.00	45.00	9.00	69.5
9	2.00	200.00	45.00	9.00	73.1
10	2.00	100.00	30.00	9.00	70.85
11	1.00	300.00	45.00	9.00	73.33
12	1.00	200.00	30.00	9.00	73.48
13	2.00	200.00	45.00	9.00	73.1
14	2.00	100.00	60.00	9.00	69.48
15	1.00	200.00	45.00	7.00	80.7
16	3.00	200.00	45.00	11.00	73.65
17	2.00	200.00	45.00	9.00	73.1
18	2.00	100.00	45.00	7.00	70.9
19	2.00	300.00	45.00	11.00	75.28
20	3.00	200.00	30.00	9.00	73.48
21	2.00	300.00	30.00	9.00	84.93
22	2.00	200.00	60.00	11.00	74.93
23	2.00	300.00	60.00	9.00	80.48
24	3.00	200.00	45.00	7.00	70.7
25	3.00	200.00	60.00	9.00	74.85
26	1.00	200.00	45.00	11.00	73.65
27	3.00	100.00	45.00	9.00	69.5
28	2.00	200.00	60.00	7.00	87.75
29	2.00	300.00	45.00	7.00	60.93

X1: level of NaOH:sample, X2: rotation speed, X3: time, X4: pH, Y:extracted protein

Table 2: Independent variables and their coded levels used in RSM studies for optimizing extraction of protein from FD-FW

Factor	Levels		
	-1	0	+1
Level NaOH:sample % (X1)	1.00	2.00	3.00
Speed rotation, rpm (X2)	100	200	300
Time, min (X3)	30	45	60
pH (X4)	7	9	11

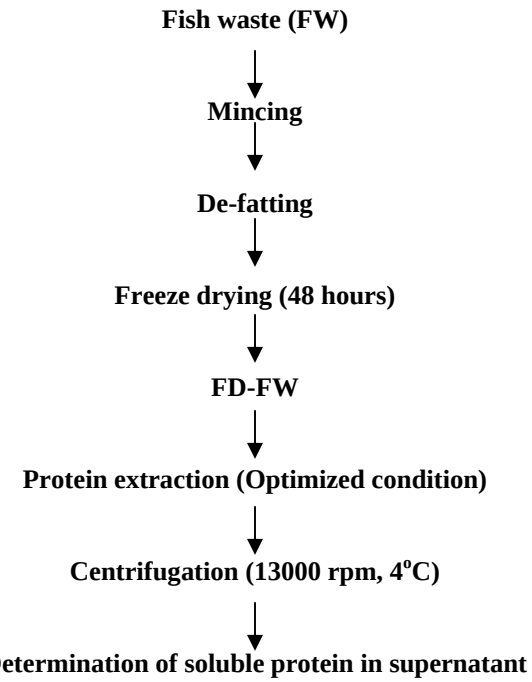

Figure 1: Flow sheet for the extraction of protein from freeze dried fish industry waste (FD-FW) under optimized condition.

RESULTS AND DISCUSSIONS

Proximate composition of the raw material and other intermediates in the study

Table 3: Protein composition of FW (g per 100 g)

	Protein	Fat
Raw FW	16.3	13.1
De-fat FW	15.5	8.3
Freeze dried FIW (FD-FW)	14.0	-[a]

[a] *not analyzed*

As can be seen in Table 3, the raw FW had a protein content of 16.3 g with a high amount of fat content. After having been de-fatted and freeze dried, the protein content of FW decreased slightly to 15.5 g and 14.0 g, respectively.

Fitting the models

The study utilized RSM to develop a prediction model for optimizing the extraction of protein from FD-FW. The experimental conditions and the corresponding response values from the experimental design are presented in Table 1. The independent and dependent values were analyzed to obtain a regression equation that could predict the response within the given range. The regression equation for protein extraction is as follows:

$$\text{Protein extracted, mg/ml} = 61.68 - 1.50\,X2 - 1.16\,X3 + 4.68\,X4 + 2.41\,X2X3 - 9.47\,X2X4 - 0.73\,X3X4 + 0.97\,X1^2 + 8.93\,X2^2 + 1.70\,X3^2 + 4.74\,X4^2 \quad (1)$$

The plot of experimental values of extracted protein (mg/ml) versus those calculated from Eq. 1 indicated a good fit, as presented in Figure 2. Colour differences in the fit plotted indicated the level of extracted protein which represents red as the highest extracted protein while narrow down to blue colour was the lowest extracted protein. The results of analysis of variance (ANOVA) gave a coefficient of determination (R^2) of 0.8980; indicating the adequacy of the applied model. The probability (P) of the regression model significance was 0.001 which is less than 0.05 and the Model F-value was 8.80; implying that the model is significant. Therefore, the developed model could adequately represent the real relationship among the parameters chosen.

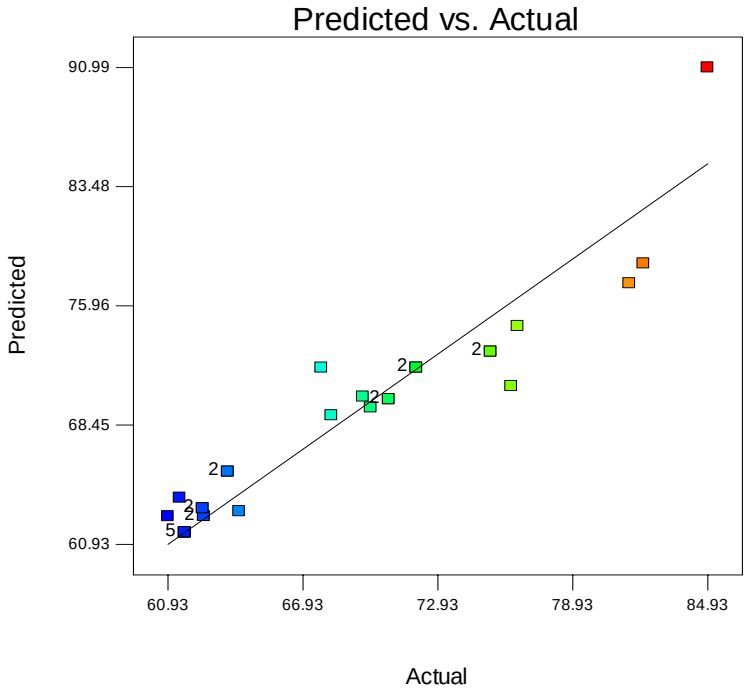

Figure 2: Correlation between calculated and experimentally extracted protein

Effects of independent variables on responses

The response surface graph for extracted protein from FD-FW as a function of time and pH, is shown in Figure 3. The graph indicates that the amount of extracted protein increased up to 70 mg/ml before reducing considerably with the decrease in pH. The extracted protein was more pronounced at the high alkaline side. Similarly with Batista (1999), for both protein extractions using NaOH from hake and monk

fish, extracted protein was at the highest solubility in the alkaline solution. In Figure 4, amount of extracted protein showed a similar decreasing pattern with decrease in time. It shows that reaction time affect the protein yield more significantly compared to the level of NaOH:sample.

Figure 5 and 6 represent the pattern of changes in protein yield as affected by pH values. As evident in Figure 5, the protein yield increased up to 72 mg/ml at higher pH and clearly reduced with decreasing pH. Figure 6 concludes that the maximum amount of protein extraction occurred at the highest pH; approximately at pH 11. Rotation speed did influence the extraction, where the protein yield increased with increasing speed of rotation.

For each graph discussed above, all other independent variables not depicted in graph were positioned at the center of their levels. From the graphs, it was clearly shown that pH value played a major role in protein extraction compared to other independent variables.

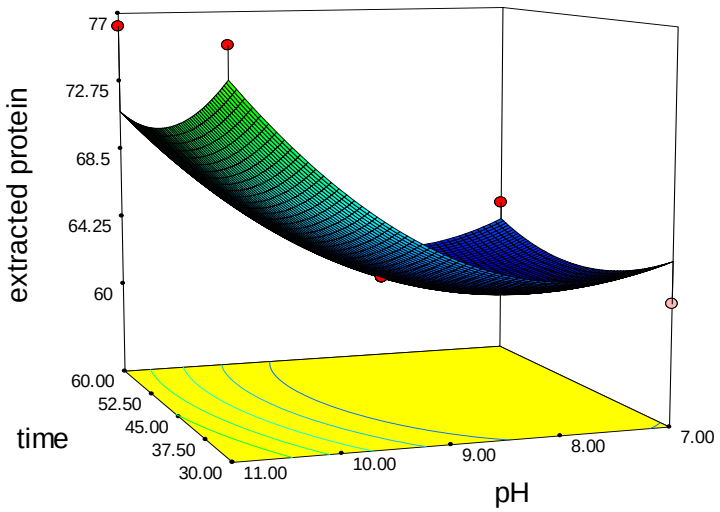

Figure 3: Response surface graph for amount of extracted protein as a function of time (minute) and pH during protein extraction from FD-FW (rotation speed and level of NaOH:sample at the center of their levels)

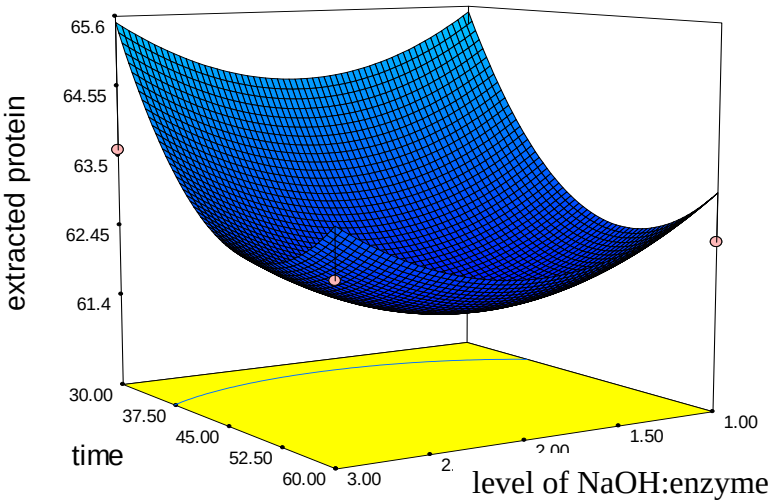

Figure 4: Response surface graph for amount of extracted protein as a function of time (minute) and level of NaOH:sample during protein extraction from FD-FW (rotation speed and level of pH at the center of their levels)

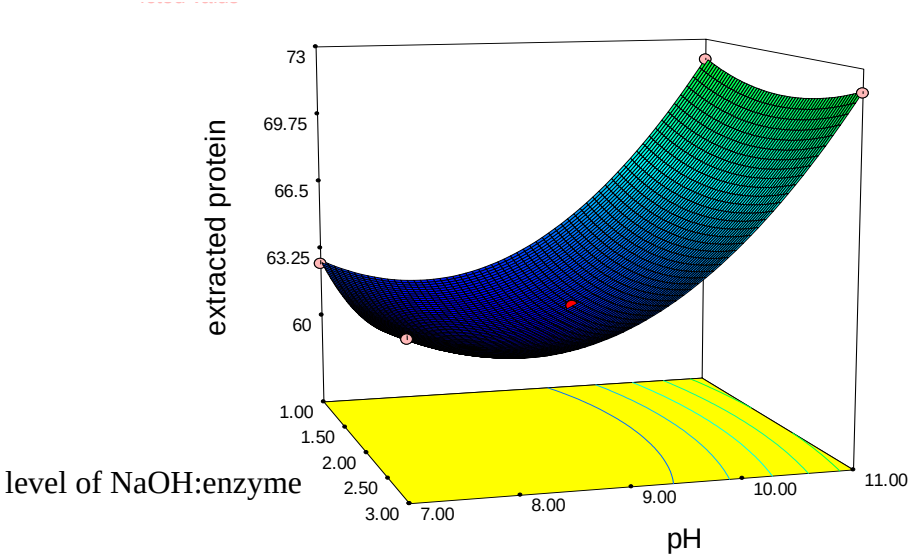

Figure 5: Response surface graph for amount of extracted protein as a function of level of NaOH:sample and pH during protein extraction from FD-FW (rotation speed and level of time at the center of their levels)

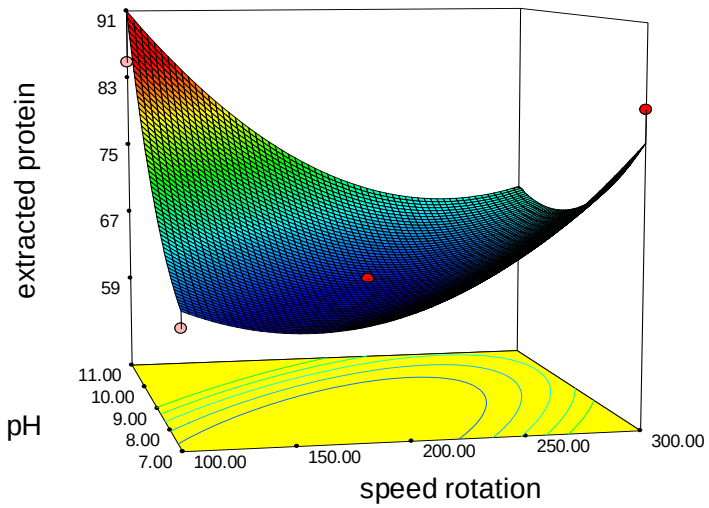

Figure 6: Response surface graph for extracted protein as a function of pH and rotation speed (rpm) during protein extraction from FD-FW (level of NaOH:sample and time at the center of their levels)

Optimum conditions for the extraction of FD-FW and model verification

From the model, optimum conditions for extraction of protein from FD-FW obtained were as presented in Table 6. Under the optimum conditions, a maximum yield of 85.02 mg/ml protein was extracted at level of NaOH:sample of 1.54, rotation speed 104.77, time 48.61 min and pH 10.56. The suitability of the model equation for predicting the optimum response value was tested by additional independent experiments using the recommended optimum conditions (Table 6). The results indicated that the amount of experimentally extracted protein was not significantly different from the predicted protein value.

Table 6: Optimum conditions for extraction of protein from FD-FW

Optimum condition				Extracted protein (mg/ml)	
Level of NaOH:sample	Speed rotation	Time	pH	Predicted value	Experimental value
1.54	104.77	48.61	10.56	85.02	83.51

CONCLUSIONS

The conditions of protein extraction (level of sodium hydroxide to sample, pH, time and rotation speed) were optimized using RSM to improve protein extraction from FD-FW. From the RSM results, the optimum conditions of level of NaOH:sample (1.54), speed rotation (104.77), time (48.61 min) and pH (10.56) were obtained with the highest predicted protein value of 85.02 mg/ml. The predicted protein value was subsequently confirmed by verification experiments. Under the optimum conditions, a protein yield of 83.51 mg/ml was obtained, which was not significantly different from the predicted value. For further study, these results can be compared with extraction of fish waste without freeze drying process on the FW and also with other types of drying on the FW before the extraction process in order to decrease the possibility of protein loses during frozen storage.

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