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Optimization of Protein Extraction from Fish Waste using Response Surface Methodology

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Abstract: The aim of present study was to optimize protein extraction from Fish Waste (FW), sardine (*Sardina pilchardus*) using enzymatic method. In this study, enzymatic method involved two combinations of enzymes which were alcalase and protamex. Response Surface Methodology (RSM) was used to study the effect of independent variables, namely temperature (35-55°C), rotation speed (100-300 rpm), time (60-1440 min) and enzyme:substrate ratio (0.5-1.5) on protein extraction from FW. From RSM-generated model, the optimum conditions for extraction of protein from FW were identified to be at temperature 35°C in 1429 min reaction time, with rotation speed of 171 rpm and enzyme:substrate ratio of 1.50. At the optimum conditions, predicted protein yield in the extraction process was 80.75 mg mL^{-1}.

Key words: *Sardine pilchardus*, fish waste, enzymatic method, response surface methodology

INTRODUCTION

In Malaysia, 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 fish waste is dumped as garbage or directly used as feedstuff. Fish waste consists of fish head that constitute approximately 20% of the fresh water fish biomass and are a rich source of protein and polyunsaturated lipids (Bhaskar, 2008). This solid waste has approximately the same protein content as fish flesh (Batista, 1999). Although, some amount of fish waste is being utilized today as feedstuff, a huge amount is still being discarded. The reason for introducing fish waste as feedstuff is due to good source of protein content in the fish waste (Arnesen and Gildberg, 2007; Bhaskar *et al*., 2007; Gildberg, 2001). However, using FW directly as feedstuff might be harmful for animal due to microbiological factor contributed by the composition of undesirable substances in the fish waste (Garcia *et al*., 2005).

In this study, FW was being extracted in order to get protein by using enzymatic method. Enzymes used to produce fish protein hydrolysate have at least one common characteristic; they should be food grade and if they are of microbial origin, the producing organism has to be non-pathogenic. The choice of substrate, protease employed and the degree to which the protein gets hydrolysed generally affects the physicochemical properties of the resulting hydrolysates (Mullally *et al*., 1995). For this study, two enzymes (alcalase and protamex) were combined for the investigation. The choice of the two enzymes was made based on the fact that these enzymes have already been widely used in the food industry for various applications and are proved to be safe (Beg *et al*., 2003). Both enzymes are belongs to protease enzyme which produced a significantly higher extraction yield. Alcalase and protamex are alkaline bacterial protease produced from *Bacillus*, have been proven to be among the best enzymes used in the preparation of fish protein hydrolysate by many researchers (Hoyle and Merrit, 1994).

Above all, several factors in the extraction process, such as extraction time, temperature, rotation speed and level of enzyme to substrate, may affect final properties of the extracted protein from 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. Experimental design technique is a very useful tool for this purpose as it provides statistical models which help

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in understanding the interactions among the parameters that have been optimized (Hameed *et al*., 2009). 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 (Batista, 1999).

With respect to the background described above, the specific objective of the study was to optimize the parameters (extraction time, temperature, rotation speed and level of enzyme to sample) in the extraction of protein from FW using combination of two enzymes which were alcalase and protamex.

MATERIALS AND METHODS

Fish waste: Fish waste was supplied by Protigam Food Industries Sdn. Bhd., which contained fish head. Fish waste was then minced using a standard electrical blender, Panasonic and treated with petroleum ether for the purpose of fat removal. De-fatted FW were placed in container for further analysis.

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

Protein extraction process: Enzyme solution was prepared with a ratio of 1:1 between alcalase and protamex. Amount of enzyme solution added to the sample and the condition of extraction were based on different combinations programmed by RSM. From RSM, 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, FW solution was centrifuged (Sigma, Model 3K18) at 13000 rpm and 4°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 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 (temperature, level of enzyme: sample, speed rotation and extraction time). 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 1. The dependent variable was known as response function.

Table 1: Independent variables and their coded levels used in RSM studies for optimizing extraction of protein from FW

	Levels		
Factors	-1	0	+1
Temperature (°C) (X1)	35	45	55
Enzyme:sample (w/w) (X2)	1	2	3
Speed rotation (rpm) (X3)	100	200	300
Extraction time (min) (X4)	60	720	1440

RESULTS AND DISCUSSION

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

As can be seen in Table 2, the raw FW had a protein content of 16.3 g with a high amount of fat content. After have been de-fatted, the protein and fat content of FW decreased slightly to 15.5 and 8.3 g, respectively.

Fitting the models: The relationship between four variables (level of enzyme:sample, speed rotation, extraction time and temperature) and the important process response (extracted protein) for protein extraction process was analyzed using Response Surface Methodology (RSM). Significance model terms are desired to obtain a good fit in a particular model (Ghafari *et al*., 2009). The study utilized RSM to develop a prediction model for optimizing the extraction of protein from FW. The experimental conditions and the corresponding response values from the experimental design are presented in Table 2. 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:

$$\text{Protein extracted (mg mL}^{-1}) = 69.08 - 2.85X1 + 3.95X2 - 0.31X3 + 1.47X4 + 1.34X1X2 + 4.47X1X3 - 6.81X1X4 + 3.63X2X3 + 1.93X2X4 + 0.88X3X4 + 0.85X12 + 0.22X22 - 1.50X32 - 5.02X42 \quad (1)$$

Effects of independent variables on responses: The response surface graph for extracted protein from FW as a function of speed rotation and level of enzyme to sample is shown in Fig. 1. The graph indicates that the amount of extracted protein increased up to 76 mg mL^{-1} before

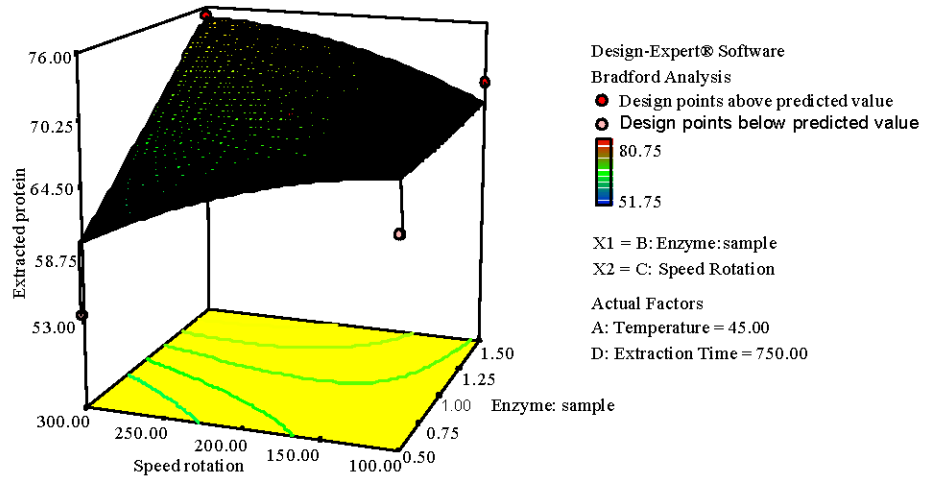

Fig. 1: Response surface graph for amount of extracted protein as a function of speed rotation and level of enzyme:sample during protein extraction from FW (extraction time and temperature at the center of their levels)

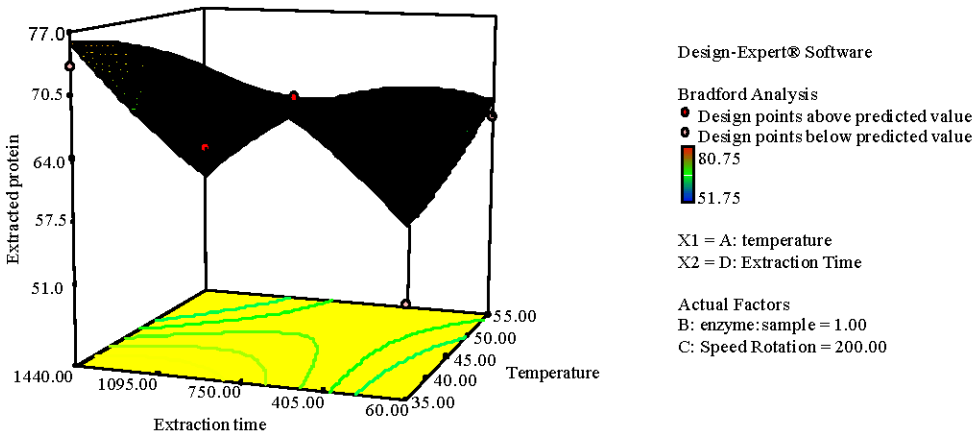

Fig. 2: Surface graph for amount of extracted protein as a function of extraction time and temperature during protein extraction from FW (rotation speed and level of enzyme:sample at the center of their levels)

Table 2: Protein composition of FW (g/100 g)

Fish waste	Protein	Fat
Raw FW	16.3	13.1
De-fat FW	15.5	8.3

reducing considerably with the decrease in level of enzyme to sample. The extracted protein was more pronounced at the high level of enzyme. In Fig. 2, amount of extracted protein showed a decreasing pattern with increase in temperature and at the same time increase in extraction time. It also showed an increasing pattern of extracted protein yield with the increase in extraction time at the lower temperature.

Figure 3 and 4 represent the pattern of changes in protein yield as affected by extraction time. In Fig. 3, the plots are approximately symmetrical in shape with circular contour. Rotation speed did influence the extraction, where the protein yield increased with increasing in the speed of rotation. Figure 4 concludes that the higher amount of extracted protein occurred at the longest extraction time and at the highest level of enzyme to sample. 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 level of enzyme to sample and extraction time values played a major role in protein extraction compared to other independent variables.

Optimum conditions for the extraction of FW and model verification: The influence of temperature, speed rotation, extraction time and level of enzyme to sample, on the

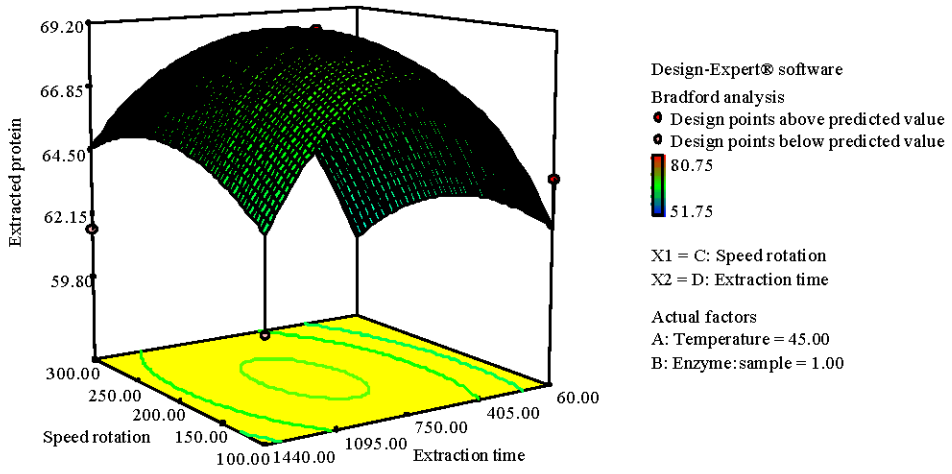

Fig. 3: Response surface graph for extracted protein as a function of speed rotation and extraction time during protein extraction from FW (level of enzyme:sample and temperature at the center of their levels)

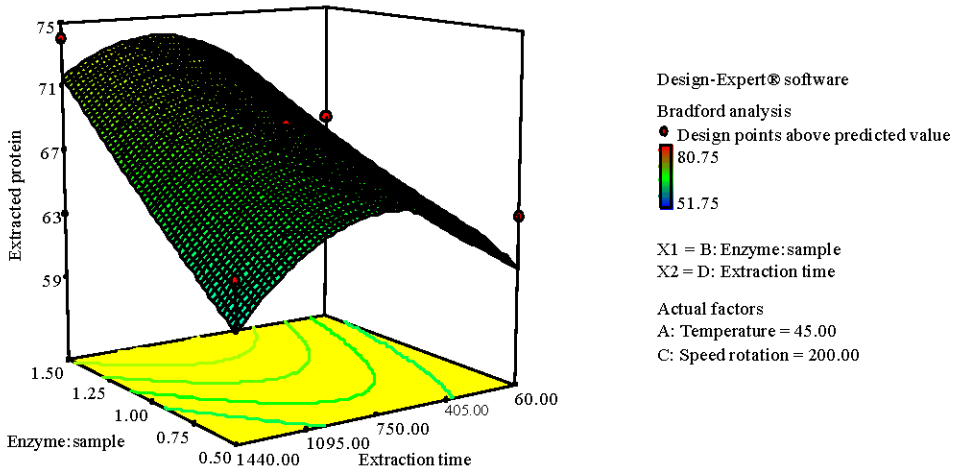

Fig. 4: Response surface graph for amount of extracted protein as a function of level of enzyme:sample and extraction time during protein extraction from FW (rotation speed and temperature at the center of their levels)

extracted protein using combination of alcalase and protamex enzymes was determined using Box-behnken design as mentioned in the previous section. From the model, under the optimum conditions, a maximum yield of 80.75 mg mL^{-1} protein was extracted at level of enzyme:sample of 1.50, rotation speed 171.24, extraction time 1429 min and temperature of 35°C.

CONCLUSION

The conditions of protein extraction (level of enzyme to sample, speed rotation, extraction time and temperature) were optimized using RSM to improve protein extraction from FW using combination of alcalase and protamex enzymes. From the RSM results, the optimum conditions of level of enzyme: sample (1.50), speed rotation (171.24), extraction time (1429 min) and temperature (35°C) were obtained with the highest predicted protein value of 80.75 mg mL^{-1}. For further study, these results can be compared with extraction of fish waste by using single enzyme or combination of enzyme in different stage of extraction.

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