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Abstract. *Hermetia illucens* or black soldier fly larvae (BSFL) have recently been used as an alternative source of protein in the processing of feed for various types of animals. Monogastric animals such as broiler, however, unable to completely consume all nutrient sources upon ingestion of the meal. The protease was then developed and used to treat the BSFL so that protein can be converted into amino acids, thereby enhancing the absorption of nutrients from broiler. In this analysis, Response Surface Methodology (RSM) was used to investigate the optimum condition in obtaining the highest decrease in protein content (%) of the BSFL. A Central Composite Model (CCD) model with three variables: water volume (5-8 mL), protease volume (100-300 µL) and incubation time (30-90 min) was used to investigate the response variable, which was a decrease in the protein content (%). At the end of this experiment, optimum conditions were established as 5 mL of water, 100 µL of protease and 30 minutes of incubation time to treat BSFL to achieve a decrease in protein content of 10.5266%.

1. Introduction

Animal feed (soybean meal, fishmeal and animal meal) accounts for about 60-70% of the total cost of production. The price of animal feed ingredients, especially fishmeal, has risen since the price has tripled from 2000 to 2015 [1]. Insects were recognised as one of the possible sources of protein for both human food and animal feed [2]. Black soldier fly larvae (BSFL), *Hermetia illucens* is a species that has gained significant popularity as feed relative to the other species of insects. A broad range of feed sources, e.g., from kitchen waste [5,6], dairy manure [5], chicken manure [6] as well as human faeces [3,9] is some of the places these insects can feed and grow. The larva is considered to have a high nutrient level of up to 35% of fat and 42.5% of protein content, depending on their diet [8].

In animal production, increasing feed costs, especially for protein sources and environmental pollution concerns, have urged nutritionists to use enzymes in poultry diets [9]. The use of protease as an enzyme in animal feed has recently gained significant interest to improve dietary protein hydrolysis. In a previous study, protease was found to be used extensively in the feeding industry as it can help to boost the growth of animals, including the digestibility of broilers against protein [10]. Approximately, one-fourth of the diet being fed cannot be digested by poultry due to indigestible deleterious factors that somehow obstruct the digestive process and inhibit the growth of the animal without any of the enzymes involved to degrade those components of the feed. Therefore, Thermostable Alkaline Protease Enzyme (TAPzyme) was introduced to treat the BSFL in enhancing the efficiency of the digestion and absorption of the nutrient as it could hydrolyse protein in detergent and dehairing applications for animals [14,15].

2. Materials and Methods

2.1 Preparation of protease

The microorganisms were thoroughly screened on Skim Milk Agar (SMA) containing (g/L) at different temperatures (50, 55 and 60 ℃): nutrient agar (g/l), 12.6 and skim milk powder, 6.0. Isolates showing only positive results (with clearing zone) on the SMA plates were further checked in liquid medium for their enzyme output. The organisms were preserved at 4 ℃ and placed on the nutrient agar plates. The stock was held at 80 ℃ in 15% of glycerol. The protease production procedure has been published elsewhere [13].

2.2 Statistical Analysis

2.2.1 Experimental Design using Response Surface Methodology (RSM). The lower (-1) and higher (+1) levels of three variables, which are the volume of water (5 and 8 mL), protease volume (100 and 300 µL) and incubation period (30 and 90 minutes), were inserted into Design Expert Software version 11.

2.2.2 Protein Analysis. Quantification of protein was determined by performing Bradford assay [14]. The absorbance values were measured at wavelength 595 nm.

2.3 Optimisation Studies

Optimisation studies were applied by using interaction plot, 2D contour plot and 3D response surface graph, which was generated by the Design Expert Software version 11.

3. Results and Discussion

3.1 Protein Analysis

From Table 1, Formulation 7 (run 7) demonstrates the highest decrease in protein content with a value of 11.78% followed by Formulation 6 and 10 (run 6 and 10) with a decrease of 9.24% and 8.41% respectively. Besides, formula 20 (run20) indicates the lowest protein content drop, which was just 0.36%. In a previous report, prepupae's total protein content was found to be 41.2%, where the stage of insect development immediately after the larvae [1]. As for the most significant reduction in protein content seen in Formulation 7 (run 7), 11.78% of 41.2% is 28.59%.

Table 1. Twenty Runs of BSFL Formulation Generated by RSM

Run	Volume of water (mL)	Volume of protease (µL)	Time of incubation (min)	Decrease in protein content (%)
1	5	300	90	5.29
2	6.5	200	60	1.07
3	6.5	300	60	1.79
4	8	200	60	1.68
5	5	200	60	2.16
6	8	100	30	9.24
7	5	100	30	11.78
8	8	300	90	6.96
9	6.5	200	60	0.60
10	5	300	30	8.41
11	6.5	200	60	0.60
12	8	300	30	6.96

Table 1. (continue)

Run	Volume of water (mL)	Volume of protease (µL)	Time of incubation (min)	Decrease in protein content (%)
13	5	100	90	4.93
14	6.5	200	60	0.6
15	6.5	100	60	0.95
16	6.5	200	60	2.51
17	8	100	90	7.32
18	6.5	200	90	3.10
19	6.5	200	60	2.27
20	6.5	200	30	0.36

3.2 Development of Regression Model Equation for Decrease in Protein Content

Table 2. Model Summary Statistics for Decrease in Protein Content (%)

Source	Std. Dev	$\mathbf{R^2}$	Adjusted $\mathbf{R^2}$	Predicted $\mathbf{R^2}$	PRESS	
Linear	3.65	0.0478	-0.1307	-0.8054	404.22	
2FI	3.93	0.1019	-0.3126	-6.1039	1590.57	
Quadratic	1.76	0.8613	0.7365	0.1933	180.62	Suggested
Cubic	1.61	0.9310	0.7814	-62.3191	14177.16	Aliased

From Table 2 and 3, the obtained correlation coefficient (R^2) was 0.8613, which is lower but close to 1.00. A more robust empirical model fits the actual data when R^2 reaches the value of 1.00, while a lower value of R^2 implies less importance to the dependent variables in the model [15]. The value of R^2 was 0.8613, and this means that 86.13% of the variability in the dependent variable may be explained. In comparison, the model would be unable to explain just 13.87% of the total variance. This interest can be inferred as an excellent indicator to use in this study. Furthermore, there was a massive disparity between the Predicted R^2 value and the Adjusted R^2 value 0.1933 and 0.7365, respectively. Predicted R^2 is a measure of how well the model predicts the value of the response [13]. The value of Predicted R^2 and Adjusted R^2 should be within an approximate of 0.20 to be fair. Nevertheless, the disparity obtained was more than 0.20 due to the occurrence of some possible problems or major impact as too many insignificant terms in the model or data.

Next, the value of Adequate Precision was 8.0253, as it is a valid signal to noise ratio as the most optimal value should be 4.00 or more [13]. Meanwhile, the Coefficient of Variation (CV) tests the repeatability and reproducibility of the models [16]. CV not exceeding 10% implies that the model can be considered relatively reproducible because the lower value of CV suggests a higher degree of accuracy and the reliability of the experimental values is a good ideal. Some errors may have existed, however, because the CV value derived from this analysis is higher than 10%, which was 44.85%.

Table 3. Standard Deviation and Quadratic Model for $\mathbf{R^2}$ for Decrease in Protein Content (%)

Std. Dev.	1.76	R^2	0.8613
Mean	3.93	Adjusted R^2	0.7365
C.V. %	44.85	Predicted R^2	0.1933
PRESS	180.62	Adequate Precision	8.0253

3.3 Statistical Analysis for Decrease in protein Content (%)

Table 4 displays the model's *F*-value is 6.90 while its p-value is 0.0029, which is statistically significant because the *p*-value is less than 0.05 ($p < 0.05$) [17]. This significance also means that due to noise, there were only 0.29% chances that the *F*-value model could be high. From the table, the result showed that coefficients A, B and C as well as the interaction of AB, AC, BC, A^2, B^2 and C^2 were considered as significant in Quadratic model for the decrease in protein content (%). The largest *F*-value recorded on variable C (incubation time), which is 2.70 indicates that it was the most crucial determinant that led to the BSFL's decrease in protein content (%).

Table 4. ANOVA Table for Surface Quadratic Model for Decrease in Protein Content (%)

Source	Sum of Square	df	Mean Square	***F*-Value**	***p*-value**	
Model	192.85	9	21.43	6.90	0.0029	significant
A-Volume of water	0.0168	1	0.0168	0.0054	0.9428	
B-Volume of protease	2.31	1	2.31	0.7451	0.4082	
C-Time of incubation	8.37	1	8.37	2.70	0.1316	
AB	0.0171	1	0.0171	0.0055	0.9423	
AC	8.10	1	8.10	2.61	0.1373	
BC	3.99	1	3.99	1.29	0.2834	
A^2	17.39	1	17.39	5.60	0.0395	
B^2	10.61	1	10.61	3.42	0.0942	
C^2	14.86	1	14.86	4.79	0.0535	

Figure 1 displays a data point which was distributed close to the straight line based on the figure. It implies that there were only subjective residual variations, and the data were normally distributed. At the other hand, Figure 2 displays the diagnostic plot of actual values versus expected protein content decreases (%), which could not be seen lying near to the straight line.

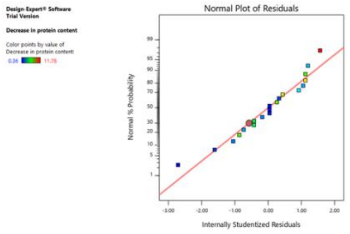

Figure 1. Normal Plot of Residual of Decrease in Protein Content (%)

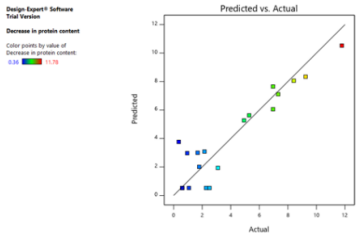

Figure 2. Diagnostic Plot of Actual Values versus Predicted Values of Decrease in Protein Content (%)

3.4 Effect of Volume of Water, Volume of Protease and Time of Incubation towards Decrease in Protein Content (%)

3.4.1 Effect of Volume of Water and Volume of Protease towards Decrease in Protein Content (%). Figures 3 and 4 demonstrate a 5% decrease in protein content when the water volume is between 5.0 mL and 5.6 mL whenever the protease amount is between 100 µL and 150 µL, respectively. It can also be shown that the protein content decrease is 1% as the water volume used is between 6.2 mL and 6.8 mL, while the protease volume is between 150 µL and 200 µL.

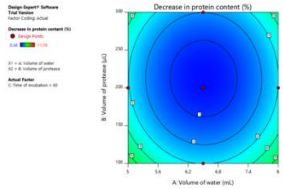

Figure 3. 2D Contour Plot of Interaction Effect of Volume of Water and Protease Volume towards Decrease in Protein Content (%)

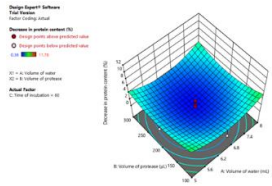

Figure 4. 3D Response Surface Graph of Interaction Effect of Volume of Water and Protease Volume towards Decrease in Protein Content (%)

3.4.2 Effect of Volume of Water and Time of Incubation towards Decrease in Protein Content. Figure 5 and 6 indicate that the decrease in protein content is 6% when the water volume is between 5.0 mL and 5.6 mL whenever the incubation period is between 30 minutes and 40 minutes. Meanwhile, the water volume is between 6.2 mL and 6.8 mL and the incubation period is between 40 minutes and 50 minutes cause the protein content decreases by 2%. This outcome indicates that low volume of water and less incubation period is more effective in reducing the higher percentage of protein content in BSFL as incubation is part of heat treatment and excessive heating or temperature may minimise the protein's accessibility to free amino acids [17].

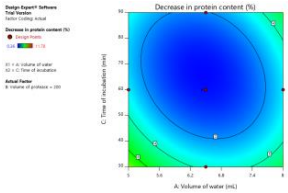

Figure 5. 2D Contour Plot of Interaction Effect of Volume of Water and Incubation Period towards Decrease in Protein Content (%)

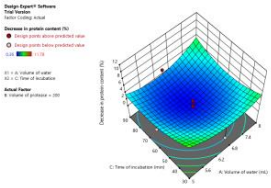

Figure 6. 3D Response Surface Graph of Interaction Effect of Volume of Water and Incubation Period towards Decrease in Protein Content (%)

3.4.3 Effect of Volume of Protease and Time of Incubation towards Decrease in Protein Content (%). Figure 7 and 8 showed that when the protease volume ranges from 100 µL to 150 µL, and the incubation period is between 30 minutes and 40 minutes, the protein content decreases by 6%. Unfortunately, when the protease volume is between 200 µL and 250 µL, and the incubation period is about 50 minutes and 60 minutes, the protein content decrease by 1%. As conclusion, less protease volume and incubation time would be enough to increase protein decrease (%) in BSFL as protease can aid to break the associated protein cell wall, which then facilitates the substrate's microbial use [18].

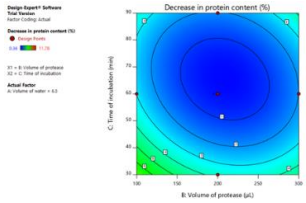

Figure 7. 2D Contour Plot of Interaction Effect of Protease Volume and Incubation Period towards Decrease in Protein Content (%)

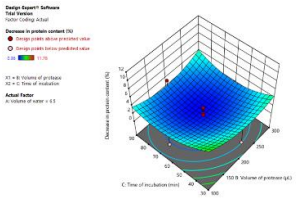

Figure 8. 3D Response Surface Graph of Interaction Effect of Protease Volume and Incubation Period towards Decrease in Protein Content (%)

3.4.4 Effect of Volume of Water and Volume of Protease towards Decrease in Protein Content (%) when Time of Incubation was Adjusted. A modification has been made by reducing the incubation time to 30 minutes (the lowest parameter), which lead to higher protein decrease up to 10%, as shown in Figures 9 and 10. This proved inability of protein to access or be converted to free amino acids after higher heating time or temperature is assisted [17].

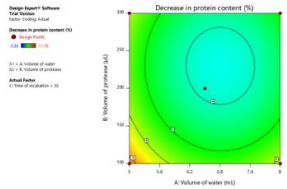

Figure 9. 2D Contour Plot of Interaction Effect of Volume of Water and Protease Volume towards Decrease in Protein Content (%) with Adjusted Incubation Period

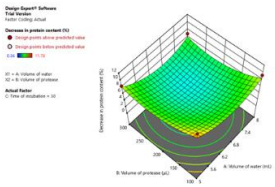

Figure 10. 3D Response Surface Graph of Interaction Effect of Volume of Water and Protease Volume towards Decrease in Protein Content (%) with Adjusted Incubation Period

3.4.5 Effect of Volume of Water and Time of Incubation towards Decrease in Protein Content (%) when Volume of Protease was Adjusted. The protease was adjusted to a lower (100 µL) to achieve a higher decrease in BSFL protein content (%). As seen in Figures 11 and 12, there was a 10% decrease in protein content after this modification as the negative impacts of inappropriate protease use on protein were evaluated by [18].

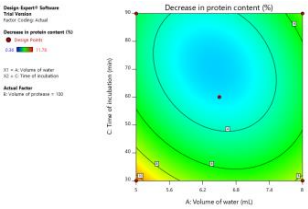

Figure 11. 2D Contour Plot of Interaction Effect of Volume of Water and Incubation Period towards Decrease in Protein Content (%) with Adjusted Protease Volume

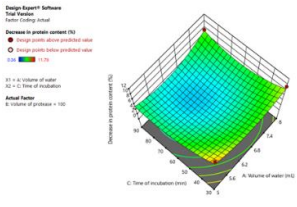

Figure 12. 3D Response Surface Graph of Interaction Effect of Volume of Water and Incubation Period towards Decrease in Protein Content (%) with Adjusted Protease Volume

3.4.6 Effect of Volume of Protease and Time of Incubation towards Decrease in Protein Content (%) when Volume of Water was Adjusted. Less water volume was found from these estimates to reduce the high percentage of protein content in the BSFL, which was 5% and 6%. Nevertheless, after modification by further reducing the volume of water to 5 mL, it was found that as shown in Figure 13 and 14, the decrease in protein content increased to 10%.

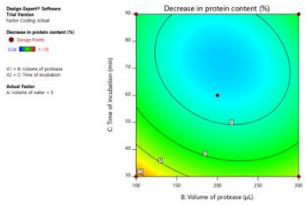

Figure 13. 2D Contour Plot of Interaction Effect of Protease Volume and Incubation Period towards Decrease in Protein Content (%) with Adjusted Volume of Water

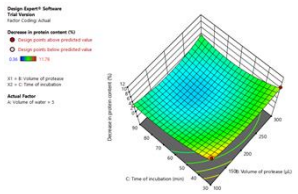

Figure 14. 3D Response Surface Graph of Interaction Effect of Protease Volume and Incubation Period towards Decrease in Protein Content (%) with Adjusted Volume of Water

4. Conclusion

In conclusion, this research presented new data and information on the optimisation of protease-treated Black Soldier Fly Larvae (BSFL) using Response Surface Methodology (RSM) and protein content before and after protease treatment by conducting protein analysis as an alternative source of protein seemed to be a promising feed ingredient for broiler chicken. Further research efforts are required to investigate in detail the effect of BSFL on broiler's growth performance through feeding trials. Additionally, the effects of BSFL on broiler's carcass characteristics and blood constituents should be studied carefully for both customer acceptance and marketing purposes.

Acknowledgements

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