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Optimizing protein yield in black soldier fly larvae through liquid milk waste utilization: A response surface methodology approach

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ABSTRACT

Black soldier fly (*Hermetia illucens*) larvae have emerged as an effective bioconversion agent capable of transforming organic waste into high-protein biomass that is suitable for animal feed applications. This study aimed to analyze the effectiveness of liquid milk waste feeding on the protein content of black soldier by optimizing feed amount and harvest age using response surface methodology (RSM). A laboratory-scale experiment was conducted using a central composite design (CCD) with 13 experimental runs. Four-day-old black soldier (30 g per treatment) were fed varying amounts of liquid milk waste (2.3–3.7 liters) and harvested at different ages (4–10 d). Environmental conditions were monitored daily, maintaining temperatures of 28.8–29.7 °C and humidity of 71–75%. Protein content was determined through laboratory analysis, and the data were analyzed using ANOVA and RSM optimization. The highest protein content achieved was 12.93% at a feed amount of 3.5 L with harvest on day 5, while the lowest was 10.18% at a feed amount of 3 liters feed with harvest on day 10. The quadratic model demonstrated an excellent fit ($R^2 = 0.9666$), with both feed amount ($p = 0.0001$) and harvest age ($p < 0.0001$) significantly affecting protein content. RSM optimization suggested optimal conditions of 3.4 liters feed with day-5 harvest, predicting 13.034% protein content. Validation experiments confirmed this prediction with a protein content of 12.96 %. In conclusion, liquid milk waste effectively supports black soldier protein production, demonstrating its potential for sustainable waste valorization and alternative protein source development.

Keywords: black soldier fly larvae, bioconversion, liquid milk waste, protein content, response surface.

INTRODUCTION

Food waste is a significant global issue, approximately one-third of all food produced is wasted, primarily due to improper consumption behaviors and socio-demographic influences (Portugal et al., 2020). Factors contributing to food waste include poor meal planning, inadequate food storage, and lack of awareness regarding expiration dates (Portugal et al., 2020). Research indicates that socio-demographic variables, such as education level and household composition, play a crucial role in shaping food consumption behaviors and attitudes towards waste (Rame et al., 2022). Addressing these issues through targeted educational campaigns and interventions can significantly mitigate food waste, promoting sustainable consumption practices (Kennedy et al., 2024).

The increase in food waste over the past two decades, estimated at approximately 3.2–7.6 million tons per year, has significantly impacted the environment, contributing to greenhouse gas emissions and global warming (Phooi et al., 2022). The complexity of food waste generation is influenced by various factors, including

consumer behavior and awareness, which can be addressed through educational initiatives and policy changes aimed at reducing waste at both household and institutional levels (Kusumaningtiar & Vionalita, 2022).

The rapid growth of the dairy industry in East Java, Indonesia, has led to significant increases in fresh milk production, from 445,213 tons in 2021 to 530,426 tons in 2023 (Wahyu Maesarini, 2023). However, this growth is accompanied by substantial waste generation, which includes not only wastewater but also reject products, damaged packaging, and production spills (Čechura & Žáková Kroupová, 2021). Reject products can constitute approximately 0.1% to 4% of daily production, while overall milk waste can reach up to 18% of total production due to various factors (Manurung, 2024). The environmental implications of such waste are severe, necessitating effective waste management strategies to mitigate its impact (Glibert, 2020). Black soldier fly (BSF) larvae can effectively convert various organic materials, including dairy industry waste, market waste, and food scraps, into high-protein biomass, achieving waste reduction rates of up to 58% (Matheka et al., 2022). The protein content of BSF larvae ranges from 31.7% to 47.6%, making them a valuable resource for animal feed (Barrera et al., 2023). The bioconversion process facilitated by BSF larvae not only reduces waste volume but also minimizes pathogenic bacteria, thereby enhancing the public health. This method is particularly promising for low- and middle-income countries, where waste management challenges are prevalent (Satori et al., 2021).

This study aimed to analyze the effect of feeding liquid milk waste on protein content by optimizing the protein content of BSF larvae. This study focused on optimizing liquid milk waste for protein production by varying the amount of feed and the harvest age.

MATERIALS AND METHODS

Materials

This study was conducted over 10 days to optimize the protein content of black soldier fly (*Hermetia illucens*) larvae using the response surface methodology (RSM) approach. Environmental parameters were measured for nine consecutive days to ensure optimal larval rearing conditions.

The research materials consisted of primary and analytical materials. The primary materials included black soldier fly larvae in the active growth phase and larval feed with varying volumes depending on the treatment (2.3–3.7 liters). The analytical materials for determining protein content using the Kjeldahl method consisted of concentrated sulfuric acid (H_2SO_4), a mixed catalyst of K_2SO_4 and $CuSO_4$, 40% NaOH solution, 4% boric acid (H_3BO_3), a mixed indicator of methyl red and methylene blue, standard HCl solution for titration, and distilled water. The larval rearing equipment consisted of a rearing container with an appropriate capacity, a digital thermometer (accuracy of 0.1°C), a hygrometer (accuracy of 1%), and a digital scale for weighing the feed and larvae. Protein analysis tools included a Kjeldahl flask, Kjeldahl digestion and distillation apparatus, burette, Erlenmeyer flask, volumetric pipette, and analytical balance (accuracy 0.0001 g). Design Expert was used for response surface methodology analysis, and Microsoft Excel was used for initial data processing.

This study used RSM with a central composite design (CCD) as the experimental design. CCD was chosen because it can produce a curved response surface (curvature), allowing the optimum point of the studied variable to be detected. The two independent variables used in this study are listed in Table 1.

The dependent variable measured in this study was the protein content of black soldier fly, expressed as a percentage (%). The experimental design applied in this study used a CCD with 13 experimental runs. The design configuration consisted of three main components such as first, four factorial points representing the combination of levels -1 and +1 of the factors studied. Second, four axial points at the level of ±1.414 (or $\pm\sqrt{2}$) allow for the estimation of quadratic effects and expansion of the exploration space beyond the factorial range. Third, five center points at level 0 were replicated to evaluate pure error and test the adequacy of the model (lack of fit). Further details are presented in Table 2.

Research procedures

Black soldier fly larva preparation

The BSF larvae used were prepared under healthy and uniform conditions, particularly during the active growth phase, to ensure consistent responses to the treatments. Before entering the

Table 1. Independent variables and coding levels

Variables	Symbol	Unit	Encoding level				
			-1,414	-1	0	+1	+1,414
Amount of feed	X_1	Liter	2.3	2.5	3.0	3.5	3.7
Harvest age	X_2	Day	4	5	7	9	10

Note: the encoding level description: Level -1.414 (negative axial point): the lowest value of the variable, Level -1 (negative factorial point): low value of the variable, Level 0 (center point): the middle value of the variable, Level +1 (positive factorial point): high value of the variable, Level +1.414 (positive axial point): the highest value of the variable.

Table 2. Central composite design experimental design matrix

Run order	Code X_1	Code X_2	Amount of feed (liters)	Harvest age (days)
1	0	0	3.0	7
2	-1	-1	2.5	5
3	0	0	3.0	7
4	0	0	3.0	7
5	-1,414	0	2.3	7
6	0	1,414	3.0	10
7	1,414	0	3.7	7
8	0	0	3.0	7
9	1	-1	3.5	5
10	0	-1,414	3.0	4
11	0	0	3.0	7
12	1	1	3.5	9
13	-1	1	2.5	9

treatment phase, the larvae were acclimatized to the research environment. After acclimatization process was complete, the larvae were grouped according to the required number of experimental units, which was 13.

The feed volume used in this study varied from 2.3 to 3.7 L. Before feeding the larvae, the feed was weighed and measured. The larval rearing process was conducted in specially prepared containers. Each experimental unit received a different volume of feed according to the predetermined treatment. Throughout the rearing period, the environmental conditions, including temperature and humidity, were consistently monitored and recorded daily. Temperature measurements were performed using a digital thermometer with an accuracy of 0.1°C. Humidity measurements were performed using a hygrometer with an accuracy of 1%. Environmental conditions were maintained within the optimal range for BSF larval growth, namely, a temperature of 24–30 °C and a humidity of 60–90%. The harvest age varied from day 4 to day 10. The selection of the harvest age range from day 4 to day 10 was based on the consideration that during this period, BSF larvae are in the active growth phase and have accumulated significant biomass. After reaching the specified harvest age, the harvested larvae are cleaned of residual feed and feces to ensure that the analyzed sample truly represents larval biomass without contamination from other materials. This cleaning process was carried out carefully to avoid damage to the larvae that could affect the measurement results. The cleaned larvae were then weighed to determine their wet weight, which was used as supporting data in the larval growth analysis. Next, the larvae were dried for protein content analysis.

Protein content analysis was conducted using the Kjeldahl method. This method consists of three main stages such as destruction, distillation, and titration, each of which has a specific function in determining the protein content. The destruction stage began with weighing a 0.5–1.0 gram sample of dried BSF larvae using an analytical balance to ensure the accuracy

of the mass of the sample being analyzed. The weighed sample was then placed in a Kjeldahl flask, which is a special container used for the digestion process. Next, a catalyst is added to the Kjeldahl flask, which is a mixture of potassium sulfate (K_2SO_4) and copper sulfate ($CuSO_4$) of 5–7 grams. After adding the catalyst, 15-20 mL of concentrated sulfuric acid (H_2SO_4) was added. The Kjeldahl flask was then heated in a digestion device until the solution became clear and greenish. The digestion process was carried out until all organic nitrogen in the sample was converted to ammonium sulfate ($(NH_4)_2SO_4$). After digestion was complete, the resulting solution was cooled to room temperature before proceeding to the next stage.

Distillation Stage, the cooled digestion solution was first added with sufficient distilled water to dilute the solution and facilitate the distillation process. The solution was then transferred to a prepared Kjeldahl distillation apparatus. A 40% sodium hydroxide (NaOH) solution was slowly added to the solution until it became alkaline, as indicated by a color change to black. The addition of NaOH converts ammonium sulfate into free ammonia (NH_3), which is volatile and can evaporate. Before the distillation process began, an Erlenmeyer flask containing 25 mL of 4% boric acid (H_3BO_3) solution and a mixed indicator were prepared to hold the distillate. The distillation process was carried out until the distillate volume reached approximately 150 mL or until the distillate was no longer alkaline, indicating that all the ammonia had evaporated and been captured. The collected distillate was bluish-green, indicating the presence of ammonia absorbed by the boric acid solution and reacting with the mixed indicator.

Titration stage, the distillate obtained from the previous stage was titrated with a standard hydrochloric acid (HCl) solution with a concentration of 0.1 N or 0.02 N, depending on the estimated protein content in the sample. The titration was carried out slowly until a color change occurred from bluish green to pink, indicating that the titration endpoint had been reached. The volume of HCl used to reach the titration endpoint was carefully recorded because this value was used to calculate the protein content. In addition to sample titration, a blank titration (without sample) was performed to correct for the possible presence of nitrogen originating from reagents or contamination during the analysis process.

Protein content calculation

The protein content in the sample was calculated based on the volume of HCl used in the titration using the standard Kjeldahl calculation formula. The calculation began by determining the percentage of nitrogen in the sample using the following formula:

$$\% \text{ Nitrogen} = (V_{\text{sample}} - V_{\text{blank}}) \times N_{HCl} \times \times 14.007 \times 100 / \text{Sample weight (mg)} \quad (1)$$

Next, the protein percentage was calculated by multiplying the nitrogen percentage by the conversion factor:

$$\% \text{ Protein} = \% \text{ Nitrogen} \times \text{Conversion factor} \quad (2)$$

In this calculation, Vsample is the volume of HCl used for sample titration in milliliters (mL), Vblank is the volume of HCl used for blank titration in milliliters (mL), NHCl is the normality of the HCl solution used, 14.007 is the atomic weight of nitrogen, and the conversion factor used is 6.25, which is a common conversion factor for protein. The conversion factor of 6.25 is based on the assumption that the average nitrogen content in protein is approximately 16%. Thus, 100/16 = 6.25. The results of the calculation of protein content from each experimental unit were then recorded and used as response variables in the response surface methodology analysis for the development of mathematical models and optimization of BSF larval production conditions with maximum protein content.

Data analysis

Response surface methodology analysis

Protein content data obtained from each run order were analyzed using response surface methodology with the help of Design Expert software through several systematic stages. The first stage is the selection of a statistical model based on two main criteria, namely the Sequential Model Sum of Squares, which compares several models (linear, 2FI, quadratic, cubic) to determine the most appropriate model based on a p-value $<\alpha$ (0.05) with the description "suggested", as well as the Model Summary Statistics, which evaluates the suitability of the model based on the highest R^2 (coefficient of determination), adjusted R^2, and predicted R^2 values. The second stage is the lack of fit test to determine the suitability of the selected model, where the model is accepted if the

p-value lack of fit> α (0.05), which indicates that there is no model gap in the response. Otherwise, the model is rejected if the p-value lack of fit <α (0.05). The third stage is the analysis of variance (ANOVA), which is carried out to test the significance of the model and each independent variable on the response, where the model or variable is declared to have a significant effect on the response if the p-value < α (0.05).

Response visualization

Response visualization in response surface analysis is performed using two main graphical approaches such as contour and surface plots. A contour *plot is* a two-dimensional graphical representation showing a cross-section of a three-dimensional curve, where the color interpretation is key to understanding. Red indicates a high response value, blue indicates a low response value, and the color gradient between the two represents a continuous change in the response value. The *surface plot presents* a visualization in the form of a three-dimensional graph that depicts the highest response point from the results of the interaction of each variable that influences the resp onse, thus providing a more comprehensive understanding of the complex relationship between the independent variables and the response variables.

Response optimization

The optimization criteria were set by maintaining the feed volume within the range of 2.3–3.7 liters and harvest time within the range of 4–10 days, while the protein content was targeted at a maximum value. Setting the "in range" criteria for the independent variables provided the software with the flexibility to explore the entire experimental space. The Design Expert software generates optimal solutions based on a numerical optimization algorithm with a desirability indicator as a measure of suitability. Desirability values range from 0 to 1, with 0 indicating a response that does not meet the criteria and 1 indicating a response that fully meets the optimization target. A solution with a desirability value close to 1 indicates that the combination of variables is capable of producing a response that is very close to the desired ideal conditions.

The optimization results were visualized using a desirability graph, which illustrates the interactions between variables to achieve optimal conditions. The graph is interpreted using color gradations, with blue and red areas representing zones of low (undesirable responses) and high desirability (desirable responses), respectively.

Model validation

Model validation in RSM is conducted using two main approaches. First, the comparison of actual and predicted values is visualized in a graph where the actual values are displayed as scattered points while the predicted values are displayed as a linear line. The model is considered good if the actual values are spread around the predicted line and form a linear pattern, with the model quality increasing as the actual values get closer to the predicted values. Second, the optimum conditions were validated through a confirmation experiment at the optimal point suggested by RSM, where the protein content from the confirmation experiment was compared with the predicted value and evaluated against a 95% prediction interval. The research results are considered valid if the validation value is within this interval. If it is outside the interval, a re-evaluation of the model or experimental procedure is required.

Statistical parameters

The p-value was used to test the significance of the model and variables at a significance level (α) of 0.05. The coefficient of determination (R^2) is used to measure the proportion of response variation that can be explained by the model, with values ranging from 0 to 1. The adjusted R^2 is the R^2 value that has been adjusted for the number of predictors, thus allowing comparisons between models with different numbers of predictors. The predicted R^2 shows the model's predictive ability to new responses, with the difference between adjusted R^2 and predicted R^2 being less than 0.2 to ensure model consistency. Desirability is used to measure the level of suitability of the solution to the optimization criteria, with values close to 1 indicating an optimal solution. Finally, the 95% prediction interval (PI) was used as a confidence interval to validate the response prediction results.

RESULTS AND DISCUSSION

Table 3 shows that the temperature measurement results ranged from 28.8 to 29.7°C, with an average temperature of 29.3°C. The temperature

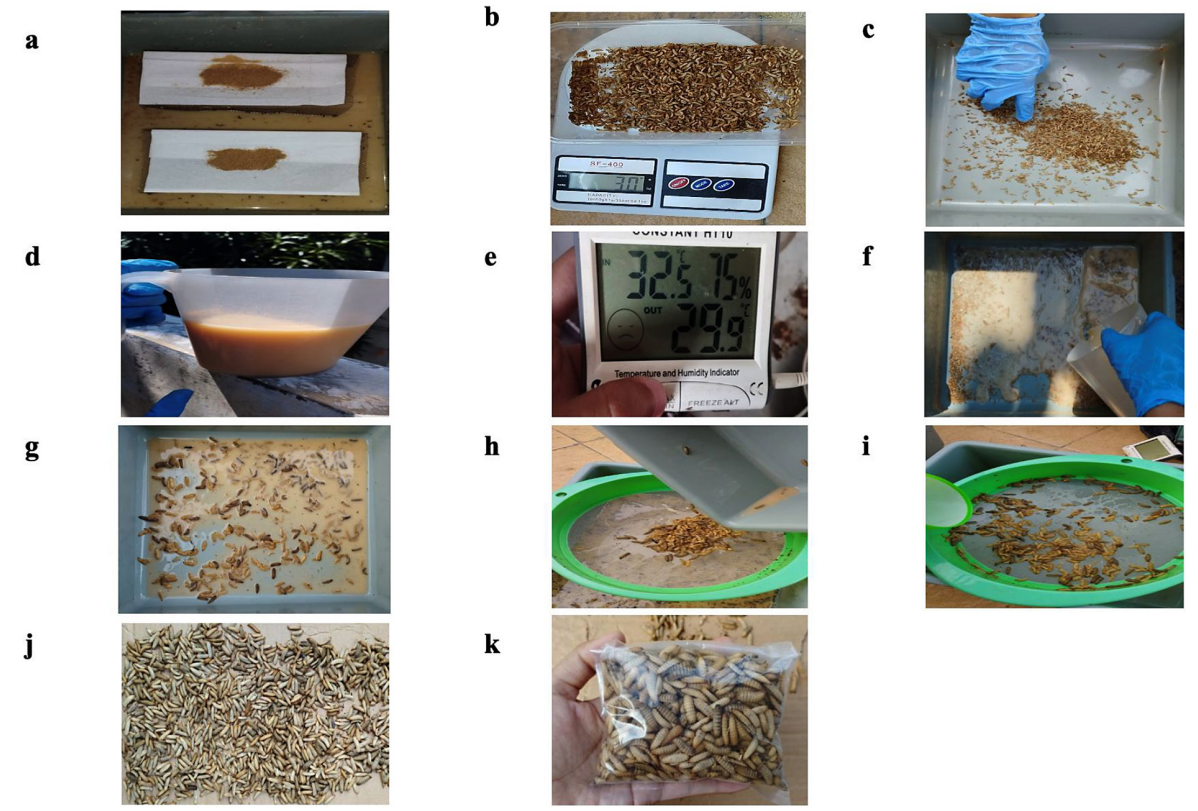

Figure 1. Procedures for maintenance and harvesting of hermetia illucens larvae in liquid milk reject media**: (**a) hatching of BSF larvae eggs on rejected liquid milk feed media; (b) weighing 30 grams of hatched BSF larvae; (c) placement of weighed BSF larvae in the breeding biopond; (d) measuring the amount of feed to be given; (e) measuring the temperature and humidity of the feed media; (f) providing rejected liquid milk to BSF larvae; (g) BSF larvae to be harvested; (h) separation of BSF larvae to be harvested from the feed media; (i) BSF larvae sanitation process; (j) harvested BSF larvae; k) BSF larvae samples ready to be sent to the testing laboratory

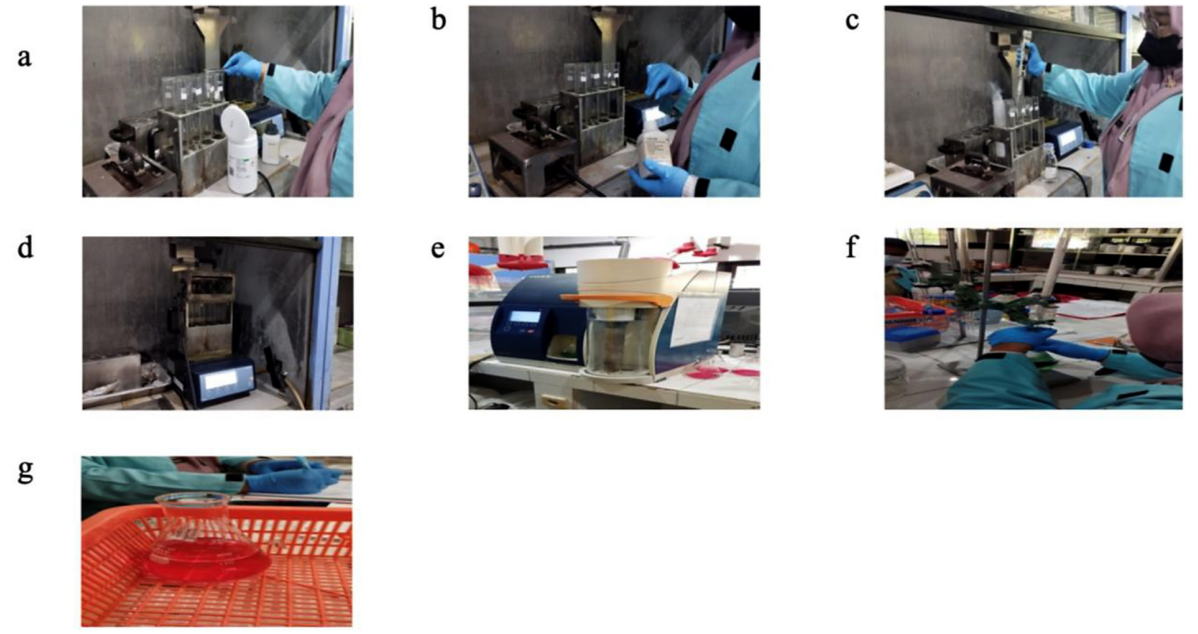

Figure 2. Stages of protein analysis using the Kjeldahl method: (a) addition of kjedahl tablets to the sample (destruction process), (b) addition of boiling chips granules to the sample (destruction process), (c) addition of concentrated H_2SO_4 to the sample, (d) sample destruction process, (e) distillation process, (f) sample titration process from green to pink, (g) final titration result

Table 3. Temperature and humidity measurement results

Day	Temperature (°C)	Humidity (%)
1	29.1	72
2	29.7	71
3	29.5	75
4	29.3	74
5	29.3	73
6	29.3	74
7	29.7	73
8	29.6	73
9	28.8	73
Average	29.3	73

measurement results obtained were still within the optimum temperature interval for Black Soldier growth, which is between 24–30°C. The humidity measurement results ranged from 71 to 75%, with an average humidity of 73%. The humidity measurement results obtained were still within the optimum humidity interval for Black Soldier larval growth, which is between 60% and 90%.

Table 4 shows that the highest protein content obtained was 12.93%, which was in the treatment of giving the amount of feed as much as 3.5 liters with the age of harvest on day 5. The lowest protein content was 10.18%, which was in the treatment of giving the amount of feed as much as 3 liters with the age of harvest on day 10.

The research design produced a curvature to create a fitted response surface. Surface fitting analysis is equivalent to the actual system analysis by RSM if the response function providing the surface fitting is a sufficiently good approximation. The response results of the protein content in black soldier fly are shown in the following graphs.

On the contour plot and surface plot response graphs, it can be seen that on the contour plot the higher the response value will show a red color, while the lower the response value, the contour plot will show a blue color. The 3D surface plot graph illustrates the highest response point from the interaction results for each variable that influences the response, whereas the contour plot is a 2D slice of the 3D curve cross-section.

Selection of a statistical model based on the sequential model sum of squares and model summary statistics and selection of the one that best fits the suggested optimum response. The selected model was then subjected to lack-of-fit tests to determine its suitability of the suggested model. The quadratic vs. 2FI model is the most suggested model based on the analysis of the response results generated with the suggested statement on the right side of the table with a p-value of 0,0002, where the p value < α (0,05) which indicating that the selected model is suitable. More details are presented in Table 5.

Table 6 shows that the quadratic model is a suggested model with suggested information on the right side of the table with an R2 value of 0.9666, which means that 96.66% of the BSF larvae sample variable is influenced by the amount of feed and harvest age, and the remaining 3.34% is influenced by other factors outside

Table 4. The percentage of protein content of black soldier fly larvae

Run order	Coded		X_1	X_2	yA
			Feed amount (liters)	Harvest age (day)	Protein content (%)
1	0	0	3	7	12.17
2	-1	-1	2.5	5	11.35
3	0	0	3	7	12.59
4	0	0	3	7	12.48
5	-1.414	0	2.3	7	10.56
6	0	1.414	3	10	10.18
7	1.414	0	3.7	7	12.79
8	0	0	3	7	12.52
9	1	-1	3.5	5	12.93
10	0	-1.414	3	4	11.91
11	0	0	3	7	12.62
12	1	1	3.5	9	10.82
13	-1	1	2.5	9	10.49

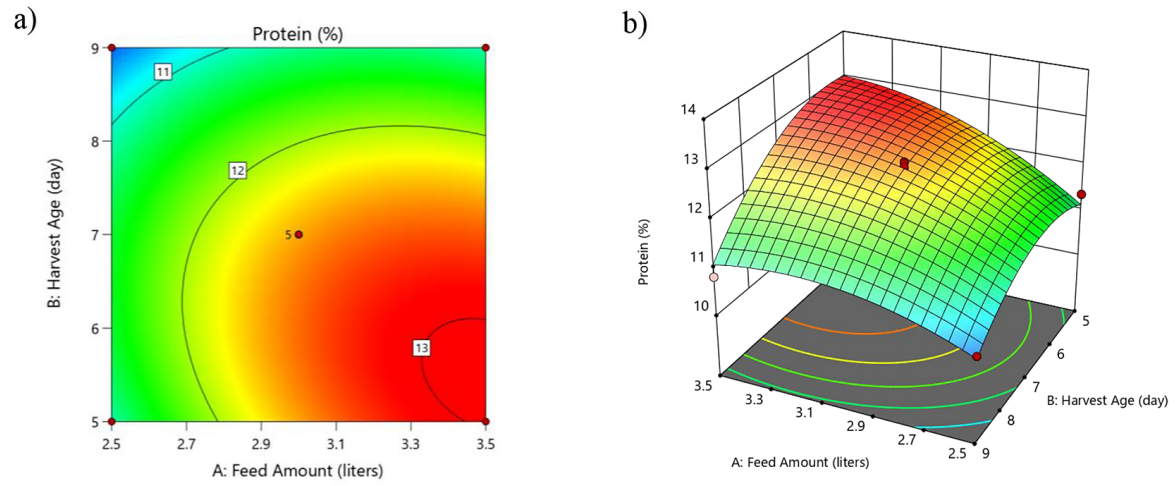

Figure 3. Protein content response in black soldier fly larvae on: (a) contour plot, (b) surface plot

Table 5. Model selection based on sequential model sum of squares

Source	p-value	Description
Mean vs total		
Linear vs mean	0.0125	
2FI vs linear	0.3994	
Quadratic vs 2FI	0.0002	Suggested
Cubic vs quadratic	0.0657	Aliased

Table 6. Model selection based on model summary statistics

Source	R^2	Adjusted R^2	Predicted R^2	Description
Linear	0.5834	0.5001	0.2871	
2FI	0.6168	0.4890	0.1421	
Quadratic	0.9666	0.9428	0.8288	Suggested
Cubic	0.9888	0.9730	0.9696	Aliased

the treatment observed in the study. The test results using the Sequential Model sum of squares and model summary statistics on the resulting response data showed that the quadratic model was the most appropriate for optimizing the response content produced. Therefore, the right ANOVA test analysis for the data presented used a quadratic model ANOVA test.

Table 7 shows that the test results of the quadratic model showed that the p-value for the lack of fit was 0.1806, which means the p value > α (0.05), so that there is no model gap on the response so that the response model is accepted. In the table of the results of the ANOVA test of the quadratic model obtained, it can be seen that the p-value for the research variables, which include the amount of feed and harvest age, is < α (0.05), which means that these variables have a significant effect on the response of protein content in BSF larvae. Model validation optimization was performed to test the accuracy of the model in representing an actual situation. Response optimization produced in the response surface methodology was performed by comparing the predicted value with the actual value of the research results obtained in Table 8.

A comparison of the predicted and actual values, which is the distribution of the resulting response distribution shown in Figure 4. The actual values are expressed as a spreading box, whereas the predicted values are expressed as a linear line. The actual values were spread around the line and formed a linear pattern. This indicates that the results obtained were very good because most of

Table 7. Anova test results of quadratic model in response surface methodology

Source model	Sum of squares	df	p-value
Model	11.33	5	<0.0001
A-feed amount	3.20	1	0.0001
B-harvest age	3.64	1	<0.0001
Lack of fit	0.2620	3	0.1806

Table 8. The results of actual and predicted protein content of black soldier fly larvae

Run order	Feed amount (liters)	Harvest age (day)	Response protein content (%)	
			Actual	Predicted
1	3	7	12.17	12.48
2	2.5	5	11.35	11.12
3	3	7	12.59	12.48
4	3	7	12.48	12.48
5	2.3	7	10.56	10.77
6	3	10	10.18	10.05
7	3.7	7	12.79	12.55
8	3	7	12.52	12.48
9	3.5	5	12.93	13.02
10	3	4	11.91	12.01
11	3	7	12.62	12.48
12	3.5	9	10.82	11.08
13	2.5	9	10.49	10.44

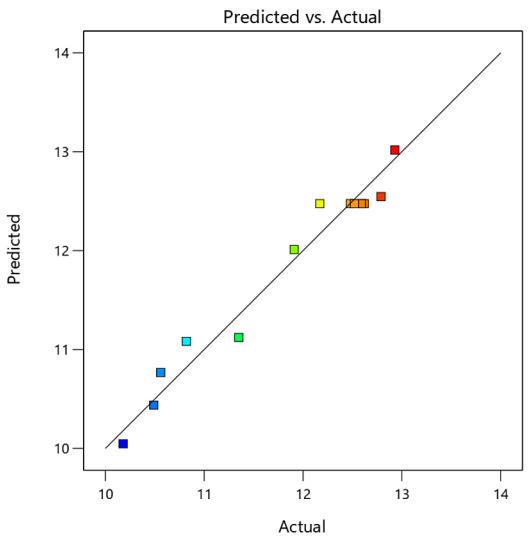

Figure 4. Comparison of actual and predicted values of protein content response in black soldier fly larvae

the actual values obtained were close to the predicted values.

Table 9 shows that RSM provides an optimum point solution with the highest protein content response, resulting in a combination of the amount of feed 3.4 l with the age of harvest on day 5, which is predicted to produce a protein content of 13.034%. The desirability value obtained for the optimum point suggested by the RSM was 1.000, where the desirability value is generally between 0 and 1. Desirability values closer to one indicate that the program's ability to produce the desired response is excellent.

The interactions between the variables that influence protein levels in optimizing the best response are presented in the desirability graph shown in Figure 5. The difference in color indicates desirable areas. The blue area represents an undesirable response because it has a low desirability, whereas the red area represents a desirable response because the desirability obtained is 1.

Table 10 shows the results of the validation of the protein content response value based on the optimal solution using RSM. BSF treated with 3.4 liters of feed at harvest age on day 5 had a protein content of 12.96%. The protein content verification value obtained was still within the 95% PI Low and High intervals. Therefore, the research results are valid. The black soldier fly, scientifically known as *Hermetia illucens*, has

Table 9. Optimal solution based on response surface methodology

Variable	Optimization point	Response protein content (%)		95% PI low	95% PI high
		Prediction	Validation		
Feed amount	3.4 liters	13.034	12.96	12.3891	13.6785
Harvest age	Day 5				

Table 10. Response validation results at optimum condition

Variable	Optimization point	Response protein content (%)		95% PI low	95% PI high
		Prediction	Validation		
Feed amount	3.4 liters	13.034	12.96	12.3891	13.6785
Harvest age	Day 5				

garnered significant attention in recent years because of its unique life cycle and nutritional profile. Importantly, BSF larvae are not vectors of disease, as their life cycle is characterized by a rapid growth phase followed by pupation and eventual death, without the capacity for disease transmission (Bogevik et al., 2022). This characteristic makes them particularly suitable for various applications, including waste management and animal feed production. The nutritional composition of BSF larvae is notably rich in proteins, fats, and other essential nutrients, making them an attractive alternative protein source for animal feed. The protein content of BSF larvae can range from 36% to 55% depending on their diet and rearing conditions (Sadykova et al., 2021). Factors influencing the nutritional quality of BSF larvae include the type of feed provided, the age of the larvae, and the environmental conditions during their growth (Yang & Tomberlin, 2020). For instance, larvae fed on high-protein substrates exhibit superior growth rates and nutritional profiles compared to those fed on lower-quality organic waste (Yang et al., 2023). Moreover, the environmental conditions during the breeding of BSF larvae are crucial for optimizing their growth and nutrient composition. Studies have shown that BSF larvae thrive in a variety of organic substrates, including food waste, agricultural by-products, and animal manure (Moyet et al., 2023).

The growth of BSF larvae is significantly influenced by environmental factors such as temperature and humidity. Research indicates that the optimal ambient temperature for the growth of BSF larvae ranges from 24°C to 30°C (Ikram et al., 2023). When temperatures exceed this range, larvae exhibit behavioral adaptations, such

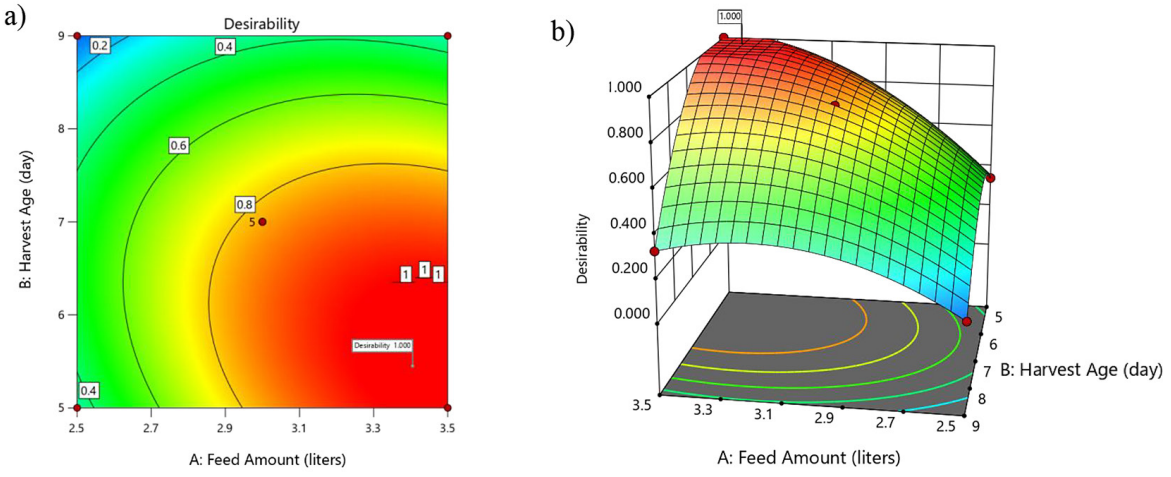

Figure 5. Desirability graph of interaction of feed amount and harvest age on protein content of BSF larvae on: (a) contour plot, (b) surface plot

as seeking cooler environments to mitigate heat stress. Conversely, lower temperatures can lead to a slowdown in metabolic processes, adversely affecting growth rates (Li et al., 2023). This temperature sensitivity is critical for maintaining the efficiency of BSF larvae in waste conversion and biomass production. Humidity also plays a vital role in the development of BSF larvae. The ideal humidity levels for optimal growth are reported to be between 60% and 90% (Logan et al., 2021). High humidity levels facilitate feed medium digestion, which is crucial for larval development. If the feed medium is not sufficiently moist, it can hinder the larvae's ability to process nutrients effectively, thereby affecting their growth and overall health. In a controlled study, the average temperature recorded was 29.3°C, with an average humidity of 73%, both of which fall within the optimal ranges for BSF larvae growth(Ikram et al., 2023).

Moreover, the substrate or feed media used for rearing BSF larvae must maintain adequate moisture levels to ensure efficient digestion by the larvae. The moisture content of the feed not only affects the larvae's growth but also influences their ability to convert organic waste into high-quality protein and fat (Amrul et al., 2022). Studies have shown that BSF larvae can thrive on various organic substrates, including food waste and agricultural by-products, provided that the environmental conditions are conducive to their growth (Amrul et al., 2022).

The BSF undergoes a life cycle that includes distinct phases, such as the egg, larval, prepupal, pupal, and adult phases. Each of these phases has specific environmental requirements, particularly concerning temperature and humidity, which significantly influence their development and overall performance. In the egg phase, the optimal temperature for hatching ranged from 28–35°C. Temperatures below 25°C can delay hatching, while temperatures below 20°C or above 40°C can lead to egg mortality (Jalil et al., 2021).

Humidity levels are also critical during this phase, with an ideal range of 30% to 40% for successful hatching (Sadykova et al., 2021). These conditions are essential for ensuring the viability and rapid development of eggs into larvae. Transitioning to the larval phase, the optimal temperature for growth is between 24°C and 30°C, with humidity levels maintained between 60% and 90% (Intayung et al., 2021). This phase is characterized by the voracious feeding behavior of larvae, which efficiently converts organic waste into biomass. The temperature and humidity not only affect growth rates but also influence the larvae's ability to digest various substrates effectively (Dzepe et al., 2021).

Research indicates that larvae thrive under these conditions, leading to improved bioconversion rates of organic materials (Nelson et al., 2020). In the adult phase, the temperature for optimal egg-laying is slightly higher, ranging from 27.5°C to 37.5°C, with humidity levels exceeding 60% (Yang et al., 2021) . This phase is crucial for reproduction, and maintaining the right environmental conditions is vital for maximizing egg production. Studies have shown that higher temperatures within this range can enhance reproductive output, thereby contributing to the sustainability of BSF populations (Moyet et al., 2023).

Overall, the environmental conditions during each life stage of the BSF are critical for their development and productivity. Temperature and humidity play pivotal roles in ensuring that the larvae grow efficiently and that adults reproduce successfully, thereby supporting their use in organic waste management and as a sustainable protein source in animal feed (Kannan et al., 2024).

Black soldier fly larvae have garnered significant attention in recent years because of their impressive nutritional profile, which is characterized by high levels of protein, fat, and dry matter. This nutritional composition makes BSF larvae an excellent candidate for use as an alternative protein source in animal feeds, particularly in aquaculture and poultry production (Ido et al., 2021). The nutritional value of BSF larvae is significantly influenced by their diet. Studies have shown that larvae fed on protein-rich substrates yield higher protein content in their biomass (Sadykova et al., 2021). This characteristic is particularly beneficial for formulating feeds for livestock and aquaculture, where protein quality and digestibility are critical for growth performance (Yang et al., 2021). Moreover, the ability of BSF larvae to thrive on a wide variety of organic waste materials enhances their potential for sustainable waste management applications. They can effectively decompose food waste, transforming it into high-quality protein and fat-rich biomass while simultaneously reducing the volume of organic waste (Jalil et al., 2021). The larvae's adaptability to different substrates allows for the engineering of their nutrient composition, which can be tailored to meet the specific dietary requirements of various animal species (Bogevik et al., 2022).

The protein content of BSF larvae, specifically those fed on liquid milk waste, demonstrates remarkable conversion efficiency. The larvae achieved a protein content of 29.59%, which was significantly higher than the 3.56% protein content of the liquid milk waste used as the feed. This indicates that BSF larvae can convert protein content from their feed into their biomass at an approximate factor of eight, showcasing their efficiency as a protein source in animal feed (Su et al., 2025). The protein content of BSF larvae varies according to their moisture content. For instance, wet BSF larvae contain about 15.83% protein, while their dry counterparts exhibit a much higher protein concentration of approximately 55.24% (Mshayisa et al., 2022). The substantial difference in protein content between wet and dry larvae is primarily attributed to their water content, as protein concentration is inversely proportional to moisture levels in biological materials (Mshayisa et al., 2022).

The selection of statistical models based on the sequential model sum of squares (SS) and model summary statistics is crucial for testing the adequacy of a model. In particular, the optimization model selection analysis indicated that a quadratic model was preferred, as evidenced by a significant p-value of 0.0002, which was less than the alpha level of 0.05, thus confirming the model's effectiveness. The use of sequential sum-of-squares programming facilitates the analysis of nonlinear systems, providing a robust framework for model evaluation (Tang et al., 2022).

From the results of the analysis of the suggested model selection, the quadratic model was the most appropriate. Therefore, the analysis of variance used was the quadratic model ANOVA. The effect of the tested variables on the model was evaluated using the quadratic model ANOVA test, and p values were obtained for the variables of the amount of feed and harvest age, which were 0,0001 and <0,0001, respectively, where the value was < α (0,05), so that the variables used in this study had a significant effect on the response model.

Lack-of-fit testing is a critical aspect of model validation, particularly in statistical modeling. A model is deemed acceptable if the p-value from the lack-of-fit test exceeds the significance level ($\alpha = 0.05$). In the context of the presented data, the p-value of 0.1806 indicates that there is no significant gap in the selected model, thus validating its adequacy. This aligns with findings from various studies where model adequacy was assessed using statistical tests such as ANOVA, confirming the reliability of the models in different contexts (Ouzidan et al., 2020).

Response Surface Methodology compares the actual and predicted values in the response optimization system to obtain the optimum point for producing the desired response. The results of the comparison of the actual and predicted values obtained are very good because most of the actual values obtained are close to the prediction values that have been set.

The optimal solution for maximizing protein content in BSF larvae, as determined by response surface methodology, suggests an ideal feed amount of 3.4 liters with a harvest age of 5 d, yielding a protein content of 13.034% and a desirability value of 1. The desirability function is a crucial aspect of RSM, enabling the optimization of multiple response variables by indicating how close a given solution is to the ideal target, with values closer to 1 signifying optimal conditions (Rheem, 2023). This method has been effectively applied in various studies to enhance protein extraction and optimize food processes, demonstrating its versatility and reliability in achieving desired outcomes across different contexts (Rios-Morales et al., 2023). The findings underscore the importance of RSM in refining production parameters to enhance nutritional quality in insect farming, particularly for sustainable protein sources like BSF larvae (Baca-Bocanegra et al., 2021).

The validation results in Table 10, in the optimum condition given 3.4 liters of feed with harvest age on day 5, resulted in a protein of 12,96%, with the value of protein content predicted by RSM being 13,034%. where the validation value is still within the interval range of 95% PI Low and 95% PI High, indicating that the results obtained are consistent with the predicted results.

The protein content of BSF larvae has significant applications in livestock nutrition, particularly in the formulation of maggot meals and milk replacers for ruminants. The incorporation of BSF larvae into livestock feed has been shown to enhance weight gain and feed conversion efficiency, making it a valuable protein source in animal husbandry (Sepriadi et al., 2022).

CONCLUSIONS

This study successfully achieved its objective in optimizing the protein content of black soldier

fly larvae using response surface methodology with liquid milk waste as feed. The optimization results showed that the combination of 3.4 liters of feed with a harvest age of 5 days resulted in the highest protein content of 13.034% (prediction) and 12.96% (validation), with a desirability value of 1.000. New scientific findings from this study include such as identification of a specific optimal point for the interaction between the amount of liquid milk waste feed and harvest age on BSF protein content, which has not been previously reported, a quadratic model with $R^2 = 0.9666$ which showed that 96.66% of the variation in protein content can be explained by these two variables. This study fills the gap in the literature regarding the utilization of liquid milk waste as an alternative substrate for BSF cultivation, while providing optimal parameters that can be practically applied. The prospects opened include the development of an efficient and sustainable bioconversion system for dairy industry waste, as well as the potential for producing high-protein animal feed from organic waste sources.

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