A project report on

“Sequential extraction and quantification of fat, protein and carbohydrate from black soldier fly larvae cultivated on organic waste for enhanced waste management”

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CERTIFICATE

This is to certify that **Mr. Suraj Sharma (23015026)**, MSc -I, Department of Chemistry, Savitribai Phule Pune University has successfully Completed On Job Training on project entitled “**Sequential extraction and quantification of fat, protein and carbohydrate from black soldier fly larvae cultivated on organic waste for enhanced waste management”** under the guidance of **Dr. Arun dixit**. Throuout the period he was sincere and hardworking student and the result obtained from the resources are duly acknowledged in the report.

Date: 07/07 /2024.
Place: Pune.

Signature of the Guide
Dr. Arun Dixit,
Vigyan Ashram Pabal.

ACKNOWLEDGEMENT

It is a genuine pleasure for me to undertake this project entitled “Sequential extraction and quantification of fat, protein and carbohydrate from black soldier fly larvae cultivated on organic waste for enhanced waste management.”

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- *Suraj Sharma*

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1.INTRODUCTION

The increase in waste production because of population growth is among the major concerns in many areas around the world. One of the most innovative technology for waste management is the bioconversion of side streams by insects. Many insects naturally feed in organic wastes, converting biomass nutrients into their own biomass and reducing the amount of waste material. *Hermetia illucens*, better known as black soldier fly (BSF), is one of the most important species proposed as a converter of organic waste. Hermetia illucens, or black soldier fly larvae, are tiny, innocuous insects that can be produced in large quantities for use in a variety of industrial and agricultural settings. They have the capacity to effectively dispose of a wide range of organic waste materials by turning them into marketable biomass that is high in fat and protein because of their efficient feed conversion ratio and nonselective rearing substrate.

H. illucens has a life cycle that includes eggs, larvae, pupae, and adults, among other developmental stages. The insect's feeding activity is restricted to the larval stage, and during the final stage of larval development, known as prepupae, it migrates away from the feed source in search of a dry location where pupation takes place. The larvae's fat content reaches its peak when they get to the prepupal stage because they need to have enough energy stores to support all of their activities in the pupal and adult stages that follow. The protein and fat composition of larvae and prepupae raised on various organic substrates is described in a number of studies. Black soldier fly larvae are reported to have protein and fat contents on a dry matter basis that range from 35 to 57% and from 15 to 49%, respectively, depending on the rearing conditions. The content of the feed has a significant impact on both the amount of fat and the fatty acid composition of the larvae. The amino acid profile of BSF larvae, however, is not subjected to such large variation when different rearing substrates are used. Insect meal has a strong nutritional profile, which makes it a promising substitute for fish or soy meal in the feed business when it comes to the creation of compound feeds. In this context, other animal species have previously been given black soldier fly larvae in an experimental setting. The meal made from black soldier fly larvae was found to be an appropriate substitute for traditional sources of protein and fat in all of the trials that were carried out. In addition to employing insect meal, extracting proteins, lipids, and other nutrients from *H. illucens* may produce a more refined product that is suitable for

use as high-value feed. In addition to employing insect meal, extracting proteins, lipids, and other nutrients from H. illucens may produce a more refined product that is suitable for use as high-value feed. For their high protein content, BSF larvae/prepupae have been proposed to be used as feed for different species as fish, chicken and pigs and as a pet food. Moreover, due to the large amount of fat in the prepupae, another application exploited for BSF biomass is the production of biodiesel. Finally, yet importantly, BSF is also a source of chitin. Chitin and its derivatives have great economical value because of their numerous applications: food, cosmetics, pharmaceuticals, textile industries etc.

As described in previous paragraphs, the different biomolecules (i.e., lipids, proteins, and chitin) have a lot of potential for several applications, making black soldier fly larvae, prepupae, and pupae a source of valuable biomass. Furthermore, this high-value biomass can be produced by rearing *H. illucens* on low-value waste streams with varying compositions such as restaurant waste and manure. When extracting the aforementioned biomolecules from insects, a better cost–benefit ratio can be achieved if all fractions are valorised. Thus, from an economic point of view, a sequential extraction delivering products with high purity is preferred.

2. LITERATURE REVIEW

The various biomolecules found in black soldier fly larvae, including as lipids, proteins, and chitin, have a wide range of possible uses, making them a valuable biomass source. Raising *H. illucens* on low-value waste streams with varied compositions, such as kitchen waste, restaurant waste and manure, can yield this high-value biomass. The previously listed biomolecules can be extracted from insects to improve the cost-benefit ratio. Therefore, it is necessary to understand the composition of biomolecules and the sequential extraction process for highly pure products from an economic standpoint.

In order to obtain data regarding the nutritional worth of black soldier fly (BSF) larvae and their appropriateness as animal feed, Fonseca et al. (2017) conducted a review of the literature. Conducted experiments on the use of BSF larvae in fish, pig, and poultry feed diets to see how much traditional feedstuff can be replaced. The performance of BSF larvae is greatly impacted by biotic and abiotic parameters, including food quality,

temperature, substrate moisture, and larval crowding. This emphasizes the necessity of specific rearing conditions. They examined the effects of high ash (9 to 28% dry matter) and fat (7 to 39% dry matter) contents on the feeding performance of BSF larvae.

Caligiani et al. (2017) explored different extraction protocols, including chemical and enzymatic methods, to separate pure fat, protein, and chitin fractions from BSF prepupae biomass, with the most challenging aspect being the separation of protein from chitin. Various extraction techniques were employed, such as alkali extraction and enzymatic assisted extraction, to recover proteins and chitin fractions efficiently while maintaining their integrity and quality. They performed protein extraction by precipitation with 10% TCA solution.

Smets et al. (2019) concentrated on extraction process across multiple life stages present in the same rearing batch. They performed detailed extraction procedure involving lipid extraction using petroleum ether, followed by protein extraction through solubilisation and precipitation methods, resulting in high protein content extracts ranging from 85% to 98%. Lipid content was determined by following Soxhlet extraction with petroleum ether. The result found that all samples contained high levels of proteins (ranging between 31.27% and 38.86%) and lipids (ranging between 39.85 and 47.65%) but the quantities varied considerably between the different developmental stages. Larvae had the highest protein content (38.86%) whereas prepupae had the highest lipid content (47.62%). The conclusion drawn from the study is that the extraction method is suitable for all life stages of H. illucens, enabling the extraction of high-value biomolecules for industrial applications.

Shumo et al. (2019) studied the nutritive value of black soldier fly larvae reared on common organic waste streams in Kenya. CP was determined using the Kjeldahl method and a nitrogen-to-protein conversion factor of 4.76 was used in the calculation of crude protein. Diethyl ether was used as an extractant in the determination of crude fat using the Velp solvent extractor. Crude protein content was found in chicken manure fed larvae (41.1%), kitchen waste fed larvae (33%) and spent grain fed larvae (41.3%).

Chia et al. (2020) main objective was to examine the nutritional makeup of black soldier fly larvae fed different agroindustrial byproducts. Oven drying, the Kjeldahl method

for determining protein content, diethyl ether extraction for fat content using the Randall technique, and muffle furnace ignition for ash content were some of the techniques used in the study.

Queiroz et al. (2021) compared the composition of flour and protein extract in terms of moisture, ash, amino acids, minerals and protein content. They performed Protein extraction from defatted insect powder included dissolution in NaOH, centrifugation, pH adjustment and isoelectric point precipitation to obtain the protein extract.

3. MATERIALS AND METHODOLOGY

3.1. Proximate Composition of Fat and Analysis:

3.1.1. Materials:

Packed and dried *H. illucens* larvae was provided by Vigyan Ashram Institute. Soxhlet extractor include three main sections: a percolator (boiler and reflux) which circulates the solvent, a thimble (usually made of thick filter paper) which retains the solid to be extracted, and a siphon mechanism, which periodically empties the condensed solvent from the thimble back into the percolator. Petroleum ether was used as extracting solvent.

3.1.2 Fat Extraction by Soxhlet Extraction Method:

10 g of the crushed BSFL were added to 120ml petroleum ether in a Soxhlet apparatus. The sample material was extracted for 6 h at 70 to 80°C on heating mantle. Then the solvent was removed by a rotary evaporator.

$$Fat\ \% = \frac{weight\ of\ oil}{weight\ of\ sample} \times 100$$

3.1.3. Iodine value of oil extracted form BSF larvae:

Took 10 ml of 2% solution of BSF larvae oil in a stoppered bottle and add 10 ml of chloroform to it. After properly mixing 20 mL iodine solution was added to it and mixed well. Kept bottle in dark for 1 hour with occasional shaking. After 1 hr 50mL of distilled water was added to the above solution and titraed with 0.2N sodium thiosulphate with 1% starch as an indicator. Same procedure was repeated without using oil for blank reading.

3.2. Proximate Composition of Protein:

3.2.1. Materials:

Defatted larvae obtained after Soxhlet extraction, Kjeldahl assembly includes Kjeldahl flask and Distillation apparatus, Concentrated Sulfuric acid (95%),0.5 N HCL, 0.5 N NaOH, Phenolphthalein Indicator, boiling chips, lowry reagent, Folin phenol reagent, colorimeter or spectrophotometer.

3.2.2. Preparation of defatted insect powder:

After fat extraction, the defatted larvae were left overnight to evaporate the residual solvent and then further ground to obtain a powder.

3.2.3. Protein Extraction and Estimation by Lowry Method:

Whole defatted BSF powder was mixed with 150 mL of 0.25 M NaOH solution and the mixture was heated to 40 °C for one hour with constant agitation at 400 rpm on a magnetic stirrer. The mixture was centrifuged at 4500 rpm for 15 min and the lipid fraction on the top was carefully separated by pipets. The pellet was reserved for further extraction (twice more), while the pH of the supernatant was adjusted to 4.0–4.3 by adding 37% HCl and 1N HCl successively, followed by centrifugation (4000 rpm, 15 min) to obtain the precipitated proteins. Precipitated protein was redissolved in 0.1 M NaOH and volume made to 100 ml by NaOH and Lowry assay was performed for protein solution to estimate the protein. Amount of protein is calculated with the help of standard graph of BSA (Bovine Serum Albumin) protein.

3.2.4. Protein Determination by Kjeldahl Method:

Digestion:

0.5 g of defatted larvae powder was transferred to digestion flask along with 6 to 7 ml of conc. H_2SO_4. 3.5 g of Potassium sulphate was added to raise the boiling point with catalyst usually copper (copper sulphate). Sample was digested for 1 hour and cooled. Then sample was filtered and volume made to 50 ml by distilled water.

Distillation:

The pH of the above solution was raised by using 45% NaOH till solution became alkaline. Then solution transferred to Kjeldahl flask and few boiling chips was added. 40 ml of traping solution (0.5 N HCl) was placed in conical flask and the end of the

condenser was immersed in the trapping solution. As distillation started, distillate was collected for 20 min.

Titration:

After 20 Min. trapping solution was titrated against the 0.5 N NaOH using phenolphthalein indicator till the appearance of pink colour as the end point.

$$\% \, Nitrogen = \frac{1.4007 \times (\mathrm{V}_b - \mathrm{V}_s) \times \mathrm{N}}{W}$$

$$\% \, \mathrm{Protein} = \% \, Nitrogen \times 6.25$$

Where,

V_b– is the volume of NaOH solution required for blank.

V_s – is the volume of NaOH solution required for sample.

N – is the normality of NaOH solution used for titration.

W – is the weight of sample taken.

3.3. Determination of total carbohydrate

3.3.1. Materials:

- Spectrophotometer
- 2.5 N HCL
- **Anthrone reagent:** Dissolve 200 mg Anthrone in 100 ml of ice cold 95% H_2SO_4.
- Standard Glucose: Stock: 1mg/ml
  Working standard: 0.1mg/ml

3.3.2. Determination of total carbohydrate by Anthrone method:

Weighed 100mg of the defatted sample into a boiling tube. Hydrolysed it by keeping it in a boiling water bath for three hours with 5mL of 2.5 N-HCl and cooled it to room temperature. Neutralized it with solid sodium carbonate until the effervescence ceased and made the volume to 100mL and centrifuged it. Collected the supernatant and took 0.2 and 0.5mL aliquots for analysis.

Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8, and 1mL of the working standard. '0' served as the blank. Made up the volume to 1mL in all the tubes including the sample tubes by adding distilled water. Added 4mL of anthrone reagent to each tube. Heated

them for eight minutes in a boiling water bath. Cooled them rapidly and read the green to dark green colour at 630nm. Drew a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. Calculated the amount of carbohydrate present in the sample tube from the graph.

Amount of carbohydrate present in 100 mg of sample,

$$= \frac{mg\ of\ glucose}{volume\ of\ test\ sample} \times 100$$

4. RESULTS AND DISCUSSION

4.1. Fat Composition:

The Oil was extracted form dried BSF larvae by Soxhlet extraction method and performed for three times to minimize the error. Petroleum ether was used for extraction of oil. Each time petroleum ether was evaporated by rotary evaporator and weight of oil was measured.

The lipid percentage was calculated by the following formula:

$$Fat\ \% = \frac{weight\ of\ oil}{weight\ of\ sample} \times 100$$

Weight of Sample taken:10 g of larvae

Table 1: Crude fat composition in BSF larvae:

Sample No.	Empty weight of round bottom flask (gm)	Weight of round bottom flask with oil (gm)	Weight of oil (gm)	% of oil
1	123.520	129.105	5.585	55.85
2	122.513	128.113	5.6	56
3	148.792	154.196	5.404	54.04

Crude fat content in BSFL, which is determined to be 55 ± 1% in the experiment.

4.2. The degree of saturation or unsaturation was determined by checking the Iodine value:

Burette:0.2 N $Na_2S_2O_3$

Indicator:1% starch solution (0.5) ml

End point: Blue to Colorless.

Table 2: Blank Titration

Oil (gm)	Chloroform (ml)	Iodine solution (ml)	Stopper the bottle and shake it intermittently and keep it in dark for 1 hr.	Starch (ml)	Reading (ml)
0	10	20		0.5	24.3

Table 3: Back Titration

Oil (ml)	Chloroform (ml)	Iodine solution (ml)	Stopper the bottle and shake it intermittently and keep it in dark for 1 hr.	Starch (ml)	Reading (ml)
10	10	20		0.5	21.5

Iodine value was calculated by following formula:

$$Iodine\ value\ of\ oil\ = \frac{(blank - \ back)\ \mathbf{x}\ N\ of\ Na_2SO_4\ \mathbf{x}\ 0.02538\ x\ 100}{Weight\ of\ oil}$$

Iodine value of BSFL oil was found to have 7.1064 g I_2/100g fat. Which indicates the moderately low level of unsaturation in the oil and high level of saturated fat in the oil.

4.3. Determination of Total Carbohydrates:

Total carbohydrate was determined by the Anthrone method.

Standard graph of glucose was prepared for calculation of carbohydrate.

Table 4: Standard graph of glucose:

Sr No.	Glucose (ml)	DW (ml)	Anthrone		Absorbance at 630 nm	Glucose (mg)
1.	0.0	1.0	4.0	**Boil in boiling water bath for 8 mins and cool rapidly.**	0.0	0.0
2.	0.2	0.8	4.0		0.0885	0.02
3.	0.4	0.6	4.0		0.1661	0.04
4.	0.6	0.4	4.0		0.2335	0.06
5.	0.8	0.2	4.0		0.3050	0.08
6.	1.0	0.0	4.0		0.3904	0.1

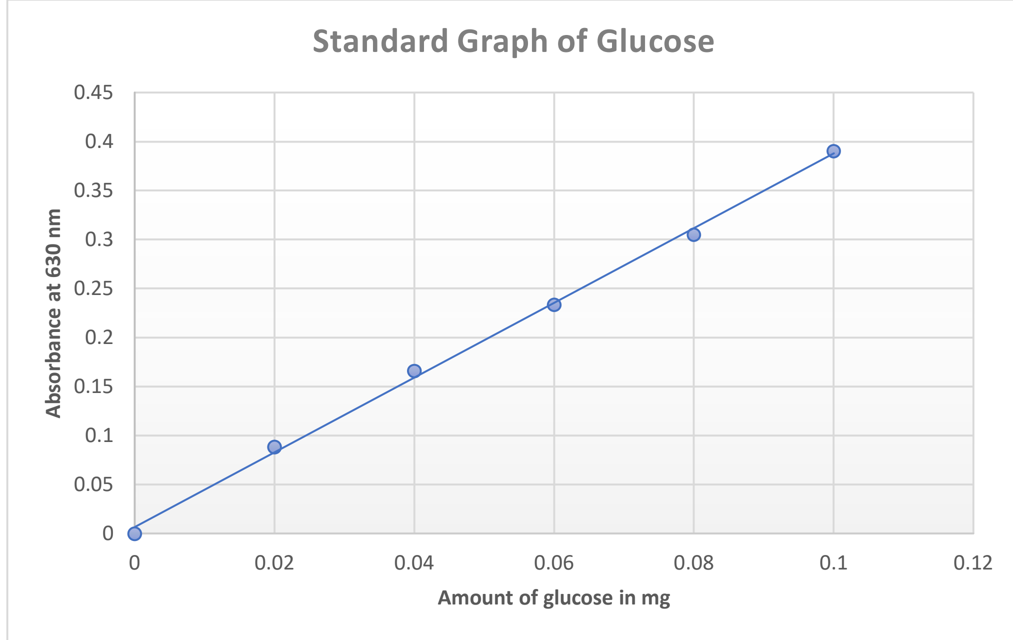

Figure 1: Standard graph of glucose

Amount of carbohydrate present in 100mg of the sample was calculated by following formula,

$$Total\ carbohydrate = \frac{mg\ of\ glucose \times 100}{Volume\ of\ the\ test\ sample}$$

Table 5: Sample 1- total weight of powdered sample - 4.487 g

Volume of solution (ml)	D.W (ml)	Anthrone (ml)		Absorbance At 630nm
0.2	0.8	4.0	Boil for 8 mins and cool	0.2019
0.5	0.5	4.0		0.5270

Total amount of carbohydrate found in sample-1 was 1.1751 g in 10 g BSF larvae which was 11.75 % of 10 g larvae.

Table 6: Sample 2- Total weight of powdered sample- 4.356 g

Volume of solution (ml)	D.W (ml)	Anthrone (ml)		Absorbance At 630nm
0.2	0.8	4.0	Boil for 8 mins and cool	0.2121
0.5	0.5	4.0		0.5440

Total amount of carbohydrate found in sample-1 was 1.1922 g in 10 g BSF larvae which was 11.92 % of 10 g larvae.

4.4. Proximate Composition of Protein:

4.4.1. Protein Estimation by Lowry Method:

The Lowry protein assay is a widely used method for quantifying a sample's protein amount. The reagents involved are copper ions and a Folin Ciocalteau reagent. The resulting color change is then measured at a specific wavelength 660 nm.

A standard graph of BSA protein was prepared for estimation of protein.

Table 7: Standard graph of BSA protein.

BSA Protein solution (ml)	Protein in mg	DW (ml)	Lowry C (ml)		Folin phenol reagent (ml)		Absorbance at 660 nm
0	0	1.0	3.0	Mix and wait for 15 mins	0.5	Mix and wait for 30 minutes in the dark.	0
0.2	0.04	0.8	3.0		0.5		0.10
0.4	0.08	0.6	3.0		0.5		0.18
0.6	0.12	0.4	3.0		0.5		0.24
0.8	0.16	0.2	3.0		0.5		0.33
1.0	0.20	0.0	3.0		0.5		0.43

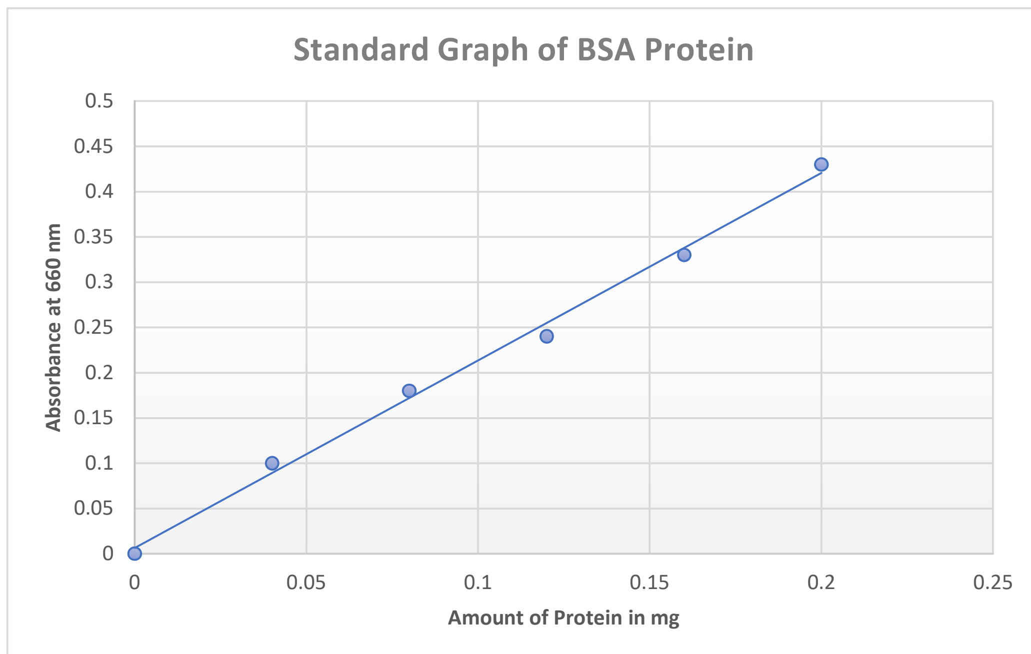

Figure 2: Standard graph of BSA protein.

Table 8: protein estimation of sample 1 by Lowry method.

Protein solution (ml)	DW (ml)	Lowry C (ml)		Follin Phenol reagent (ml)		Absorbance at 660 nm	Dilution factor
0.2	0.8	3.0	Mix and wait for 15 mins.	0.5	Mix and wait for 30 mins. in dark.	0.54	20
0.2	0.8	3.0		0.5		0.37	30

From calculation done using standard graph of BSA, the amount of protein found in 10 g of BSF larvae is 2.5758 g.

i.e. 25.75% of protein found in 10 g of BSF larvae.

4.4.2. Protein Determination by Kjeldahl Method:

Total protein content of BSF larvae was determined by Kjeldahl method. This method was performed for two samples.

Percent Nitrogen was calculated by formula,

$$\% \, Nitrogen = \frac{1.4007 \times (\mathrm{V}_b - \mathrm{V}_s) \times \mathrm{N}}{W}$$

Percent Protein was calculated by multiplying % nitrogen with nitrogen to protein conversion factor 6.25.

$$\% \, \text{Protein} = \% \, Nitrogen \times 6.25$$

The percentage of nitrogen obtained in sample- 1 and sample- 2 was 5.32% and 5.04% respectively. Thus, percentage of protein came out to be 33.25% and 31.5% for sample- 1 and sample- 2 respectively.

Table 9: Proximate composition of Fat, Protein and carbohydrate in BSF larvae

	Sample 1	**Sample 2**	**Sample 3**
Fat	55.85 %	56%	54.04%
Protein by Lowry method	25.75%		
Protein by Kjeldahl method	33.25%	31.5%	
Carbohydrate	11.75%	11.92%	

5. CONCLUSIONS

The study presented a systematic approach to estimate valuable biomolecules from BSF larvae, resulting in three major products: proteins, lipids, and carbohydrate.

By following extraction and estimation procedure successfully found composition lipids, proteins, and carbohydrate from Black Soldier Fly *(Hermetia illucens*) larvae.

Fat content of black soldier fly larvae (BSFL) was determined to be 55± 1.0 %. Iodine value of BSFL oil was found to have 7.1064 g I_2/100g fat. Which indicates the moderately low level of unsaturation in the oil and high level of saturated fat in the oil.

Protein estimated from the larvae was found to be 25.75% by Lowry method and 32.37% by Kjeldahl method. From difference in the protein content obtained by Lowry method and Kjeldahl method can be concluded that there may had been some loss of protein by lowry method or that some nonprotein nitrogen might be present in Kjeldahl method.

Carbohydrate content of BSF larvae was determined to be 11.84%.

6. LEARNING OUTCOMES

- The raw fat content of black soldier fly larvae (BSFL) was found to be 55± 1.0%. on a dry matter basis, a key metric for understanding the lipid content of BSFL.
- Soxhlet method proved to be effective in lipid extraction from BSFL.
- There is a potential for further exploration of innovative techniques to increase the overall yield of biomolecules from Black Soldier Fly at different developmental stages, opening up opportunities for diverse industrial applications in the future.
- It is evident from the available studies that while BSF larvae can partially replace traditional feedstuff in diets for animals like poultry, pigs, and fish, complete replacement may lead to reduced performance due to factors such as high fat and ash content.
- Protein extraction from BSF larvae resulted in high protein content, indicating the richness of proteins in these life stages.
- The contribution of BSF larvae in waste management is remarkable and they are highly efficient in converting organic matter into biomass. Their efficiency as decomposers and nutrient-rich composition makes them an eco-friendly solution to the environment.

7. FUTURE SCOPE

- **Exploration of Novel Extraction Methods**: Future research could focus on developing innovative extraction techniques that enhance the efficiency and sustainability of recovering biomolecules from BSF larvae, potentially improving yields and purity levels.
- **Enhanced Protein Recovery**: Investigating advanced separation technologies to improve protein recovery rates while maintaining protein integrity will be crucial for maximizing the value obtained from BSF biomass.
- **Application Development**: Future studies could focus on the development of diverse applications for the extracted biomolecules, such as utilizing BSF proteins in food supplements, functional foods, or biodegradable packaging materials, thereby expanding the market potential of BSF-derived products.
- **Sustainability and Circular Economy**: Research efforts could be directed towards assessing the environmental impact and sustainability of large-scale BSF biomass conversion processes, aiming to establish BSF-based biorefineries as a sustainable and economically viable solution for waste valorisation
- The use of insect oil as a raw material for biodiesel production shows promise as an attractive alternative for bioenergy in the future, meeting industry standards and sustainability goals.
- Future research endeavours should focus on enhancing protein recovery rates while maintaining the high purity of the extracts, aiming to optimize the extraction process for improved efficiency and sustainability.
- Further studies could explore innovative techniques to increase the overall yield of biomolecules from Black Soldier Fly across its developmental stages, potentially expanding the applications of these valuable compounds in various industrial sectors.

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