A Project Report On

Sequential Extraction and Quantification of Fat, Protein, and Carbohydrates from Black Soldier Fly Larvae cultivated on organic waste for Enhanced Waste Management.

Submitted in partial fulfillment of the requirement for the

Master of Science in Biochemistry

Submitted by

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CERTIFICATE

This is to certify that the project entitled "**Sequential Extraction and Quantification of Fat, protein, and Carbohydrates from black soldier fly larvae cultivated on Organic Waste for Enhanced Waste Management**" is a bonafide record work done by **Ms. Mitali Sharma** (Roll No:23012016) under the guidance of **Dr. Arun Dixit** and submitted to Savitribai Phule Pune University in partial fulfillment of the requirement for the On Job Training course for Master of Science in Biochemistry.

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

Signature of the Guide
Dr. Arun Dixit,
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Mitali Sharma

Sequential Extraction and Quantification of Fat, protein, and Carbohydrates from black soldier fly larvae cultivated on Organic Waste for Enhanced Waste Management

1.1 Introduction

Hermetia illucens, the black soldier fly, is a widespread fly of the family Stratiomyidae. The family Stratiomyidae comprises 260 known species in North America (Triplehorn 2005). In the southeastern United States, the black soldier fly is abundant during late spring and early fall, and has three generations per year in Georgia (Tomberlin et al. 2002). While common in the continental United States, this fly is found throughout the Western Hemisphere.

Since the late 20th century, H. illucens has increasingly been gaining attention because of its usefulness for recycling organic waste and generating animal feed. The adults of H. illucens measure about 16 millimeters (⅝ in) long. These medium-sized flies have a predominantly black body, with metallic reflections ranging from blue to green on the thorax and sometimes with a reddish end of the abdomen.

An adult female lays approximately 200 to 600 eggs at a time. These eggs are typically deposited in crevices or on surfaces above or adjacent to decaying matter such as manure or compost, and hatch in about 4 days. Freshly emerged larvae are 1.0 millimeters (0.04 in) long, being able to reach a length of 25 millimeters (1 in) and weight of 0.10 to 0.22 grams (1.5 to 3.4 gr) by the end of the larval stage. The larvae and adults are considered neither pests nor vectors. Instead, black soldier fly larvae play a role similar to that of redworms as essential decomposers in breaking down organic substrates and returning nutrients to the soil. The larvae have voracious appetites and can be used for composting household food scraps and agricultural waste products.

The larvae contain 42% crude protein and 29% fat on average in the dry matter. Although the protein content in black soldier fly (BSF) larvae is lower than in insects from the orthoptera species such as adult locusts, grasshoppers, and crickets which were reported to have up to 77% protein content in the dry matter, the advantage of BSF is the survival rate and the efficiency of converting organic materials into their own biomass.

Black soldier fly (*Hermetia illucens*) life cycle (45 days total). They spend two stages of their life in the growing media as eggs (4 days) and larva stage (18 days). Only when they are transforming from pre-pupa (14 days) to adults (9 days) do they move away from the media to find a dry place to complete the metamorphosis.

1.2 Review of Literature

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 technologies for waste management is the bioconversion of side streams by insects (Čičková et al., 2015). Many insects naturally feed in organic wastes, converting biomass nutrients into their own biomass and reducing the amount of waste material. *Hermetia illucens* Linnaeus 1758 (Diptera: Stratiomyidae), better known as black soldier fly (BSF), is one of the most important species proposed as a converter of organic waste. BSF larvae can develop on a wide range of substrates, including agricultural by-products and organic waste (Tockner et al., 2011). One of the main advantages of using BSF as waste bio converter is that adult flies do not eat, thus avoiding any disease transmission risks (Sumner et al., 2002). BSF is reported as a good source of nutrients like proteins, lipids, minerals (Spranghers et al., 2016).

Recently Schlüter et al., 2016) compared different protein extraction methods from T. Molitor and *Hermetia illucens*. Most of these studies focused on protein extraction only. However, insects and especially BSF, contain other valuable biomolecules such as lipids and chitin.

The present work explores, for the first time, several systematic approaches to separate lipid, protein and chitin from BSF prepupae into three usable fractions, with homogeneous methodologies in a biorefinery-like cascade. Extraction methods, here developed at a laboratory scale, are based on total chemical extractions or enzymatic assisted extraction, and are designed and optimized in order to obtain the three fractions at the maximum level of purity in a subsequential homogeneous process.

BSF is not a pest, so its rearing requires no specific precautionary measures and it reduces the presence of harmful bacteria (Erickson et al., 2004; Liu et al., 2008) in contrast to other dipteran species such as the house fly, M. domestica. The list of 'services' that have been developed includes the conversion of liquid manure and other domestic and agro-industrial waste types into a source of animal proteins (Caruso et al., 2014). Because of the valuable nutrient content of the BSF larvae, they can be employed as the basis of a highly promising technology to sustain a circular economy, which is the concept of an economy that is producing no waste and reducing consumption of raw materials and energy by improving their utilization, based on the interrelationships between the environment and economics.

1.3 Instrumentation

A total of 4 instruments were used viz. Soxhlet apparatus, Rotary evaporator, Centrifuge, Kjeldhal apparatus. A Soxhlet extractor has 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. The centrifuge substitutes a similar, stronger, force for that of gravity. Every centrifuge contains a spinning vessel; there are many configurations, depending on use. Kjeldahl Apparatus determines organic nitrogen (n2) and protein contents in chemical substances. This estimation is done by the Kjeldahl digestion method. These units are widely used in the food, environmental, urea, and chemical industries. A rotary evaporator is a device used in labs to efficiently and gently remove solvents from samples by evaporation.

1.4 Results and Discussion

Oil was extracted and performed for 3 consecutive days using the Soxhlet apparatus. Hexane or petroleum ether was used. The heating mantle was set at 80 degrees. There was a continuous water supply.

A porcelain piece was also added to the solvent to avoid superheating of solvent and to make the distillation flask more stable. After three days the RB was attached to a rotary evaporator to separate hexane and oil. Weight was measured separately for each of the 3 RB. Sample Taken:10 g of larvae.

Table 1. Weight and % of oil.

Sample	Empty RB (gm)	Oil (gm)	%
1	123.520	129.105	55.8%
2	122.513	128.113	56.0%
3	148.792	154.196	54.04%

The iodine value experiment checks whether the oil is saturated or unsaturated. 10 g of oil was taken and chloroform was added to ensure proper mixing. Iodine solution was prepared in 15% KI solution. Burette was filled with 0.25 N sodium thiosulphate, and 1 % starch was used as an indicator.

Burette:0.25N $Na_2S_2O_3$

Indicator:1% starch solution (0.5) ml

End point: Blue to colorless.

Table 2. Blank Titration

Oil (gm)	Chloroform (ml)	Iodine (ml)	Starch (ml)	Reading (ml)
0	10	20	0.5	21.5

Table 3. Back Titration

Oil (gm)	Chloroform (ml)	Iodine (ml)	Starch (ml)	Reading (ml)
10	10	20	0.5	24.3

Result: By observing the readings and calculation the iodine value came out to be 7.1064 g, and by this we can conclude that the oil contains more saturated fat.

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

$$A = \frac{(24.3 - 21.5)\ \mathbf{x}\ 0.2\ \mathbf{x}\ 0.02538\ \mathbf{x}\ 100}{0.2}$$

$$A = 7.1064\mathrm{g}\ \frac{\mathrm{I}_2}{100\ g\ of\ fat}$$

1.4.1 Determination of Carbohydrates

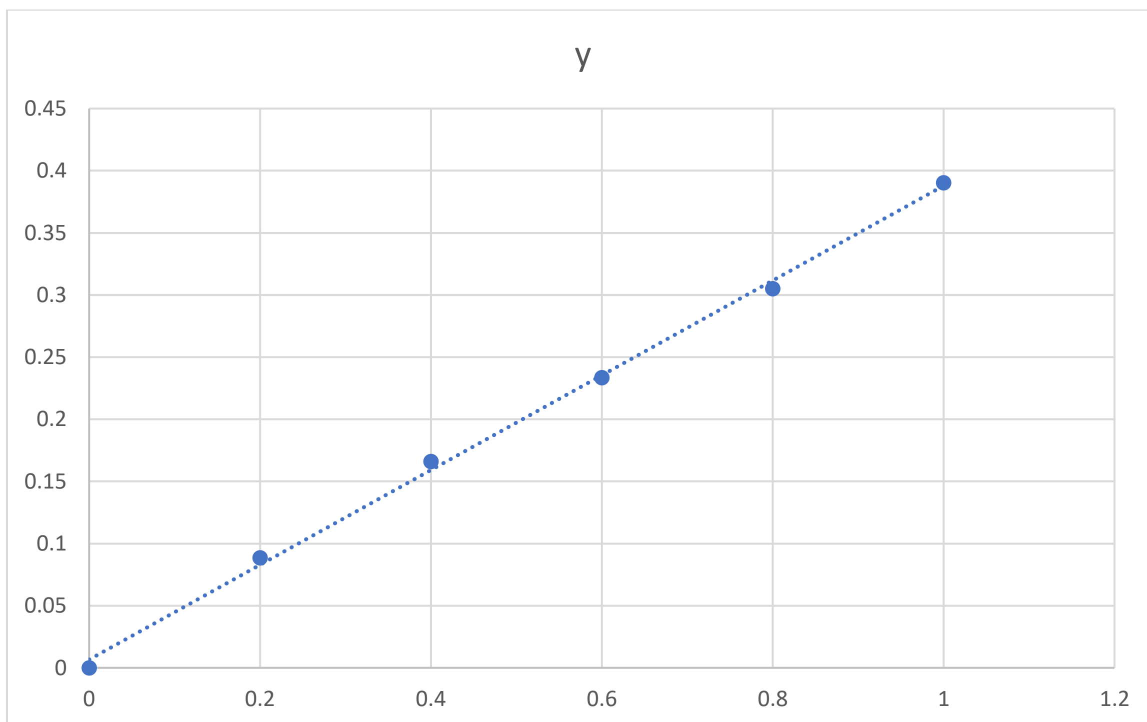
It was determined by the Anthrone method. Standard glucose was prepared along with the Anthrone reagent. Weighed 100mg of the defatted sample into a boiling tube. Hydrolysed it by keeping it in a boiling water bath for three hours with 5mL of 2.5 N-HCl and cooled it to room temperature. Neutralized it 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.

A boiling tube was required which was kept in the boiling water bath for 3 hours and hydrolyzed by 2.5 N HCl.

Table 4 . Observation table

<table>
<tr><th>Glucose (ml)</th><th>DW (ml)</th><th>Anthrone</th><th rowspan="4">Boil in bwb for 8 mins and cool rapidly.</th><th>OD at 630 (nm)</th><th>Glucose (mg)</th></tr>
<tr><td>0.0</td><td>1.0</td><td>4.0</td><td>0.0</td><td>0.0</td></tr>
<tr><td>0.2</td><td>0.8</td><td>4.0</td><td>0.0885</td><td>0.02</td></tr>
<tr><td>0.4</td><td>0.6</td><td>4.0</td><td>0.1661</td><td>0.04</td></tr>
</table>

0.6	0.4	4.0		0.2335	0.06
0.8	0.2	4.0		0.3050	0.08
1.0	0.0	4.0		0.3904	0.1



Amount of carbohydrate present in 100mg of the sample:

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

Table 5.Readings of Sample 1

Sample	D.W	Anthrone		Od At 630nm
0.2	0.8	4.0	Boil for 8 mins and cool	0.2019
0.5	0.5	4.0		0.5270

10 g of larvae contains 1.1751 g of carbohydrate.

Table 6.Readings of Sample 2

Sample	D.W	Anthrone		OD at 630nm
0.2	0.8	4.0	Boil for 8 mins and cool	0.2121
0.5	0.5	4.0		0.5440

10 g larvae contain 1.1922 g of carbohydrate.

% of carbohydrate

$$Sample\ 1 = \frac{1.1751 \times 100}{10} = 11.75\%$$

$$Sample\ 2 = \frac{1.192 \times 100}{10} = 11.92\%$$

1.4.2 Protein Estimation.

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. The amount of protein is calculated with the help of a standard graph of BSA (Bovine Serum Albumin) protein.

1.4.2.1 Kjeldhal method

0.5 N HCL

0.5 N NaOH

Potassium sulfate K_2SO_4, catalyst ($CuSO_4.5H_2O$), 45% NaOH – 45 g NaOH in 100 ml D.W., and phenolphthalein indicator were used.

Procedure:

A. Digestion:

Weigh 0.5 g of sample and add 6-7 ml of conc. H_2SO_4 in digestion flask. Add 3.5 g of K_2SO_4 and a pinch of copper sulfate. Heat the mixture for 1 hour(direct heating), cool, add 10-20 ml of DW, and filter it. Make the volume to 50 ml in a volumetric flask.

B. **Distillation**:

Take 10 ml of the above solution and raise the pH of the mixture using 45% NaOH solution. Transfer this mixture to the Kjeldhal flask. Place 100 ml of the conical flask containing 40 ml of 0.5 N HCL with the condenser's tip dipping below the trapping solution's surface (0.5N HCL). Distill and collect the ammonia in HCL solution for 20 mins. Rinse the tip of the condenser.

C. Titration:

Titrate the trapping solution against the 0.5 N NaOH using a phenolphthalein indicator, till the appearance of a pink color which is the endpoint.

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

Table 7.

Sample	**NaOH (ml)**
1	35.9
2	36.1

$$Crude\ protein\ =\ \%\ Nitrogen\ \mathbf{x}\ 6.25 = 34.125\ \%$$

Sample 1:

$$\%\ Nitrogen\ \ = \frac{1.4007\ x\ (V1 - V5)\ x\ N}{W}$$

$$\%\ Nitrogen\ \ = \frac{1.4007\ \mathrm{x}\ (39.7 -\ 35.9)\ \mathrm{x}\ 0.5}{0.5}$$

$$\% \, Nitrogen = 5.32\%$$

$$Crude\ protein = 5.32\ x\ 6.25 = 33.25\ \%$$

Sample 2:

$$\% \, Nitrogen = \frac{1.4007 \text{ x } (39.7 - 36.1) \text{ x } 0.5}{0.5}$$

$$\% \, Nitrogen = 5.042\%$$

$$Crude\ protein = 5.04\ x\ 6.25 = 31.5\%$$

1.4.2.2 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. A standard graph is also prepared.

Table 8. Standard graph of BSA protein.

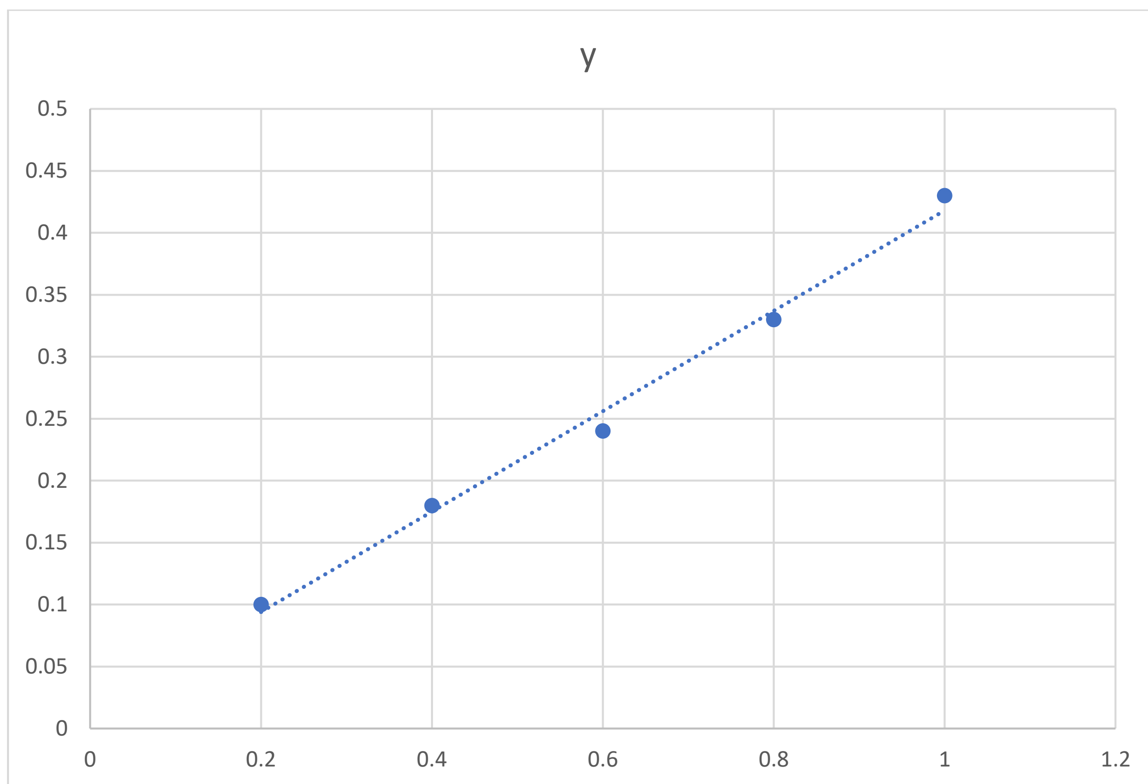
Protein (ml)	Protein (mg)	DW (ml)	Lowry (ml)	Mix and wait for 15 mins	Folin (ml)	Mix and wait for 30 minutes in the dark.	OD at 660 nm
0	0	1.0	3		0.5		0
0.2	0.04	0.8	3		0.5		0.10
0.4	0.08	0.6	3		0.5		0.18
0.6	0.12	0.4	3		0.5		0.24
0.8	0.16	0.2	3		0.5		0.33
1.0	0.20	0.0	3		0.5		0.43

The protein came out to be:

For 20 dilution times, the protein is **2.570 g**

For 30 dilution times, the protein is **2.5815 g**

Figure 1. Standard graph of BSA



1.5. Learning Outcomes

1)Understanding the life cycle and morphology of BSF larvae, which included the different colors they possess like yellow, green, black, or blue. Adult BSF can also mimic insects like bees and wasps. Female black soldier fly lays up to 500 eggs in crevices or organic matter and the eggs hatch only in 4 days. The life cycle is short we can say.

2)The larvae are not considered pests and act as effective manure recyclers, generating food for fish and other animals.

3) 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 make them an eco-friendly solution to the environment.

4) Soxhlet method proved to be effective in lipid extraction from BSFL.

1.6. Future Scope

BSF larvae have significant potential as a sustainable protein source for various applications like animal feed which is rich in protein and can be used for livestock, and poultry.

Also, they are efficient organic waste decomposers including food scraps and agriculture by-products into compost and reducing landfills.

By converting waste into high-quality biomass, they also contribute to the soil and promote sustainable agriculture practices. There can be more chances to enhance techniques like growth rate, protein content, and waste conversion efficiency for more robust production.

Future research endeavors 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.

1.7. Conclusions

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

1) By oil extraction method, the percentage of oil came out to be: a) 55.8% b) 56% c) 54.04%

2) BSF larvae oil is saturated by conducting the iodine experiment which came out to be 35.53 g /I2/ 100 g of fat.

3)The protein content in the BSF larvae was:

a) In sample 1: 33.25%

b) In sample 2: 31.5%

4)Carbohydrate present in the larvae which were measured by the Anthrone method came out to be, along with the %:

a) Sample 1: 1.1751 g (11.75%)

b) Sample 2: 1.1922 g (11.92%)

1.8. References

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