BIOLOGICAL RECOVERY OF PHA FROM BACTERIAL CELLS USING MEALWORMS (Tenebrio molitor) AND BLACK SOLDIER FLY LARVAE (Hermetia illucens)

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

3HHx	3-hydroxyalkanoate
α	Alpha
AOAC	Association of official analytical chemists
Å	Angstrom
ATPS	Aqueous two-phase system
NH_4Cl	Ammonium chloride
Na_2SO_4	Anhydrous sodium sulphate
BSF	Black soldier fly
β	Beta
cm	Centimetre
CME	Caprylic methyl ester
C: N	Carbon: nitrogen
°C	Degree Celsius
°C/min	Degree per minute
DSC	Differential scanning calorimeter
DM	Dry matter
FTIR	Fourier-transform infrared
FESEM	Field emission scanning electron microscope
g	Gram
GC	Gas chromatography
T_g	Glass transition temperature
h	Hour
HDPE	High density polyethylene

Hz	Hertz
JH	Juvenile hormone
Kg	Kilogram
kPa	Kilopascal
kV	kilovolt
λ	Lambda
L	Litre
LDPE	Low-density polyethylene
M	Molarity
mol%	Molar percentage
mL	Millilitre
mL/min	Millilitre per minute
min	Minute
m	Metre
mm	Millimetre
mg	Milligram
MM	Mineral media
mcl	Medium chain length
µL	Microlitre
θ	Theta
%	Percentage
±	Plus-minus
P: C	Protein: carbohydrate
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoates

PLA	Poly (lactic acid)
PTFE	Poly (tetrafluoroethylene)
PVC	Polyvinyl chloride
PP	Polypropylene
PBS	Polybutylene succinate
RNA	Ribonucleic acid
R.H	Relative humidity
cm^{-1}	Reciprocal centimetre
rpm	Rotation per minute
s	Seconds
scl	Short chain length
SEM	Scanning electron microscopy
$NaNO_3$	Sodium nitrate
SDS	Sodium dodecyl sulfate
SDBS	Sodium dodecyl benzenesulfonate
NaOH	Sodium hydroxide
NaClO	Sodium hypochlorite
SCF	Supercritical fluid
SD	Sprague Dawley
T_m	Melting temperature
v/v	Volume per volume
W	Watts
w/v	Weight per volume
w/w	Weight per weight
wt%	Weight percentage

µm	Micrometre
HA	Hydroxyalkanoates
P(3HB)	Poly (3-hydroxybutyrate)
P(3HB-*co*-mol% 3HHx)	Poly (3-hydroxybutyrate-*co*-mol% 3-hydroxyhexanoate)
QC	Quality control
WAXD	Wide-angle X-ray diffraction

PENULENAN BIOLOGI PHA DARIPADA SEL BAKTERIA DENGAN MENGGUNAKAN ULAT ROTI (*Tenebrio molitor*) DAN LARVA LALAT ASKAR HITAM (*Hermetia illucens*)

ABSTRAK

Pengeluaran polimer polihidroksialkanoat (PHA) boleh dihasilkan dalam kuantiti yang banyak melalui fermentasi dan boleh diekstrak secara kimia dan biologi daripada sel-sel bakteria untuk menghasilkan barangan plastik terbiodegradasi yang bernilai. Walau bagaimanapun, penulenan PHA daripada sel-sel bakteria menggunakan kloroform adalah berbahaya dan mahal. Selain itu, kaedah penyediaan sel-sel bakteria sebagai bahan makanan yang sesuai diberi kepada serangga, melalui proses pengeringan beku dan pengeringan menggunakan haba adalah tidak mesra alam, oleh itu, menghalang pengeluaran PHA secara komersial. Agen penulenan PHA yang pernah digunakan adalah tikus albino dan ulat roti (*Tenebrio molitor*) dimana ulat roti (kaedah semasa), mempunyai ~100% penulenan dengan kandungan PHA melebihi 90%. Walau bagaimanapun, keadah ini memerlukan sel-sel bakteria dalam keadaan kering sebelum diberi kepada serangga. Oleh itu, ia adalah berfaedah untuk menggunakan sel-sel bakteria dalam keadaan basah (selepas fermentasi) untuk diberi kepada serangga untuk penulenan PHA. Larva lalat askar hitam (BSF) (*Hermetia illucens*) merupakan serangga yang sangat menarik. Ia merupakan pemakan yang rakus yang boleh menghadam pelbagai jenis bahan organik yang mengandungi kelembapan antara 50-80%, secara balasannya bertukar menjadi biojisim serangga. Dalam penyelidikan ini, larva BSF diberi makan dengan sel-sel bakteria (selepas fermentasi) manakala ulat roti diberi makan dengan sel-sel bakteria yang kering. Fras serangga yang mengandungi PHA, diperolehi melalui kaedah penulenan

menggunakan air dan natrium hidroksida dan kemudiannya dicirikan dan dibandingkan dengan PHA yang diekstrak menggunakan kloroform. Pengimbasan mikrograf menggunakan FESEM (mikroskop elektron pengimbasan pelepasan medan) menunjukkan kandungan PHA serta sel-sel bakteria yang tidak dihadam dalam fras larva BSF manakala fras ulat roti tidak menunjukkan sebarang kesan sel-sel bakteria. Analisis GC (kromatografi gas) menunjukkan kandungan PHA sebanyak 66 ± 2.6% dalam fras larva BSF dan meningkat sehingga 90 ± 3.3% selepas dicuci. Sebagai perbandingan, fras ulat roti mengandungi 74 ± 2.6% PHA, dan meningkat sehingga 92 ± 1.1%, selepas dicuci. Analisis WAXD (pengurai cahaya sinar-X sudut lebar) menunjukkan bahawa granul PHA daripada larva BSF adalah lebih amorfus daripada granul PHA ulat roti. Polimer yang dibersihkan daripada larva BSF menunjukkan dua titik peleburan (Tm), dengan titik peleburan pertama pada 124°C dan titik kedua pada 136°C, berbanding dengan ulat roti yang mempunyai hanya satu titik peleburan pada 137°C. Di samping itu, kandungan lipid larva BSF meningkat sehingga 26.1 ± 0.0% daripada nilai asas 21.9 ± 0.0% apabila diberi makan sel-sel bakteria yang berasaskan protein, berbanding dengan ulat roti, yang berkurang daripada 17.8 ± 0.4% sehingga 8.3 ± 0.2%. Hasil kajian ini mengesahkan bahawa larva lalat askar hitam merupakan agen biologi yang sesuai dalam meringankan proses bawah aliran yang mahal untuk penulenan PHA daripada sel-sel bakteria (selepas fermentasi) tanpa melibatkan proses pengeringan.

BIOLOGICAL RECOVERY OF PHA FROM BACTERIAL CELLS USING MEALWORMS (*Tenebrio molitor*) AND BLACK SOLDIER FLY LARVAE (*Hermetia illucens*)

ABSTRACT

The production of polyhydroxyalkanoates (PHA) can be upscaled through fermentation and be chemically and biologically recovered from the bacterial cells to produce valuable plastic wares. PHA can be chemically recovered by using chloroform, but it is hazardous and costly. For biological recovery, the bacterial cells must be dry prior, to be a suitable insect feed through freeze-drying or drum-drying. These drying processes are not environmentally friendly, thus, hindering the commercial productions of PHA. The biological methods of recovering PHA is by using agents such as rats and mealworms (*Tenebrio molitor*), in which PHA recovered using mealworms (current method) had ~100% recovery with PHA purity >90%. However, this method of recovery requires cells to be dry prior to feeding. It is advantageous to utilize cells in wet form (post-fermentation) to feed directly to insects for PHA recovery. The Black Soldier Fly (BSF) larvae (*Hermetia illucens*) is an organism of interest. They are voracious eaters that can convert a wide array of organic matter with moisture content ranging between 50-80% into insect biomass. In this research, the BSF larvae were fed with wet bacterial cells (post-fermentation), while mealworms were fed with dried bacterial cells. The PHA was recovered from its faecal pellets and purified using water and diluted sodium hydroxide. The characteristics and purity of PHA recovered were compared to chloroform-extracted PHA. Field emission scanning electron microscope (FESEM) micrographs revealed the presence of PHA and undigested bacterial cells in the faecal pellets of BSF larvae, meanwhile,

undigested bacterial cells were absent in mealworm faecal pellets. Gas chromatography (GC) analysis showed a 66 ± 2.6% PHA content in the faecal pellets of BSF larvae, which increased to 90 ± 3.3% post-purification. In comparison, faecal pellets of mealworms contained about 74 ± 2.6% PHA, and up to 99 ± 1.1%, after purification. Wide-angle X-ray diffraction (WAXD) analysis indicated that the recovered granules from BSF larvae were more amorphous than those from mealworms. The purified polymers from BSF larvae exhibited two melting points (Tm), with the first melting point at 124°C and the second point at 136°C when annealed, contrary to mealworms with only one melting point at 137°C. Additionally, the lipid content of BSF larvae increased up to 26.1 ± 0.0% from a baseline value of 21.9 ± 0.0% upon being fed with bacterial cells., In contrast, the lipid content of mealworms decreased from 17.8 ± 0.4% to 8.3 ± 0.2%. This study indicates that BSF larvae can be a suitable candidate in alleviating the costly downstream processes, by the recovery of PHA from post-fermentation bacterial cells without the need to dry the cells.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Over the years, several modes of recovering polyhydroxyalkanoates (PHA) from bacterial cells have been explored and established, from using halogenated solvents and ketones to digestion methods involving surfactants and enzymes (Berger et al., 1989; Yasotha et al., 2006). The extraction of PHA using halogenated solvents were greatly effective, yielding PHAs with purities >90% while retaining its high molecular weight. However, large quantities of highly volatile solvents are needed for extraction, which inevitably, increases capital cost and operational expenses. Furthermore, the process is time-consuming and requires high energy throughput. The heat and chemical process during solvent extractions could cause depolymerization, reduce the molecular weight of PHA and a loss of mechanical properties, thus affecting the quality of PHA. In an attempt to overcome these setbacks, various digestion methods were tested but the results were varying from one detergent/solution to another. Certain solutions resulted in the severe reduction of molecular weight of the PHAs extracted, while having a purity >80%. Although these methods proved effective to a certain extent, the commercialization of PHA is hindered as these processes are not sustainable or economical. With the increase in demand due to their biodegradable properties and the prospect of overturning the petroleum-based plastic economy, a sustainable production must be achieved. In the last decade, researchers (Kunasundari et al., 2013; Murugan et al., 2016; Ong, Kho, et al., 2018; Zainab-L & Sudesh, 2019) have successfully demonstrated the recovery of PHA using biological agents such as Sprague Dawley rats and mealworms by feeding them with freeze-dried bacterial cells. Mealworms are researched extensively in aquaculture and in poultry farming as feed,

for its high protein content (Bovera et al., 2016; Ng et al., 2001; Ramos-Elorduy et al., 2002). At present, the recovery works using mealworms are in the process of upscaling by feeding and obtaining the undigestible PHA granules from dried bacterial cells in large quantities of kilograms (*unpublished data*). The efficiency of recovery is almost 100% as mealworms are incapable of digesting the PHA. The process of producing dried bacterial cells involves the use of commercial drum dryer which is more effective than freeze-dryers. However, both drying processes are expensive and require high energy, that is deemed unsustainable in the long haul. This research reports the idea of recovering PHA from wet bacterial cells (post-fermentation) using Black Soldier Fly (BSF) larvae (*Hermetia illucens*) and comparing these findings with mealworms. This insect species undergoes complete metamorphosis much like mealworms (*Tenebrio molitor*), going through 4 main stages of development: egg, larva, pupa, and adult, with the larval stage being the longest phase of development. The BSF larvae are high-profile insects making headlines in the animal feed industry. They are highly sought-after for sustainable animal feed, chitosan, and biodiesel production, not to mention its lower land space usage for cultivation, low carbon emission, and exponential growth. This insect species is also used in organic waste treatments. They are also voracious eaters that can convert a wide array of organic matter with moisture content ranging between 50-80% into insect biomass (Tomberlin et al., 2005; Banks et al., 2014; Nguyen et al., 2015), which makes them suitable candidate for this research. In this study, the BSF larvae were fed with wet bacterial cells, and their faecal pellets were examined under a scanning electron microscope to determine the capability of the larvae to digest the bacterial cell wall and recover PHA granules from the cell cytoplasm. The faecal pellets obtained were further subjected to purification steps

using water and dilute solutions of sodium hydroxide and sodium hypochlorite. The resulting PHA was then characterized to determine its purity and properties.

1.2 Research Objectives

The objectives of this study are as follows:

a) To evaluate the capability of black soldier fly larvae in recovering PHA from wet bacterial cells in respective to mealworms.
b) To characterize the recovered PHA from black soldier fly larvae and mealworms.
c) To conduct proximate analysis of the black soldier fly larvae.

CHAPTER 2

LITERATURE REVIEW

2.1 Plastics: Petroleum based-, biobased-, and biodegradable

Plastics are accessible and useful materials for everyday utilization. There are several petroleum hydrocarbon derivatives such as polyethylene terephthalate (PET), high density polyethylene (HDPE), polyvinyl chloride (PVC), low-density polyethylene (LDPE) and polypropylene (PP) that are used extensively in the automotive industry, in consumer products, construction, and packaging (Marsh & Bugusu, 2007). The importance of plastics was emphasized greatly during the pandemic with the rise in need of personal protective equipment such as face masks, single-use surgical tools and gloves (Hamann et al., 2014). Plastics are durable, lightweight, and cheaper due to mass production and consumerism. Its many chemical and physical properties make it an ever more desirable product. However, these plastics are non-biodegradable. Their persistence in the environment harms the ecosystem, polluting the ocean, further endangering wildlife. In addition, plastics are produced from crude oil (non-renewable) found on the ocean floor. The excavation of this resource emits greenhouse gases, contributing to global warming. The Center for Biological Diversity released an article stating that the demand for petroleum-based plastics is expected to rise by 40% in 10 years, which will only overflow landfills. In an attempt to tackle environmental concerns caused by petroleum-based plastics, bio-based polymers were developed. A few examples of widely studied bio-based polymers are polyhydroxyalkanoates (PHA) that are synthesized in bacteria, polylactic acid (PLA) through bio-chemical synthesis, and polybutylene succinate (PBS) synthesized from renewable raw materials such as cassava or corn. Other bio-based plastics also include starch-based plastics, protein (soybean protein) based plastics,

and cellulose-blended plastics. The current pursuit of producing bio-based and biodegradable plastics is geared towards sustainable production. However, it is to be clear that not all bio-based plastics are biodegradable. In the year 2009, Coca-Cola, a big-name company, endeavored a plastic-reducing scheme by incorporating their packaging with 70% PET and 30% plant material. Although companies like Dasani and Heinz have also implemented similar ideas, only the plant material portion of the packaging will degrade, and the PET will still remain. An effective solution would be to create biodegradable biopolymers that mimic conventional plastics. More than a decade later, Coca-Cola presented its first prototype plant-based bottle made out of 100% plant-based paraxylene (bPX) (Coca-Cola collaborates, 2024).

2.2 Polyhydroxyalkanoates (PHA)

In the early 1900s, lipid-like inclusions were discovered in *Azotobacter chroococcum*. Soon after, many more bacterial species such as *Bacillus megaterium*, *Pseudomonas putida* KT2440, *Bacillus cereus*, and *Cupriavidus necator* were identified to have similar inclusions and the term P(3HB) was coined by French microbiologist, Maurice Lemoigne. By the late 1980s, it was discovered that gram-positive, gram-negative and archaebacteria amongst others could produce P(3HB) and different types of hydroxyalkanoates (HA) collectively known as polyhydroxyalkanoates (PHA). PHA, which is found in the bacterial cell cytoplasm is in the form of granules and is produced under certain nutrient deficit such as nitrogen, phosphorus, magnesium, sulfur, or oxygen (Madison & Huisman, 1999; Valdés-García et al., 2017). The granules consist of a PHA core, and it is surrounded by phospholipid monolayer, and granule-associated proteins such as Phasins, PHA synthases, and depolymerases (Pötter et al., 2002; Pötter & Steinbüchel, 2005). The Phasins influence

granular size, distribution, and quantity while the enzymes influence PHA synthesis and degradation. The PHA granules ranges at an average size of 0.2 – 0.5 μm in diameter. Although many wild type bacteria, and recombinant strains are able to produce PHA, not all are well-suited for upscaled production of PHA. The *C. necator* H16 has been one of the most studied strains to date. Researchers have explored various substrates for the production of PHA such as waste cooking oil, crude palm kernel oil, jatropha oil, rapeseed oil, glucose, fructose, and so forth (Kahar et al., 2004; Loo et al., 2005; Obruca et al., 2010; Verlinden et al., 2011; Zainab-L et al., 2018). However, vegetable oils are preferred over sugars because it is cost-effective, contains high carbon and ferments easily (Guo-Qiang, 2010). The visualization of PHA using stains such as Sudan Black B, Nile red and Nile blue (Ostle & Holt, 1982), under a phase-contrast microscope and transmission electron microscope (TEM) (Doi et al., 1990; Sudesh et al., 2000). Bacterial cells can accumulate about 8 to 12 PHA granules without having adverse effect on its osmotic pressure (Sudesh et al., 2000).

2.2.1 Challenges in commercialization of PHA

There are several drawbacks involved in the commercialization of PHA in the upstream, midstream, and downstream processes. A bottleneck in the upstream process, is the use of pure cultures cultivated in a sterile environment that is vital to initiate fermentation. Contamination in any stage of cultivation process could halt production and affect the quality of cell biomass produced. The conventional pure-culture fermentation can be replaced with open mixed cultures that enables continuous fermentation under non-sterile conditions (Kleerebezem & van Loosdrecht, 2007; Temudo et al., 2008). Through open mixed cultures, a low-cost production of PHA can be achieved without the need to sterilise the bioreactor (Reis et al., 2003). Other

researchers have reported production of PHA up to 89 wt% (Moita et al., 2014) and the successful cultivation of open mixed cultures on lactate (Y. Jiang et al., 2011). Apart from that, recombinant strains can reduce production cost. In the midstream process, carbon sources are a financial bottleneck. Replacing expensive substrates with cheaper alternatives such as molasses, sugarcane bagasse, waste cooking oil, volatile fatty acids waste, and crude palm kernel oil (Bhubalan et al., 2010; H. Chen et al., 2013; Kumar et al., 2009; Silva et al., 2004) would lessen the cost tremendously. Aside from that, the downstream process is another costly process that involves large quantities of solvents for PHA recovery (Choi & Lee, 1997; Kourmentza et al., 2017). Although, chemical recovery of PHA yield higher purity of polymer, but it is at the expense of the environment and well-being of the personnel. The current production cost of conventional plastics is still cheaper than that of PHA production. However, researchers have been attempting to utilize renewable, low-cost carbon sources to achieve mass production of PHA (Sudesh, 2012b).

2.3 Conventional methods of PHA recovery

There are several techniques to isolate and purify PHA from bacterial cells. The two common methods of PHA recovery are extraction and digestion. Extraction methods involve solvents which solubilize PHA granules, followed by precipitation using non-solvents such as methanol or ethanol (Jacquel et al., 2008; Ramsay et al., 1990). Some of the solubilizing agents used by researchers are chloroform, 1,2-dichloroethane, ethylene carbonate, methylene chloride and even ketones such as acetone (Fiorese et al., 2009; Ramsay et al., 1994b; Valappil et al., 2007; Zinn et al., 2003). Digestion methods involving chemicals or enzymes are used to solubilize cellular materials enclosing the PHA granules. Chemicals such as sodium hypochlorite

were reported to yield up to 86% PHA with 93-98% purity (Hahn et al., 1994). Similar results were mentioned in other reports using a combination of surfactants such as sodium dodecyl sulfate (SDS), Triton X-100, or betaine paired with chelates, sodium hypochlorite or chloroform (Dong & Sun, 2000; Lakshman & Shamala, 2006; Ramsay et al., 1990; Valappil et al., 2007). Although chemicals and solvents would result in higher purity of PHA, these methods however, are not environmentally friendly or cost efficient. The chemicals are volatile and toxic and must be used in large volume to obtain a certain amount of PHA.

Apart from this, bead mills and homogenizers can be used (Tamer et al., 1998). These methods do not require chemicals, and the chance of contamination is less. However, this process is time consuming. Homogenizers may also cause thermal degradation of PHA granules as well as micronization that would affect the downstream process. Other unique methods of recovery include supercritical fluid (SCF), cell fragility, gamma radiation, floatation, and aqueous two-phase system (ATPS). SCF is a straight-forward method technique that is gentle to the environment and less expensive. However, it is challenging to work with natural materials (Hejazi et al., 2003). Cell fragility on the other hand is a simple method but requires skills to strike a balance between cell wall softening and cell wall rupture (Divyashree & Shamala, 2010). Likewise, ATPS also requires a skillset to understand the complex mechanism and improve parameters. This method yields a high amount of PHA, has a short process time, and has a low energy throughput (Divyashree et al., 2009). Floatation methods work when substantial amounts of volatile solvents are used even though it is a fairly easy method (Ibrahim & Steinbüchel, 2009). Gamma irradiation is a method that uses less chemicals but has a high capital cost (Divyashree & Shamala, 2009).

Table 2.1: The various methods of PHA recovery

Extraction Methods	Comments	Results	Reference
Solvent extraction	Mixed solvent, microwave irradiation	Purity: 98%	(Yu & Chen, 2006)
	Methyl *tert*-butyl ether	Yield: 15017 wt%	(Wampfler et al., 2010)
	Acetone/isopropanol for P(HB-*co*-HHx)	Up to 98–99% yield	(García et al., 2024)
	Non-halogenated green solvents (e.g., ketones, lactones)	Eco-friendly alternative	(Liang et al., 2025)
	SDS, Triton X-100	Purity: 94%, 80% yield	(Hahn et al., 1994)
Digestion method	Common method, low cost, damages PHA	Purity: 75%, 50% yield	(Choi & Lee, 1999)
Surfactant	Digestion of mixed microbial culture biomass	High purity, ~74% yield	(Inoue et al., 2025)
Sodium hypochlorite	Enzyme combined with SDS-EDTA	Purity: 93%	(Kathiraser et al., 2007)

NaOH Digestion (MMC)	Proteases (scalability issues)	High purity (~97–98%)	(García et al., 2024)
Mechanical disruption	Bead milling, sonication, scalable solvent-free	Moderate to high yield	(García-Chumillas et al., 2024)
	Pestle/mortar, bead vortex, sonication	34–45% PHB yield	(Bhat et al., 2024)

2.4 Biological recovery of PHA

The recent advancement in the recovery of PHA is by using insects/animals, first published by Kunasundari and coworkers (Kunasundari et al., 2013, 2016). This novel method marks the end of using harsh and expensive chemicals and solvents. In their research, Sprague Dawley (SD) rats were fed with lyophilized cells of *C. necator* H16, as a single cell protein feed (SCP) containing 39 wt% poly (3-hydroxybutyrate) [P(3HB)]. The resulting faecal pellets were white-coloured and contained 87-90 wt% P(3HB). The P(3HB) purity increased significantly after washing with water and 2 wt% sodium dodecyl sulfate (SDS) or sodium dodecyl benzenesulfonate (SDBS). Further characterization analysis conducted by the research team, revealed that the P(3HB) recovered were comparable to P(3HB) extracted using chloroform. Although, PHA was successfully recovered, the rats, however, had poor weight gain compared to the control group.

In the coming years, Murugan and coworkers (Murugan et al., 2016) ventured further by using yellow mealworms to replace SD rats. In his research, mealworms were fed with lyophilized cells of recombinant *C. necator* Re2058/pCB113 containing

54 wt% Poly (3-hydroxybutyrate-*co*-25 mol% 3-hydroxyhexanoate) [P(3HB-*co*-25 mol% 3HHx)]. The resulting faecal pellets were washed with water and 1% SDS which resulted in a purity of 89% and 100%, respectively. This result proved that mealworms were able to digest the cell biomass and excrete PHA granules.

Over the next few years, Ong and colleagues (Ong, Kho, et al., 2018), ventured into synthesizing cells biomass from waste animal fats as primary carbon source to be fed to mealworms. Additionally, optimisation of purification methods was also conducted. In their study, faecal pellets from mealworms were first washed with distilled water and afterwards with 0.01 M NaOH, and 0.05 M NaOH respectively. Later, Zainab and colleagues improved that consumption of cell biomass by mealworms and subsequently the PHA purity (Zainab-L & Sudesh, 2019). The research hypothesized that the high salt content was the cause for the low feeding efficiency and low PHA purity. Upon, removal of excess salt, the mealworms increased its feeding efficiency and turned into a high protein biomass. Furthermore, a maximum PHA recovery was achieved through this process. The selection of an appropriate animal model for the recovery of PHA was done carefully.

The use of large organism like ruminants requires large space to breed and raise, which would incur high cost. Moreover, this organism possesses complex digestive systems that may severely reduce the molecular size of PHA. Also, the high levels of ribonucleic acid (RNA) in bacterial cells may not be stomached by various organism (Edozien et al., 1970). Hence, a much smaller organism would be the wiser choice. Insects such as superworms, mealworms, cockroaches and cricket were tried and tested in the recovery of PHA and produced favourable results (Ong, Zainab-L, et al., 2018). These insects can be reared in large quantities in small area setting.

Mealworms were extensively used in the recovery of PHA as highlighted in the various published works. There are many types of mealworms; the yellow mealworm (*Tenebrio molitor* L.), dark mealworm (*Tenebrio obscurus*) and giant mealworm (*Zophobas morio*). The yellow mealworm is of great interest partly because it is commonly found in the grain business (Howard, 1955). Yellow mealworms hereafter denoted as mealworms are larvae of darkling beetles from the order Coleoptera, genus Tenebrio. The name 'darkling' is derived from the Latin word 'darkness' and is found typically in dark places and are nocturnal in nature. They are found across Europe and Asia. This 'pest' mostly consumes wheat bran, cereals, barley, maize, flour, oats and even chaffs. However, mealworm larvae have grown to be a popular feed in the aquaculture (Ng et al., 2001) industry including recreational fishing, in broiler feed industry and even aviculture (Makkar et al., 2014; Ramos-Elorduy et al., 2002; Sánchez-Muros et al., 2014).

The application of mealworms is seen in in-silico and food-safety screening. In silico and LC-MS studies on mealworm's hydrolysed proteins identified peptides with predicted antimicrobial action against bacteria, fungi, and viruses—suggesting its applications in food safety and healthcare (Gonzalez-de la Rosa et al., 2025). Mealworms are also an efficient and sustainable source of chitin and chitosan that is widely used in biomedicine, wastewater treatment and food packaging with extractions yields that are comparable to crustacean by-products (Son et al., 2021). Mealworms are also used as a functional ingredient in human food. Its protein hydrolysates contains bioactive peptides with antioxidant, anti-inflammatory, ACE-inhibitory, antithrombotic, lipase-inhibitory, hepatoprotective, and anti-adipogenic activities which enhances the nutritional value and protein digestibility in breads, pasta and meat analogs (Chewaka et al., 2023; Muñoz-Seijas et al., 2025). Mealworms are also a rich

protein substitute for fishmeal and soybean meal- promoting gut health and increased immune function in poultry and fish (Jeong et al., 2020; Zacharis et al., 2024).

Mealworms have 4 main stages of growth and development: eggs, larvae, pupae and finally adults with each stage being unique from one another (complete metamorphosis) (Figure 2.1). The eggs of mealworms are whitish, oval in-shape and is minute to be seen with the naked eye (Cotton, 1940). The eggs are approximately 2 mm in length and is often laid individually or in cluster (Lyon, 1930). The eggs hatch within 10 to 14 days of incubation.

The newly emerged larvae feeds on the food source it was laid in, moults up to 12-20 times before reaching approximately 10-28 mm in length before pupating. With each moulting, the larvae shed its old cuticle and appears white before progressively forming its honey-coloured cuticles, hence its name, yellow mealworms. The larval phase lasts between 3 to 4 months. When the larvae are satiated and ready to become pupae, they moult one last time and curl into a foetus-like position. The pupae in its dormant feeding state, are approximately 10-15 mm in length and incubates for 12 to 20 days before pupating into beetles (Lyon, 1930). The pupae undergo rhythmic contractions followed by intense contractions before the exuviae is shed completely (Howard, 1955). The newly emerged beetles are always white in-colour before gradually changing into brown and finally black when sexually matured. The beetles live about 1 to 3 months before the female oviposits anywhere between 200 to 600 eggs (Cotton, 1940).

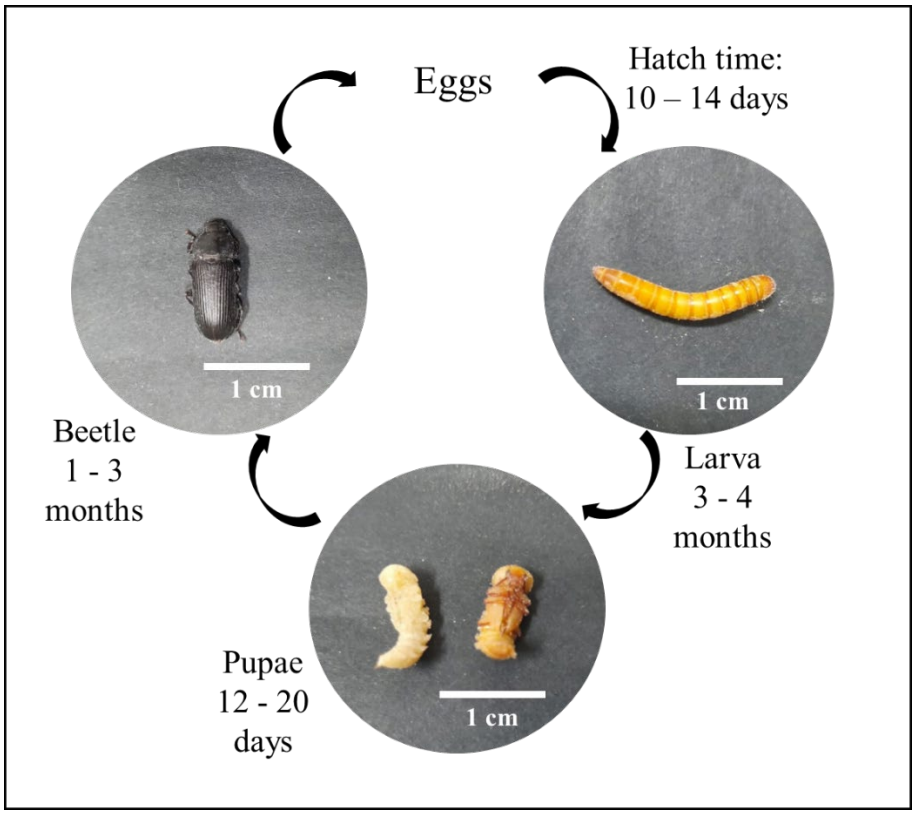

Figure 2.1: The life cycle of darkling beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae) with 4 important stages of development: eggs, larvae, pupae, and adult. The larval stage is used in experimental feeding due to its long development and active feeding phase.

The growth and development of mealworms are influenced by environmental temperature, relative humidity, diet, population density (Morales-Ramos et al., 2010) and photoperiodism. In terms of diet, mealworms have chewing mouthparts; therefore, it typically feeds on various grains which is usually dry in nature. It was reported by researchers (Murray, 1960), that mealworms responds differently to various dry feeds but have a strong affinity towards wheat bran. In present day, a vast majority of mealworm breeders use wheat brans as primary feedstock due to its affordability. Additionally, mealworms also respond positively on wheat bran in terms of weight gain and survivability. In industrial settings, mealworms are provided with water

through damp cloth, water sprayed over the feed or through vegetables. These larvae can thrive in dry conditions and obtain water from the air and feed (Dossey et al., 2016). However, the water content in feed must be carefully regulated as it encourages the growth of moulds that will decrease mealworms appetite, resulting in smaller pupae and adults (Howard, 1955). Furthermore, the relative humidity (RH) is an important parameter to be considered. A RH more than 85% could also cause moulds to grow meanwhile RH less than 13% could stunt mealworm growth and cannibalism (Fraenkel, 1950). Besides that, suitable temperatures are vital for the optimal growth of mealworms. Temperatures between 15 to 40°C gives better feed conversion ratio. Mealworms have been reported to have developed optimally at temperatures between 25 to 30°C (Kim et al., 2015; Koo et al., 2013) meanwhile other reports mentioned temperatures between 10 to 35°C (Ludwig & Fiore, 1960; Punzo & Mutchmor, 1980).

2.5 Black Soldier Fly

There are over 1000 dipteran species across the world, but the black soldier fly (BSF) is one of the most sought-after fly species. In earlier times, the BSF was considered a pest and contaminant, commonly infesting food wastes and causing myiasis in humans and animals with untreated wounds. In recent times, the larvae of this species have caught headlines for its ability to feed on various organic wastes such as food wastes and manure and converting its frass into fertilizer which is rich in nitrogen, phosphorus and beneficial microbes. Also, the frass is used as soil amendment/ biofertilizer in the agriculture industry. Additionally, the larvae itself become a high-protein feed. The application of the frass fertilizer and high-protein feedstock is extensive in agriculture and aquaculture industries. The BSF larvae also

produces antimicrobial peptides (AMP), enzymes (lipase and protease) which is useful in biotechnology application, pharmaceutical and cosmetic industry.

AMPs derived from BSF larvae haemolymph have been incorporated into biodegradable packaging films (e.g., starch-based), providing active antimicrobial protection in foods. Extracts from BSF larvae also exhibited strong activity against Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, and even drug-resistant pathogens, while remaining stable against proteases—highlighting their potential as next-gen antibiotics or feed additives (Di Somma et al., 2021). Among other applications, a recombinant defensin-like AMP (C-15867) from BSF larvae has been structurally characterized and shown to destroy bacterial cell membranes, making it a promising therapeutic candidate (Lee et al., 2020). Additionally, studies have shown that adding certain microbes into BSF larvae diets could boost the activity of gut lipase and protease, thus enhancing the breakdown of nutrients which greatly influence insect rearing and bioconversion process (Fahmy et al., 2023; Meng et al., 2023).

The BSF was originally from South America but made its way to the temperate, tropical countries across Europe, Africa, and Asia. The life cycle of BSF is divided into 4 phases: eggs, larvae, pupae, and adult stage (Figure 1.1) (Q. Li et al., 2011; Tomberlin et al., 2002).

The life span of an adult BSF is approximately 4 to 9 days during which it actively copulates and lay eggs. The mating requirements of BSF adult flies are sunlight, UV, temperature, humidity, and water (Holmes et al., 2012; Tomberlin & Sheppard, 2002; Park et al., 2010). Male BSF has visual receptors to detect female BSF entering their mating region, therefore, mating is successful when there is direct

sunlight. Also, wavelengths between 332 to 535nm were shown to increase mating success and fertile eggs (Tomberlin et al., 2009; Oonincx et al., 2016). Furthermore, successful mating occurs at an optimum temperature of 27°C at a relative humidity of 60% (Shumo, Khamis, et al., 2019; Tomberlin et al., 2009). However, temperatures more than 13°C is required for BSF to keep flying. In addition to that, adult BSF feeds on nothing except water (X. Liu et al., 2017; Paulk & Gilbert, 2006). In an experimental setting, the oviposition of BSF is encouraged by preparing flute space using cardboards or wood blocks held together by a rubber band and placing it on top of attractants such as bananas, cornmeal, or wheat bran (Chia et al., 2018; Shumo, Khamis, et al., 2019).

These organic matters act as an indicator for the adult female flies to lay their eggs. The food is kept moist to prevent the female flies from laying their eggs on top of the food but around the edges (Booth & Sheppard, 1984; Tomberlin & Sheppard, 2002). The food produces mould after 2-3 days and must be replaced regularly to avoid foul smell. To minimize frequent replacement, the food may be treated with antibiotics or introduced with larvae to consume the mould-causing fungi. The eggs laid is to be harvested every 1 or 2 days to make empty flute space for oviposition of new sexually matured flies. The eggs are then introduced to compost for hatching. The hatching of BSF eggs takes approximately 4 days and requires high moisture, the humidity of 70% and temperatures ranging from 27-30°C (Chia et al., 2018). The critical factors involving hatching failure is low humidity accompanied by high temperatures and/or unfertilized eggs. The female flies have eggs developed in their ovaries regardless of mating. Therefore, during copulation (about 10-30 minutes), if there are interruptions before finished such as human activities or poor light source, there will be unfertilised eggs present when the female fly lays all her eggs resulting in no hatching of those

unfertilized eggs. After the hatching of fertilized eggs, the neonates take about 8 days to reach visible size. The diet for neonatal BSF is specifically formulated to increase the survivability of neonates and to ensure it has all the nutrients necessary for growth (Hogsette, 1992; Sheppard et al., 2002; Woods et al., 2019).

Once neonates are visible, the larvae are transferred to compost food scraps. The air humidity and substrate moisture should be maintained at 70% (Chia et al., 2018; Shumo, Khamis, et al., 2019). The critical factors that cause a decrease in larvae population are high temperature and poor substrate aeration. Research conducted by Chia and coworkers (Chia et al., 2018) has observed that the shortest larval development occurred between 32-40°C. Temperatures more than 44°C, causes the larvae to stop feeding and cluster at the top of the substrate to release heat. In an industrial setting, the larvae are placed on trays with substrates not more than 3-inches thick. The larvae can dig deep into the substrate. However, too deep of a substrate pile causes the larvae to die due to high internal substrate heat and lack of oxygen. Furthermore, the rapid movement of larvae causes the food to sink to the bottom of the tray. Therefore, the larvae have no food available at the surface. Hence, a 3-inch substrate pile is recommended for efficient bioconversion and larval growth. In addition to that, the substrates must be aerated to ensure thorough mixing (Shumo, Khamis, et al., 2019). Apart from that, the diet of BSF larvae is formulated specifically to increase larval weight and fecundity of female BSF after the larval stage (Gobbi et al., 2013; Meneguz et al., 2018; Pastor et al., 2015).

The stage whereby the larvae moults and grow are known as the larval stage. This process takes approximately 10 to 18 days. The moulting of larvae between instar is known as ecdysis. This process is controlled by the hormone, ecdysone. Ecdysone acts on genes that synthesize enzymes to catalyse digestion, reabsorption of certain

materials in the inner parts of the old cuticle and formation of new soft cuticles. The BSFL sheds its outer chitinous cuticle. The newly formed soft cuticle emerges. The larvae then actively feed, absorbs water and air. The larvae size increases and the new cuticle gradually harden with chitin. The process repeats several times until the larvae are in their 5th instar. In an industrial sector, after food substrate is converted to larval frass and larval mass, a sifting system comprising of vacuum transporter and frass separator separates the larvae from the frass. A certain percentage of larvae is turned into the product while the remaining larvae in their 5th instar, enters the pre-pupal stage (6th instar). The pre-pupal stage lasts up to 14 days (Barros-Cordeiro et al., 2014). The prepupae move to a dry area and enters the pupal stage. The pupae no longer feed but undergoes metamorphosis into adult BSF.

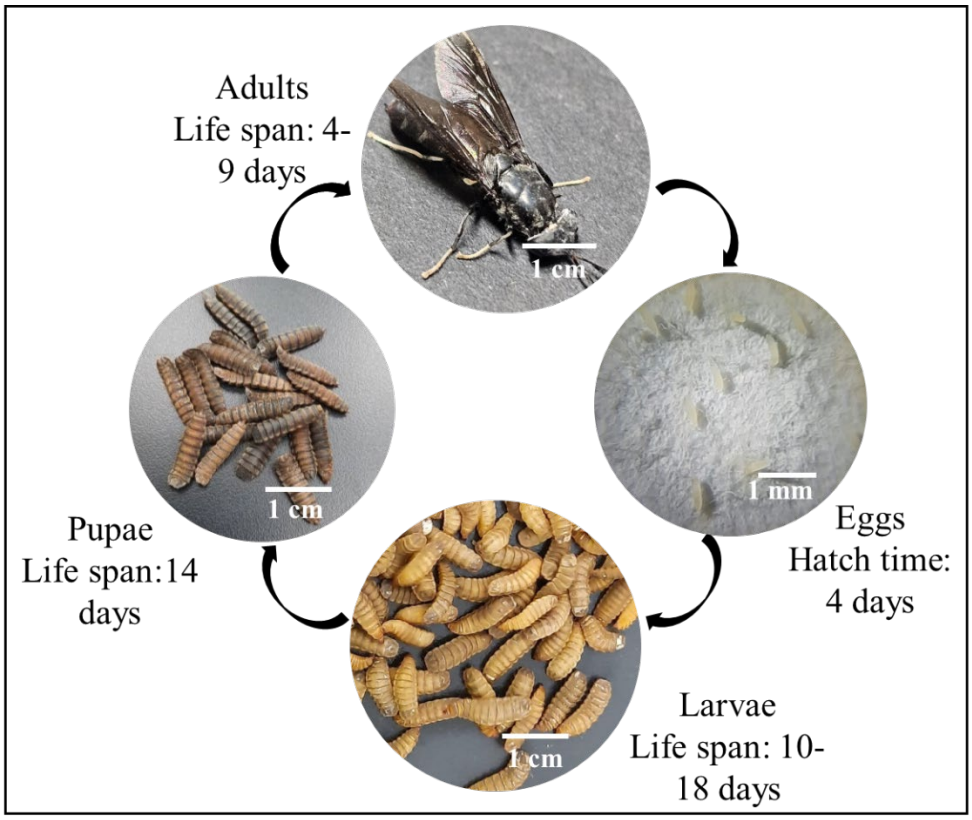

Figure 2.2: The life cycle of black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae) with 4 important stages of development: eggs, larvae, pupae, and adult.

The larval stage was used in experimental feeding due to its long development and active feeding phase.

2.6 Nutritional requirements of black soldier fly larvae

BSF larvae are used as an alternative protein source in animal feed industry. The larvae are capable of reducing 50-75% of organic waste into high protein biomass. The BSF larvae can feed on a variety of organic matter such as vegetable waste, coffee bean pulp, food residues, municipal waste including manure, and fish offal. BSF larvae consists of approximately 50% of crude protein and 35% of lipids. Also, its amino acid profile is similar to fishmeal. The utilization of BSF larvae as an alternative protein source in aquaculture, poultry, pigs is a growing trend (Craig Sheppard et al., 1994; Lalander et al., 2020; Shumo, Osuga, et al., 2019; Zhou et al., 2013). However, different diet formula influences the growth of BSF larvae.

2.6.1 Carbohydrate-based diet

Simple and complex forms of carbohydrates are not limited to bread and grains but can be found in other food including fruits, and vegetables. Even cellulose, which provides tensile and structural strength will break down to its simpler forms during composting. Amino acids are required to produce enzymes, protein and even build tissues, however, carbohydrates are the driving force that fuels the biosynthesis of these compounds. Carbohydrates are necessary for the growth, development, survivability, and reproduction activity of insects. Most industries formulate the diet of BSF larvae with a certain percentage of protein and carbohydrates. In the year 2018, researchers (Beniers & Graham, 2019), conducted two experiments. Firstly, to determine protein and fat accumulation in BSF larvae at its pre-pupal stage when placed on a chicken feed-based diet with 70% moisture, 48.8% carbohydrate and 17.5% protein. Secondly, a two-part

experiment to determine the impact of varying protein and carbohydrate concentration on the growth of BSF larvae of which the protein ratio in wet weight was increased to 71.4% with 5.9% of carbohydrate. Meanwhile, for the second part, the carbohydrate ratio was increased to 77.4% and 7.2% of protein.

The results from experiment 1 demonstrated that the protein levels were higher in the early instar level. However, researchers believe that control substrates with a wide variety of carbohydrates from leftover cereals, grinding dust, spilt grains and broken pellets could be used to compare to the chicken-feed diet used. The second experiment highlighted that carbohydrate had little effect on larval growth when compared to the enhanced protein percentage in the diet.

According to Danieli and colleagues (Danieli et al., 2019), BSF prepupae reared on non-fibre carbohydrates were found to be highest in fat and saturated fatty acids in comparison to cornmeal and fibre diets. However, the fatty acids in BSF prepupae are believed to be dependent on the amount contained in the given diet, which suggests the influence of the fatty acid profile. A paper by Cammack and team (Cammack & Tomberlin, 2017), revealed that BSF prepupae placed on a high carbohydrate diet had protein and fat content that were much lower than BSF prepupae that were given a high protein diet. Further study revealed that the BSF larvae developed slower by 6 days when places on carbohydrate-based diets. Cammack and team also presented that the development rate of larvae was faster, about 32 to 38 days and had 57-62% chances of surviving on a balanced protein: carbohydrate diet (21:21).

In a separate study conducted by Barragan and coworkers (Barragan-Fonseca et al., 2018), involving protein: carbohydrate ratios, P: C, various concentrations were formulated to test the development and reproduction of BSF. It was observed that survival of BSF was high in all types of P: C formulas, however, the influence of protein and carbohydrate content individually were impactful. In high carbohydrates content, the

larval yield and crude fat were low. However, pupal development was faster in high levels of carbohydrates. It was concluded that high contents of macronutrient with low P: C ratio, affects the BSF performance positively. The ratio of P: C of 17:55 has been shown to produce high body protein content in larvae with an average crude fat content.

Besides that, carbohydrates are much needed for the formation of chitin, a principal structure that consists of β-(1,4)-2-deoxy-D-glucose (N-acetyl-D-glucosamine). Chitin also forms the integral structure such as the trachea, intestines, reproductive tract as well as endo and exocuticle. In addition to that, carbohydrates are essential for metamorphosis. BSF obtains its highest carbohydrates as mature non-feeding larvae. The carbohydrates are then stored as glycogen in the fat body and trehalose in its haemolymph. These components are utilized as an energy source for the synthesis of pupae and adult tissues. Also, carbohydrates are vital for the reproduction of BSF. They are an energy source for male BSF to maintain its sperms in its seminal vesicle. In female BSF, carbohydrates are accumulated in oocytes for embryo development. Moreover, BSF relies on carbohydrates as flight fuel. Their flights require high oxidative metabolisms provide by carbohydrates. However, a high level of carbohydrates inhibits enzymatic reactions including glycolysis and gluconeogenesis.

2.6.2 Protein-based diet

Proteins are beneficial for the various physiological processes that occur in insects in general, among which are digestive, circulatory, respiratory, muscular, and reproductive. One such protein that is involved is enzymes. In the digestive tract, enzymes amylase, invertase, lipase, nucleases and trypsin break down complex molecules into their basic constituents to be utilised by the insect (Lehane & Billingsley, 2012). Apart from digestion, the circulatory system of insects uses proenzyme, phenoloxidase for moulting and transporting nutrients, hormones, and wastes in the haemolymph since insects lack

distinct arteries or veins (Chapman, 1998; Iwama & Ashida, 1986). Furthermore, insect locomotion is dependent on the enzyme alpha-glycerophosphate dehydrogenase that is actively involved in the glycerophosphate cycle of an insect flight muscle, producing force and movement (Healy et al., 2004). Additionally, the two key hormones, ecdysone, and juvenile hormone (JH) are potent for metamorphosis and moulting in an insect life cycle (Shinoda & Itoyama, 2003). Moreover, neurotransmitters, acetylcholine and dopamine are essential in regulating insect metabolism (Thany et al., 2010).

Proteins can be found in almost all types of food and wastes. Fruits, vegetables, and grains contains protein as well. Several researchers (Liland et al., 2017; Shumo, Osuga, et al., 2019; Spranghers et al., 2017; Surendra et al., 2016; Tschirner & Simon, 2015) have uncovered that the highest levels of isoleucine, leucine, aspartate, and glutamate were found in BSF larvae reared on fruits and vegetables, wheat and brown algae, wheat, and food waste respectively. The BSF larvae total protein content was 4.3%, 6.9%, 9.4% and 9.8% respectively. However, the levels of amino acids may vary due to the protein content of rearing substrates. Researchers (Danieli et al., 2019) also, discovered that fatty acids found in BSF prepupae are dependent on the levels found in the diet, that suggests the influence of fatty acid profile of substrates. This notion may apply to the amino acids found in BSFL placed on varying protein substrates.

A review article by Hopkins and team (Hopkins et al., 2021), described that BSF larvae reared on fish waste had the highest protein content of 78.8% in comparison to BSF larvae reared on fruits and vegetables. In a research study by Barragan-Fonseca and team (Barragan-Fonseca et al., 2018), larval body and adult BSF traits were studied in regard to dietary protein. Three protein-based diets with increasing protein percentages (10%, 17% and 24%) were formulated to encourage the growth, development, and reproduction of the fly species. It was noted that the survival rate was high for all three protein levels. In addition to that, pupal development was fast when protein content was low. However, the

egg yield increased when protein levels increased. Besides that, the crude protein of larvae increased when protein levels were at 10% and 17%. However, it is also observed that the crude fat levels in larvae were high in 24% protein level. The paper concluded that protein content of 17% was appropriate to achieve a high crude protein level and intermediate crude fat level.

In a separate study by (Beniers & Graham, 2019), BSF larvae were fed 25 diets with various protein levels. The protein ratio was enhanced to 71.4% with added protein concentrations of 0g, 0.7g, 1.4g and 2.1g respectively for each replicate. In the study conducted, the highest recorded protein content was recorded in early instars of BSF larvae. Further study revealed that protein influences the growth of BSF larvae. In a separate study conducted (Cammack & Tomberlin, 2017), it was uncovered that BSF larvae grew the fastest and had a high survival rate up to its prepupal stage when reared on a balanced protein: carbohydrate (21:21) ratio with 70% moisture. Another elaborate study (Nguyen et al., 2015), revealed that high protein levels in the swine liver diet resulted in the production of BSF prepupae with high crude protein content. The results were supported by other researchers as well (Oonincx et al., 2015).

Food wastes are an excellent source of nitrogen. Nitrogen is essential to produce amino acids. In a study conducted by Lu and team (Lu et al., 2022), 9 types of nitrogen elements (NH_4Cl, $NaNO_3$, urea, uric acid, Gly, L-Glu, L-Glu: L-Asp (1:1, w/w), soybean flour, and fish meal) were used to determine its effect on the development of BSF larvae. In the study, it was unveiled that the carbon to nitrogen ratio affects larval development and the bioconversion process. Nitrogen species $NaNO_3$ and NH_4CL were harmful to the BSF larvae whereas the remaining nitrogen species did not pose any harm. The C: N ratio of 18:1 and 14:1 yields high levels of larvae meanwhile ratio of 18:1 and 16:1 was appropriate for high larval protein yield. Further study implies that C: N ratio may not