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Dual application of black soldier fly (*Hermetia illucens*): Protein-rich animal feed and biological extraction agent for polyhydroxybutyrate

Lydia Mohamad[1], Hadura Abu Hasan[2], Kumar Sudesh[2] and Siti Baidurah[1]*

[1]School of Industrial Technology, Universiti Sains Malaysia, 11800 Gelugor, Penang, Malaysia.
[2]School of Biological Sciences, Universiti Sains Malaysia, 11800 Gelugor, Penang, Malaysia.
Email: sitibaidurah@usm.my

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ABSTRACT

Aims: The primary aim of the present study is to evaluate the effect of rearing substrates on the nutritional content of black soldier fly larvae (BSFL) by incorporating *Cupriavidus necator* cells containing intracellular polyhydroxybutyrate (PHB) in BSFL diet to further increase the protein content and simultaneously to biologically extract the polymer by utilizing the digestive system of BSFL. The potential application of BSFL as a biological PHB extraction agent was determined.
Methodology and results: Two feeding strategies consists of a mixture of protein (P) to carbohydrate (C) with a ratio of P50:C50 food waste (control feeding) and feed with bacterial cells (modified feeding). A comparison on the proximate analysis between this research and two commercially available products were conducted. Feeding BSFL with P50:C50 food waste revealed the highest crude protein content of 81.3 ± 0.2%. Additional bacteria cells in the BSFL diet, however, showed a negligible decrease in crude protein content of 0.67% as compared to the control feeding. Howbeit, this results comparably higher in contrast to the commercial products, with increment of crude protein content by 12.1% and 40.8%, respectively.
Conclusion, significance and impact of study: Two desirable products were obtained from the feeding with cells: (1) high protein content of BSFL and (2) biologically extracted polymer. This is the first study to demonstrate the utilization of BSFL as a biological extraction agent to partially extract biopolymer and increase the protein content by feeding with cells.

Keywords: Biodegradable plastics, polyhydroxyalkanoates, biological extraction, black soldier fly

INTRODUCTION

The black soldier fly (BSF), *Hermetia illucens* (Diptera: Stratiomyidae) undergoes a complete life cycle consisting of five stages: egg, larva, pre-pupae, pupa and adult (flies). Egg-to-adult development time is characterized as short life cycle of 40 to 43 days (Tomberlin *et al*., 2002; Marshall *et al*., 2015). The larval stage comprised of first to fifth instar larvae, whereby this is the only phase which the BSF feeds throughout the entire life cycle to reserve sufficient fat and protein prior turning into adults. This period could be extended to a maximum of four months in the event of food shortage (Ortiz *et al*., 2016; Dortmans *et al*., 2017). Black soldier fly pre-pupae (BSFP) can be considered as animal feed due to its high protein and fat content which is necessary for a complete diet to support good growth in poultry and aquaculture.

Recent interest in this species is deemed as a promising source for bioconversion of organic substance into valuable protein-rich to produce animal feed and supplement (Diener *et al*., 2009; Gold *et al*., 2018; Putri *et al*., 2019). Black soldier fly larvae (BSFL) are regarded as a saprophagous species which can grow on a wide range of decaying biowaste such as vegetable scraps, fruit waste, kitchen waste, animal manure and abattoir waste to obtain nourishment for the larvae to grow and convert into insect larval biomass that can be applied for other beneficial purposes. Rearing BSF is an economical way of reducing waste as the BSFL are voracious feeders of organic materials. BSF ability to convert waste into useful nutritious animal feed makes them a high-demanding alternative as a sustainable and inexpensive option to conventional animal feed as well to reduce pollution, creating an environmentally conscious closed-loop waste treatment system (Putri *et al*., 2019; Wong *et al*., 2019).

Several studies have focused on the influence of rearing substrate on the nutritional content of the BSFP. Feeding method applied by Spranghers *et al.* (2017) is feed with restaurant waste containing random food waste resulted in BSFP crude protein content of 43.1%. Ewald

*Corresponding author

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et al. (2020) explored broader feeding strategy of bread, fish, food waste, mussels and mixture of bread with mussels, with the highest protein content of 52.6%. The effect of food waste as a rearing substrate on the nutritional value of the insect has vastly been investigated, however, limited research publication related to bacterial cells as an additional diet for BSF rearing. Non-pathogenic bacterial cells can play an essential role in enhancing the protein content of BSFP due to its properties as a single-cell protein (SCP) (Kunasundari *et al.*, 2013; Ong *et al.*, 2018).

Polyhydroxybutyrate (PHB) and its co-polymer is a type of intracellular biodegradable polymer with a great potential as an alternative to petroleum-derived plastics due do its properties which are like conventional polymers and additionally, possess other important benefits such as biodegradability, biocompatibility and non-toxic characteristics (Baidurah *et al*., 2018; Sen *et al*., 2019). These unique properties of PHB and its co-polymer gave rise to diverse applications ranging from biodegradable packaging materials to biocompatible medical devices and tissue engineering materials (Chaijamrus and Udpuay, 2008; Bhagowati *et al*., 2015; Baidurah *et al*., 2018; Boey *et al*., 2021). The current PHB extraction method is by physicochemical and mechanical means (Kunasundari and Sudesh, 2011). Chemical extraction involves the use of organic solvent such as chloroform in large volumes with the purposes to modify the cell membrane permeability and to dissolve the polymer (Fiorese *et al*., 2009; Ong *et al*., 2018). The PHB recovery from biomass using chemical pretreatment process has raised an unfavourable concern to the environment and high consumption of solvent consequently increase the costs of extraction (Fiorese *et al*., 2009; Low *et al*., 2021). On the contrary, Murugan *et al*. (2016) reported on the biological extraction of the polymer by utilizing mealworm as an alternative approach to minimize the usage of solvents and chemicals (Murugan *et al*., 2016; Ong *et al.*, 2018; Zainab-L *et al.*, 2018). However, during rearing stages, mealworms eat limited type of food wastes such as grains, fruits and vegetables. Therefore, insect with the broader range preference of food waste is required.

The primary aim of the present study is to evaluate the effect of rearing substrates on the nutritional content of BSFP by incorporating bacterial cells containing intracellular PHB in BSFL diet to further increase the protein content and simultaneously to biologically extract the polymer by utilizing the digestive system of BSFL. The changes of the BSFP chemical compositions based on the two-diet pattern: [1] conventional feeding with 50% protein:50% carbohydrate food waste (control feeding), and [2] same diet as control feeding with additional bacterial cells were evaluated. Furthermore, the obtained proximate data were compared with commercially available animal feed products and the PHB content secreted with the frass was quantitatively determined. The second objective is to determine the potential application of BSFL as a biological PHB extraction agent. This is the first study reporting utilization of BSF for dual application of high-protein content for animal feed and biological PHB extraction agent.

MATERIALS AND METHODS

Bacterial strain and growth media

Cupriavidus necator wild type (ATTC 17699) was cultured in Nutrient Rich (NR) broth at pH 7.0 (Mohamad *et al*., 2020). For short-term storage, the cells were sub-cultured on the NR agar plate. The bacterial cells were stored with 40% (v/v) glycerol solution at –80 °C for long-term preservation.

Bacterial growth and cultivation condition

For cell activation, *C. necator* bacterial colony grown for 16-18 h from the agar plate transferred into NR broth and grown overnight at 30 ± 1 °C and 200 ± 10 rpm (Incubator model: Infors Ecotron, Switzerland). A total volume of 15 mL inoculum was pipetted into 100 mL of mineral medium (MM) broth in 500 mL shake flask and incubated in the incubator shaker with the same temperature and agitation speed as cell activation stage for 48 h (Mohamad *et al*., 2020). The fermentation medium consisted of MM and trace elements solution (Murugan *et al*., 2017) with a fructose (Brand: HmbG) concentration of 20 g/L as the sole carbon source. The source of carbon, nitrogen, magnesium sulfate, calcium chloride and trace element solution were prepared and autoclaved separately to avoid Millard reaction and precipitation (Baidurah *et al*., 2016; Sen *et al*., 2019). After the designated 48 h of fermentation, the culture was harvested at 4 °C, 8 min at 8590× *g* using benchtop centrifuge (Model: KUBOTA 5100, Japan). The obtained cell pellets were washed with approximately 50 mL distilled water and kept at –20 °C prior lyophilization process. The frozen cell pellets were freeze-dried using freeze dryer machine (Model: LABCONCO, US) at temperature –50 °C with pressure range from 0.06 to 0.09 Torr for 48 h. The obtained freeze-dried bacterial cells were subjected to feeding experiment.

Feeding strategy of black soldier fly pre-pupae

The BSFL with average size 3-4 mm, aging 5-6 days (Figure 1) were acquired from School of Biological Sciences, Universiti Sains Malaysia (USM) and placed in a net-covered plastic container (43 × 29 × 9 cm). The temperature and humidity of the room is 26 °C and 60-70%, respectively. Figure 2 delineates the schematic diagram of the two-diet patterns with protein (P) to carbohydrate (C) ratio of P50:C50 (control feeding) (Barragan-Fonseca, 2019) and additional bacterial cells with 41.2 wt % PHB content (modified feeding). Applying an equal ratio of protein to carbohydrate in BSFL diet will provide optimal larval development (Cammack and Tomberlin, 2017). The newly hatched first instar larvae were fed with coconut waste containing effective

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Figure 1: Photograph of BSFL.

microorganism (EM) mixture as the growing media for larvae development. The EM mixture was purchased from local Malaysian supplier consists of EM, de-chlorinated water and molasses with a ratio of 1:20:1. The mixture was mixed with coconut waste with a ratio of 1:1 and utilized as BSF growing media (Hasan and Dina, 2019). Once it reached the second instar stage, the larvae were fed with the conventional diet comprising food waste collected from Bakti Cafeteria, USM, as the essential nutrient for survival and larval growth. The composition of food waste was given with an equal ratio of protein and carbohydrate by manually weight the protein source and carbohydrate source. The food waste was manually segregated and weighed according to their nutritional content. The protein food waste comprised of beans, chicken, meat, fish and tofu scrap while for carbohydrate, the food waste includes rice, bread, pasta, starchy vegetables such as potatoes and barley leftover. The food waste was added when there is a reduction of feed in the container. The experiment was conducted until larvae reached its fifth instar and washed with distilled water to remove food residue and frass. During the washing process, BSFL were placed on a mesh strainer prior rinsing. Upon rinsing, the food residue and frass pass through the mesh strainer and BSFL collected for next stage process.

At this stage, two sets of feeding strategies were conducted; [1] prolong feeding with P50:C50 food waste as a control experiment until it turns into pre-pupae and [2] feeding with the same diet as control experiment until late stage of BSFL and continue with additional feeding of bacterial cells (modified feeding). For the latter set of feeding strategy, a total of 1000 late stage of BSFL weighing approximately 152.64 g were collected and starved for 48 h in a different container containing sawdust for drying. Prior to the one-time feeding with lyophilized cells, the fifth instar BSFL were transferred into a clean plastic container (20 × 10 × 18 cm) and were fed with lyophilised *C. necator* in powdered form with a feeding rate of 5% of the total body weight (Murugan *et al*., 2016). The larvae turned into pre-pupae were harvested on day four and placed on the mesh strainer prior rinsing to separate the mixture of frass and extracted polymer. The extracted polymer appears in white powder secreted from the BSFP. The BSFP were kept in –20 °C overnight followed by lyophilization using the aforementioned setting. The obtained BSFP sample was subjected to proximate analysis and PHB content secreted together with the frass was used for PHB quantification.

Crude fat content

The initial analysis procedure involves measuring the total fat content by means of Soxhlet extraction method following AOAC Official Method 2003.06 using approximately 150 mL of petroleum ether as the solvent at 60 °C. About 1-5 g of ground freeze-dried BSFP sample was extracted continuously for 6 h and the remaining defatted residue was dried in the fume hood and weighed. Percentage of the crude fat were calculated using Equation 1:

$$\text{Crude fat, \%} = [(F - T)/S] \times 100\% \qquad \text{Equation 1}$$

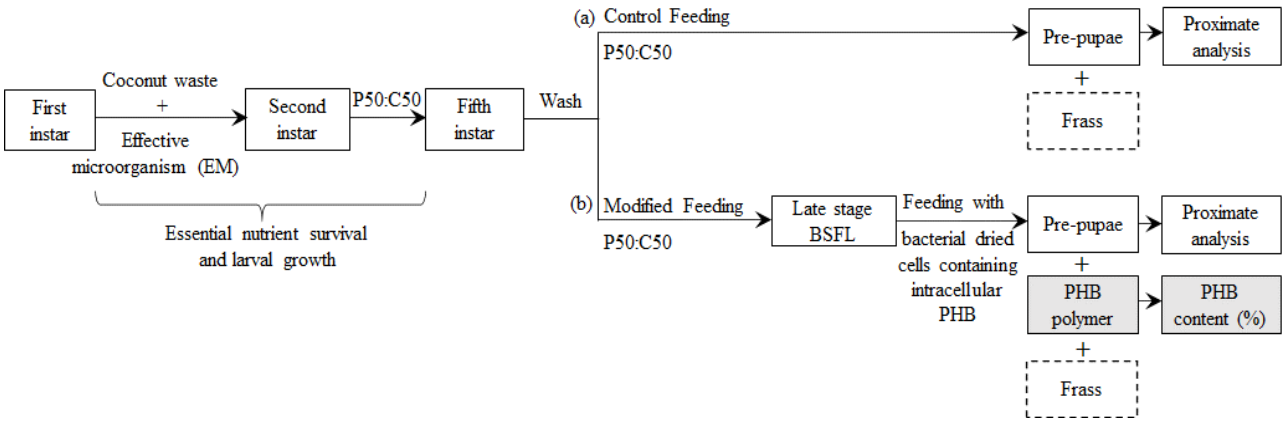

Figure 2: Schematic diagram of the two-diet patterns; (a) P50:C50 and (b) additional bacterial dried cells of *C. necator* H16 with intracellular PHB.

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where F = weight (g) of round bottom flask + fat residue; T = weight (g) of empty round bottom flask; S = weight (g) of freeze-dried BSFP. Furthermore, the defatted sample was subjected to crude protein evaluation and amino acids profiling while the extracted oil sample was further analysed for fatty acid methyl ester (FAME) profiling.

Preparation of fatty acid methyl ester (FAME) derived from BSF

FAME was synthesized from lipid sample by heating 0.3 g of the oil with 5 mL of methanolic sodium hydroxide (NaOH) for esterification. The sample was boiled for 5–10 min under reflux until the fat droplets disappear. Upon esterified, 7 mL of methanolic boron trifluoride solution was added and boiled for 2 min. *Circa* 3 mL of *n*-heptane was added and boiled for 1 min to recover methyl ester in the organic phase. The mixture was cooled at room temperature and followed by phase separation of the aqueous and organic layer by adding saturated sodium chloride (NaCl). The upper layer of *n*-heptane phase was pipetted out into test tube, and a small amount of anhydrous sodium sulphate (Na_2SO_4) was added to remove the traces of water from organic solution. The methyl ester solution was transferred into a vial and subjected to gas chromatography-mass spectrometer (GC-MS) to determine the fatty acids composition (Indarti *et al*., 2005).

Analysis of fatty acid methyl ester using gas chromatography mass spectroscopy

FAME profiling was performed using GCMS-QP2010 Ultra Gas Chromatograph Mass Spectrometer (Shimadzu) and equipped with SUPELCOWAX® 10 Capillary GC Column (30 m × 0.25 mm i.d; 0.25 µm film thickness). The oven temperature was set at 115 °C, raised to 160 °C at a rate of 10 °C/min, held for 2 min, and finally raised to 225 °C at a rate of 10 °C/min whereby it was held for another 12 min. The injection volume was 0.2 µL using helium as the carrier gas at a rate of 1.2 mL/min with a split ratio of 50:1 at the inlet. Identification of methyl esters was made by comparing the obtained mass spectra with the database (Indarti *et al*., 2005).

Crude protein content

Nitrogen determination of BSFP crude protein was performed using the Kjeldahl method according to 2001.11 of the AOAC International. The percentage of nitrogen and crude protein were calculated using Equation 2 and 3:

$$\text{Kjeldahl nitrogen (\%)} = [(V_S - V_B) \times M \times 14.01]/W \times 100\% \quad \text{Equation 2}$$

$$\text{Crude protein (\%)} = \text{Kjeldahl nitrogen (\%)} \times 6.25 \quad \text{Equation 3}$$

where V_S = volume (mL) of standardized acid used to titrate a sample; V_B = volume (mL) of standardized acid used to titrate reagent blank; M = molarity of standard HCl; 14.01 = atomic weight of N; W = weight (g) of test portion or standard; and 6.25 = factor for feed materials. Furthermore, amino acid profile analysis for the freeze-dried prepupae obtained from modified feeding also conducted (Dorresteijn *et al*., 1996).

Crude fiber content

Crude fiber analysis was performed using AOAC Official Method 973.18. The crude fiber was measured as the weight loss on the incineration of the remaining residue after digestion of the sample. The percentage of crude fiber was calculated using Equation 4:

$$\text{Crude fiber, \%} = [(A - C) - B]/S \times 100\% \quad \text{Equation 4}$$

where S = weight (g) of sample (dry and defatted); C = weight (g) of ashless filter paper; A = weight (g) of crucible + filter paper + dried precipitate; B = weight (g) of crucible and ash.

Ash content

Crude ash which comprised of non-combustible minerals analysed following AOAC Official Method 942.05. The percentage of ash content was calculated using Equation 5:

$$\text{Ash content, \%} = (\text{Weight of residue, g}/\text{Weight of sample, g}) \times 100\% \quad \text{Equation 5}$$

Moisture and dry matter content

The moisture content and dry matter were determined by means of oven-drying the lyophilized ground BSFP in an air circulating oven at 105 °C overnight until the weight is constant (Mohamad *et al*., 2020). The dry matter was calculated using Equation 6:

$$\text{Dry matter, \%} = 100 - \text{moisture content, \%} \quad \text{Equation 6}$$

Separation of the mixture of frass and extracted PHB

The secreted mixture containing frass and PHB were collected. PHB appears in white powder form weighing approximately 1.99 g was further purified by immersing the mixture in 250 mL of distilled water and stirred at 250 rpm at ambient temperature up to 10 h (Murugan *et al.*, 2016). The frass will dissolve in water *in lieu* of PHB. The solution was centrifuged at 8590× *g*, 4 °C for 8 min (Model: KUBOTA 5100, Japan) and the white pellets were collected and oven-dried at 60 °C overnight followed by PHB quantification.

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Methanolysis and PHB quantification by gas chromatography

The PHB were quantify using methanolysis followed by gas chromatography (GC) analysis (Baidurah *et al.*, 2016; 2018; Sen *et al.*, 2019; Khok *et al*., 2020). The methyl ester of PHB was prepared by weighing approximately 10 mg extracted PHB in a screw-capped borosilicate tube. Two mL of 85% acidified methanol and 2 mL of chloroform was added in the tube and tightly screwed. Methanolysis solution was prepared by adding methanol and sulfuric acid in the ratio 85:15 (v/v). The solution was heated in a digital dry bath at 100 °C for 3.5 hours. One mL of distilled water was added into the cooled solution and vigorously vortexed for phase separation. Anhydrous sodium sulphate was added to the lower organic phase containing methyl ester of 3-hydroxybutyrate to remove the remaining trace water. The organic layer was filtered using 0.22 µm syringe filter into a vial and subjected to GC analysis. The standard sample of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)] were used to construct the standard calibration curve.

A GC system (Shimadzu model GC-2010 Plus, Shimadzu Corp., Japan) equipped with an auto-injector (Shimadzu AOC-5000 Plus) and capillary column (Merck SPB-1, 30 m × 0.25 mm ID, 0.25 µm film thickness) was employed. Helium was used as the carrier gas with a flow rate of 0.91 mL/min. The injector and flame ionization detector temperature were set at 270 °C and 280 °C, respectively with a pressure of 84.2 kPa and flow rate at 13.0 mL/min. The column temperature was set from 70 °C to 280 °C with rate of 5 °C/min.

Statistical analysis

Data was recorded in GraphPad Prism 6 for all types of feeding strategy. The differences among dietary substrate means were assessed using a one-way analysis of variance (ANOVA) with a statistical significance at $p \leq 0.05$.

RESULTS AND DISCUSSION

This study investigated the effects of the larval diet on the BSF nutritional composition. Table 1 delineates the comparison of nutrient compositions and PHB content. The obtained proximate analysis data of BSF were compared with the commercially available feed products in Malaysia namely Product A contain solely ground and dried BSFL and Product B contains a mixture of chicken feed grains and dried BSFL.

Crude protein content

Protein is an essential nutrient required by poultry for growth, egg development, as well as to support the construction of body tissues such as muscles, nerve, skin, feather, blood plasma proteins, enzymes, hormones and antibodies (Abbasi *et al.*, 2014). The crude protein content of lyophilized BSFP fed with P50:C50 showed a value of 81.3 ± 0.2%, which is the highest as compared to the BSFL fed with bacterial cells (80.6 ± 1.2%), Product A (64.6 ± 2.9%) and Product B (37.1 ± 2.7%). These results indicated that the protein content did not vary between the control and modified feeding (with only 0.67% decrease) which is negligible. Howbeit, the crude protein of the BSFP fed with additional lyophilized bacterial cells evinced significant increase by 12.1% and 40.9% as compared to Product A and B, respectively. According to Malaysian Poultry Feeds Specification, the minimum level of total protein content of poultry feed must be at least 21% and protein content data obtained in this study comply with the standard specification.

Factors such as nutrient concentration, rearing density and insect's interaction can influence the crude protein content (Barragan-Fonseca *et al.*, 2018). The proximate and nutrient composition of the BSFP also influenced by the rearing substrate (Spranghers *et al.*, 2017). Several studies reported protein and carbohydrate are the most crucial nutrients for insect's survival, growth and reproduction (Aguila *et al.*, 2013; Nash and Chapman, 2014; Cammack and Tomberlin, 2017). Akin to this study, results reported in literature utilizing mealworms fed with bacterial cells shows high protein content in mealworms (Murugan *et al*., 2016) as compared to fed with oats. Furthermore, Kunasundari *et al*. (2013) utilized lyophilized cells of *C. necator* accumulating approximately 43 ± 3% (wt) of crude protein content.

Effect of feeding strategy on the BSF amino acid profile

Amino acid (AA) profile analysis was conducted to determine the essential and non-essential amino acids present in the BSFP biomass. Essential amino acids (EAA) which are deemed vital for the poultry birds, cannot be synthesized by them, thus need to be supply through diets (Ullah *et al*., 2015). In general, there are 20 AA that involved in normal body physiological functions and among them, there are 10 types of EAA for poultry, includes, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Ullah *et al*., 2015). However, the ones critical in poultry diets are cystine, lysine, methionine, threonine, tryptophan and valine (Surendra *et al*., 2016).

The AA profile of BSF harvested from modified feeding are delineates in Table 2 and compared with previous studies with various feeding substrates. Referring to Table 2, there were 17 AA detected in the BSF, whereby ten of the AA were classified as EAA. Data reported by Surendra *et al*. (2016) and Smets *et al*. (2020) which the BSFL reared on food wastes, the most dominant AA is 9% glutamic acid, akin to the BSF biomass fed with P50:C50 and lyophilized cells in this study. Moreover, the BSFP from modified feeding also contained relatively high amount of leucine (6.5%) and aspartic acid (6.2%).

The most prevalent EAA in the BSFP biomass obtained from modified feeding, which are suitable for

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Table 1: Comparison of nutrient compositional analyses of black soldier fly (BSF) and PHB content.

Nutrient content (%)	This study		Commercial products		*Standard specification (%)
	Control feeding	Modified feeding	Product A	Product B	
	P50:C50 (%)	Bacterial cells (%)	Dried powder BSFL (%)	Chicken feed grains and BSFL (%)	
**Proximate analysis					
Crude protein	81.3 ± 0.2^{c}	80.6 ± 1.2^{c}	64.6 ± 2.9^{b}	37.1 ± 2.7^{a}	Min. 21
Crude fat	40.7 ± 1.1^{e}	34.6 ± 1.9^{d}	15.1 ± 1.2^{b}	4.2 ± 3.1^{a}	Min. 5
Crude fibre	7.4 ± 1.3	7.7 ± 4.9	15.7 ± 6.8	7.6 ± 2.0	Max. 5
Ash	5.6 ± 1.4	6.6 ± 1.1	17.5 ± 0.2	7.5 ± 3.6	Max. 8
Moisture	17.6 ± 1.6	21.4 ± 3.4	8.9 ± 3.2	10.7 ± 1.7	Max. 13
Dry matter	82.4 ± 0.3	78.6 ± 0.9	91.1 ± 0.3	89.3 ± 0.2	NA
PHB content (%)	NA	73.8	NA	NA	NA

*The 4th Revision of Poultry Feeds Specification, Malaysian Standards MS 20:2008 (Department of Standards Malaysia, 2008).
**Data in percentage dry weight, except for moisture content.
Different superscript of alphabets indicates significant different (P<0.05) obtained using one-way ANOVA.
NA: Not available.

Table 2: Comparison of amino acids content (%) in BSF fed with various feeding substrates.

		This study Modified feeding	Surendra *et al*. (2016)	Smets *et al*. (2020)	Makkar *et al*. (2014)
No.	Amino acids (%)	Lyophilized cells	Food wastes	Food wastes	Soybean meal
1.	Alanine	6.0	7.9	6.5	4.5
2.	Arginine	4.4	6.6	5.4	7.6
3.	Asparagine	0	0	0	0
4.	Aspartic acid	6.2	7.8	10.5	14.1
5.	Cysteine*	1.7	3.3	0.9	1.4
6.	Glutamic acid	9.0	8.4	13.4	19.9
7.	Glutamine	0	0	0	0
8.	Glycine	5.0	7.3	6.1	4.5
9.	Histidine*	3.4	5.0	3.1	3.1
10.	Isoleucine*	3.8	4.5	4.5	4.2
11.	Leucine*	6.5	6.9	7.5	7.6
12.	Lysine*	6.0	6.5	6.5	6.2
13.	Methionine*	1.5	2.6	1.6	1.3
14.	Phenylalanine*	3.6	4.4	4.1	5.2
15.	Proline	3.0	6.2	6.2	6
16.	Serine	3.6	4.5	4.7	5.2
17.	Threonine*	3.2	4.4	4.2	3.8
18.	Tryptophan*	0	0	1.6	1.4
19.	Tyrosine	3.8	7.0	6.8	3.4
20.	Valine*	4.2	7.2	6.5	4.5

*Essential amino acids, EAA.

poultry diets were leucine (6.5%), lysine (6.0%), valine (4.2%), isoleucine (3.8%), phenylalanine (3.6%), histidine (3.4%), threonine (3.2%), cystine (1.7%) and methionine (1.5%). Lysine and methionine make a substantial contribution in enhancing the tissue protein synthesis and poultry growth (Makkar *et al.*, 2014). The lysine composition in BSFP from modified feeding is comparatively the same with the previous literature (Surendra *et al*., 2016; Smets *et al.*, 2020). These results evinced addition of lyophilized bacterial cells in the BSFL diet did not inflict the composition of EAA of the BSFP except for threonine, tryptophan and valine, whereby negligibly lower composition of AA was observed. AA are critical dietary components for a good quality livestock production owing to its significant role in normal physiological, metabolic and structural functions of the body (Ullah *et al*., 2015; Shumo *et al*., 2019). Growth performance in broilers was affected by the levels of AA present in the feed. It is therefore important to supply sufficient AA in their diets, align with the poultry's dietary requirement to achieve better feed efficiency (Ullah *et al*., 2015).

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Crude fat content

Fat added in poultry feed formulation does not only supply the energy, but also boosts the absorption of fat-soluble vitamins, reduces the pulverulence, enhance the palatability of the rations, improve productivity as well as feed efficiency (Poorghasemi *et al*., 2013). The highest crude fat content was observed on BSFP reared with P50:C50 food waste (40.7 ± 1.1%) followed by BSFP reared with lyophilized bacterial cells (34.6 ± 1.9%), Product A (15.1 ± 1.2%) and Product B (4.2 ± 3.1%) (Table 1). These results evince larval crude fat content was also affected by the protein and carbohydrate contents of the feeding substrate. This is consistent with literature as several studies on *Hermetia ilucens* larvae reported increased in larval crude fat composition of 7-39 % (dry matter) upon fed with high dietary of protein and carbohydrate which serves as stimulation for lipogenic activity and lipid storage (Nestel and Nemny-Lavy, 2008; Spranghers *et al*., 2017; Barragan-Fonseca, 2019). Referring to Table 1, the crude fat content in both feedings met the standard nutrient requirements.

The gap differences between crude protein and fat content of BSFP in the present study and the available commercial product is due to the variation of the harvesting stage. The BSF in this study were analysed at the pre-pupal stage, whereas the commercial products were investigated at the larvae stage. The results obtained in the current study is in line with findings by Liu *et al*. (2017) whereby the highest level of crude protein and crude fat content appeared to be in the early pre-pupae stage. The crude protein content varied greatly across insect species and life stages (Veldkamp *et al*., 2012). In addition, Product B shows the lowest crude protein and crude fat content due to the ingredients consist of chicken feed grains mixture, which is low in protein, instead of 100% dried BSFL.

Effect of feeding strategy on the BFS fatty acid profile

The composition of fatty acid profile was identified by means of GC-MS and the obtained FAME profile were illustrated in Figure 3 for the (a) control and (b) modified feeding. The data obtained from Figure 3 (a) and (b) were tabulated into Table 3 and compared with other recent published articles. FAME contents from both feeding strategy in this current study evinced the BSFP-derived oil contains myriad of saturated fatty acid (68.1% in control feeding and 68.6% in BSFP fed with additional lyophilized bacterial cells) as compared to unsaturated fatty acid (28.3% in control feeding and 28.4% in modified feeding). Furthermore, this study evinced lauric acid (C12:0) was present at the highest amount *circa* 39.5-39.8%, followed by elaidic acid (C18:1) 14.8-16.0% and myristic acid (C14:0) 12.9-13.1%. Irrespective of rearing substrate, lauric acid (C12:0) was found to be the most abundant in all BSF samples in this current study and align with the other references (Leong *et al*., 2016; Hong *et al*., 2018; Wong *et al*., 2019; Ewald *et al*., 2020; Mohamad *et al.*, 2020). Lauric acid (C12:0) has a great impact in poultry feed to promote not only the poultry growth and weight gain, but also exerts positive effects on health performance of poultry (Fortuoso *et al*., 2019). Withal, lauric acid can be employed as a replacement of conventional antimicrobial used in the diets of broiler chicken (Fortuoso *et al*., 2019).

It is interesting to note that modified feeding with bacterial cells evinced considerably high amount of palmitic acid (11.7%) and palmitoleic acid (4.9%) (Table 3). In contrast, palmitic and palmitoleic acid were not observed in the control feedings. In animal studies, palmitoleic acid shown to have anti-inflammatory and lipid-lowering effects. Palmitic acid support normal cellular membrane function and help body to store energy for metabolic function.

Table 3: FAME composition (%) derived from BSF fed with various rearing substrates.

FAME Composition	Value (%)				
	This study		Mohamad *et al*. (2020)		Leong *et al*. (2016)
	Control feeding	Modified feeding	Control feeding	Modified feeding	Pre-consumer waste
	P50:C50	Bacterial cells	Random kitchen waste	Bacterial cells	
Capric acid (C10:0)	2.5	2.3	1.0	1.6	0.6
Lauric acid (C12:0)	39.5	39.8	37.0	48.7	27.8
Myristic acid (C14:0)	13.1	12.9	8.0	14.1	5.9
Palmitic acid (C16:0)	0	11.7	15.3	9.8	20.4
Palmitoleic acid (C16:1)	0	4.9	2.66	4.2	4.0
Cyclopropyl acid (C17:0)	0.6	0	0	0	0
Stearic acid (C18:0)	12.4	1.9	1.6	1.2	3.0
Oleic acid (C18:1)	4.7	0	20.6	13.5	24.8
Elaidic acid (C18:1)	14.8	16.0	0	0	0
Linoleic acid (C18:2)	8.3	7.5	10.5	5.2	13.2
Linolenic acid (18:3)	0.5	0	0.4	0	0

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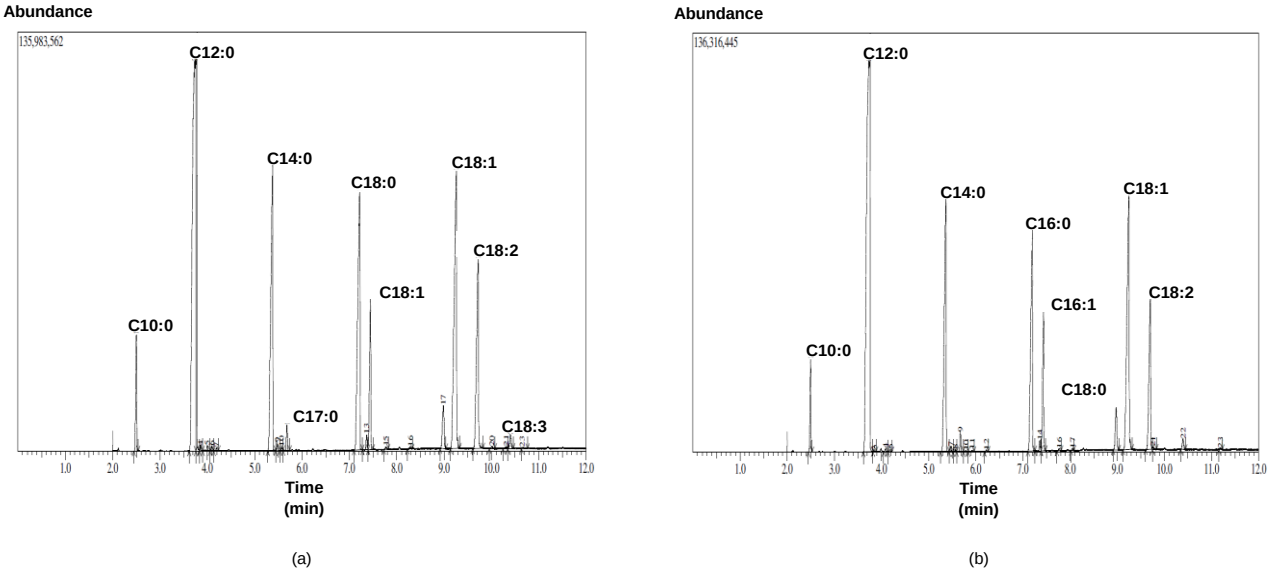

Figure 3: GC FAME profile derived from BSFL fed with two-diet patterns: (a) P50:C50 and (b) additional bacterial cells.

Crude fibre content

The crude fiber is a fraction contains the indigestible carbohydrates and cellulose, which are insoluble in alkali solutions or weak acid. Diet contains high fiber content will inflict undesirable digestion problem. The standard specification of crude fiber content in poultry feed must not exceed 5% in total. The crude fiber content obtained in this study is 7.39-7.67%, with slight increase of *circa* 2% as compared to the standard specification. Treatment may deem necessary to reduce the fibre content prior any application for animal feed. Product A contained at least twice (15.73 ± 6.78%) of the total crude fiber content in control and modified feeding. Extremely high dietary fiber in animal feed will causes delayed feeding, decreased intake of feed, inefficient digestibility and reduced nutrient utilization which will lead to the poor growth of animal (Nyakeri *et al*., 2017).

Crude ash content

The ash content of poultry feed represents the inorganic minerals content of the feed (Rahman *et al.*, 2014; Ofori *et al.*, 2019). Minerals play an important role in the formation of stronger bone and other important functions such as the development of blood cells, blood clotting, energy metabolism and enzyme activation. In this study, the crude ash content in both feedings were found in the range of 5.6% to 6.6% (Table 1). Additional feeding with cells at late stage of the BSFL in modified feeding increase the crude ash content of the BSFP and resulted in desirable amount set by Ministry of Agriculture Malaysia. The ash content of Product A is the highest at 17.5 ± 0.2%, exceeding the standard specification *viz*. maximum value of 8%.

Moisture and dry matter content

The moisture content (MC) is representing by the amount of water in the feed. Maximum standard specification value for MC is 13%. The MC of BSFP in control and modified feeding were 17.6 ± 1.6% and 21.4 ± 3.4% (Table 1). Both data exceed the specification value. Different drying method will affect the MC of the harvested BSF. Our study shows high MC due to the combination of two drying method used, *viz*. lyophilization (to prevent protein deterioration at the initial stage of analysis) followed by oven-drying in an air circulating oven. Drying method for animal feed at industrial scale is achieved oven-drying. Achieving optimum MC is important to prolong the storage and shelf life. High MC will lead to the production of mycotoxins and spoilage during storage and may elevate the risk of health problem upon consumption (Ofori *et al.*, 2019). Howbeit, this can be deterred by adding an antifungal additive into the feed ingredients. Hence, it is very crucial to ensure the MC of the feed is keep at minimum (less than 13%) to impede fungal growth. Therefore, other method of drying such as oven drying at prolong low temperature should be applied to further reduced the MC of the harvested BSFP. The dry matter is 82.4 ± 0.3% and 78.6 ± 0.9%, for control and modified feeding, respectively.

PHB content

Figure 4 shows the photographs of excreted frass black in colour mixed with PHB polymer appeared in whitish colour. Feeding BSFL with additional bacterial cells resulted in two desirable outputs which are high protein content of BSFP and extracted biopolymer of PHB. PHB content of 73.8% was obtained from the biologically recovered PHB granules washed with distilled water

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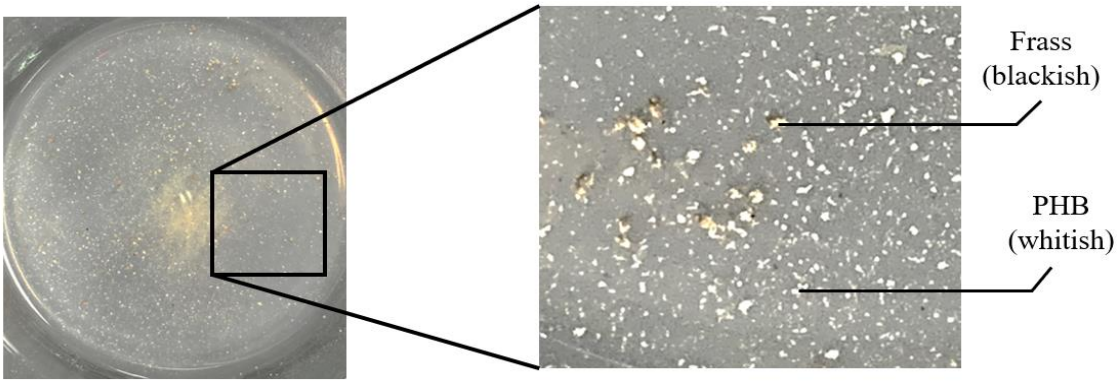

Figure 4: Photographs of excreted frass mixed with PHB polymer.

(Table 1). The observation demonstrated that BSF holds the potential application as biological extraction agent of PHB without negatively affects BSF crude protein content. This result is comparable with the findings by Murugan *et al*. (2016) which obtained 89% PHA from biological extraction using mealworm. The current studies resulted in negligibly low PHB content due to the frass collected contain remaining food waste which was in wet form. The wet condition of the frass has incorporated with the food waste and BSFL skin which contributed to the low PHB content.

CONCLUSION

The crude protein content obtained in this study is higher as compared to the commercially available products, which is desirable to be applied in poultry and aquaculture feeds. Moreover, this study demonstrated that BSFL can consume lyophilized cells, and two desirable outputs were obtained which are high protein content of BSFP and biologically extracted PHB in white pellet form secreted with the mixture of frass. Feeding BSF with cells did not negatively affect the protein content. A potential biological method of extracting PHB granules using BSF as extraction agent was successfully ascertained. Furthermore, the present study provides an alternative approach to minimize the use of hazardous solvents during polymer extraction process.

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