

Effect of feeding strategy on the protein and fatty acid contents of black soldier fly prepupae (*Hermetia illucens*) for the potential applications as animal feed and promising alternative protein-rich food

L Mohamad¹, F Dina², H A Hasan², K Sudesh², S Baidurah^{*1}

¹School of Industrial Technology, Universiti Sains Malaysia, 11800, Minden, Penang, Malaysia

²School of Biological Sciences, Universiti Sains Malaysia, 11800, Minden, Penang, Malaysia

*Email: sitibaidurah@usm.my

Abstract. There is considerable interest in the exploring of an alternative protein source due to ever-growing population in the world. Black soldier fly (BSF), *Hermetia illucens*, can be a promising alternative protein-rich food due to its high protein content as compared to livestock. This study investigated the effect of different feeding strategies on the crude protein of BSF prepupae (BSFP) to further enhance the protein contents. The feeding strategy was done at BSFP stage using two different diets which were a mixture of food waste as a control, and bacterial dried cells. Protein and fatty acid content analyses were done on the freeze-dried BSFP samples harvested on day 4 after feeding in order to determine the crude protein and fatty acid. BSFP fed with lyophilized cells showed enhancement in the nutritional contents, compared to the conventional feeding strategy using food waste, with increased crude protein content by 17%. This study demonstrated that the bacterial dried cells can be utilized as a single cell protein to further increase the protein content in the BSFP body which can be applied for the animal feed and potential human consumption.

1. Introduction

The global food production system is facing challenges due to the ever-growing population in the world. According to the Food and Agriculture Organization of the United Nations (FAO), with the exponential growth of the global population, it is expected to reach the threshold of 9 billion people by 2050 [1]. This will result in a greater need for food, especially for animal protein sources such as cattle, poultry and fish. In addition, the FAO has foreseen that global meat production and consumption will double by 2050 compared to 2000 [2]. Food production supply will continuously decrease and this situation will be unable to satisfy the demand of the global population, hence, will lead to the food-security crisis. Livestock farming is deemed unsustainable and resulted in many environmental impacts such as the



accumulation of all anthropogenic greenhouse gases (GHG) [3], space competition with human and contribute to higher water footprint than plant-based foods [4].

To mediate this, a sustainable diet consists of alternative protein sources need to be considered. Insect meals may serve as one of the options to achieve more sustainable production due to their potential food source, high feed conversion efficiency compared to traditional livestock, short period of breeding, less space required during breeding, and high protein content [5]. Moreover, insects are considered the best alternative as a partial or complete substitution for fish meal due to their nutritional contents, ease of rearing and high biomass production of larvae or pupae [6]. Table 1 shows the composition of commonly consumed insects as compared to conventional foods [7,8,9]. Overall, most of the insects contain high amount of protein content which is comparatively higher than the conventional food up to 41%.

Table 1. Protein content of conventional foods and commonly consumed insects [5,7,8,9].

Organism	Protein (%)
Conventional foods	
Beef	18.4
Lamb	15.4
Pork	14.6
Chicken	22.0
Fish	18.3
Insects	
Termites	23.3
Caterpillars	38.1
Adult weevils	30.3
House fly pupae	63.1
May beetle larvae	11.1
Adult female ants	7.4
Adult male ants	25.2
Bee	18.1
Silkworm	23.1
Grasshopper	46.1
Black soldier fly	40.2

Insects such as *Hermetia illucens*, commonly known as black soldier fly (BSF) can be a promising alternative protein-rich food due to its high protein content as compared to livestock [7]. Black soldier fly larvae (BSFL) is regarded as a great potential in the feed sector whereby insect-meal could substitute in part fish-meal and soybean-meal as a protein source in the animal and aquaculture feed industry [8]. The BSFL can consume a wide range of biowaste, such as fruits and vegetable waste, kitchen waste, abattoir waste and animal manure, which will directly affect the nutritional value of the black soldier fly prepupae (BSFP) [9].

The aim of this study is to further increase the protein content of BSFP for the applications of animal feed and promising alternative protein-rich food. The feeding strategy was modified by feeding the BSFP with bacterial dried cells instead of conventional feeding with food waste, with the aim to increase the crude protein content of BSFP. The obtained BSFP was further subjected to the proximate analysis to determine the protein and lipid content. In detail, the feeding strategy was done at BSFP stage using two different diets which were a mixture of food waste as a control, and bacterial dried cells containing intracellular polyhydroxybutyrate (PHB), a type of biodegradable polymer. *Cupriavidus necator* wild type H16 (ATCC 17699), formerly known as *Ralstonia eutropha* was the strain used in this modified feeding strategy. This strain is capable of accumulating intracellular granules of PHB under growth media rich with carbon source and limiting nitrogen source [10]. PHB and its copolymers are biodegradable polymers that have similar properties as conventional petrochemical plastics with non-

toxic and biocompatibility behaviour which are of particular interest [10]. In the latter feeding strategy, two products were obtained: (1) the BSFP as a source for animal feed and as a promising alternative protein-rich food, and (2) the extracted PHB through biological extraction method, by utilizing the intestine of BSFP.

2. MATERIALS AND METHODS

2.1 Organism and culture media

Cupriavidus necator wild type H16 (ATCC 17699) was used throughout this study. The bacterial cell was sub-cultured on the agar plate for short-term storage. For long-term preservation, the bacterial cells were maintained in a 40% (v/v) glycerol stock solution. The strain was sub-cultured on the nutrient-rich (NR) agar medium consisting per liter: 2 g yeast extract, 10 g peptone, 10 g meat extract and 15 g of bacteriological agar powder [11]. The pH of the medium was adjusted to 7 by using 1 M of hydrochloric acid (HCl) and 1 M of sodium hydroxide (NaOH). The medium was sterilized by autoclaving at 121 °C for 15 minutes using the autoclave machine (Model: TOMY-ES-315, Japan). NR broth was prepared using the same method as the NR agar medium, but without adding bacteriological agar powder.

2.2 Bacterial cultivation

Bacterial cultivation used in this study was basically the same as that described in our previous paper with a slight modification [10]. *C. necator* bacterial colony on the NR agar plate was inoculated into NR broth and grown in the incubator shaker (Model: Infors Ecotron, New Zealand) at 200 rpm at 30 °C, 24 hours for cell activation. After the cell activation, 15 mL of the NR culture was inoculated into 300 mL of mineral medium (MM) in 500 mL shake flask. The fermentation media consists of MM and minor element solutions with fructose concentration of 20 g/L as the sole carbon source. The MM composed of 4.6 g/L Na₂HPO₄, 4.0 g/L NaH₂PO₄, 0.39 g/L MgSO₄, 62 mg/L CaCl₂, 0.45 g/L K₂SO₄, and 1 mL/L of trace element solution. The trace element solution was prepared by dissolving 2.4 g/L MnSO₄·H₂O, 0.48 g/L CuSO₄·5H₂O, 15 g/L FeSO₄·7H₂O, and 2.4 g/L ZnSO₄·7H₂O, in 1 L of 0.1 M HCl [11]. The culture was incubated in an incubator shaker at 200 rpm at 30 °C, 48 hours for production of polyhydroxybutyrate (PHB). After 48 hours of fermentation, the culture was centrifuged at 8590 g and 4 °C for 8 minutes using bench top centrifuge (Model: KUBOTA 5100, Japan). The cell pellets collected were washed with distilled water and kept at -20 °C prior to freeze-drying process [11].

2.3 Cell freeze-drying

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 hours. The obtained freeze-dried bacterial cells were subjected to feeding experiment.

2.4 Feeding strategy of black soldier flies prepupae

The BSF were obtained from School of Biological Sciences, Universiti Sains Malaysia (USM) and placed in plastic containers (20 x 10 x 18 cm) covered with a net. BSFL were fed with the conventional diet consisting of food waste collected from Bakti Cafeteria, USM for the survival and larval development. Once it turns into pre-pupae, the BSFP will crawl out of the food waste container to find a hiding place. A total of 400 BSFP was collected and starved for 48 hours in a different container containing sawdust. Prior to the feeding experiment, the BSFP were transferred into a clean plastic container and was fed with the freeze-dried *C. necator* once a day for a consecutive 3 days with a feeding rate of 5% of the total body weight per day as described by Murugan et al. [12], utilizing mealworm as a model organism. The BSFP were harvested on day 4 and washed with distilled water to remove the remaining food residue and fecal pellets. Next, the BSFP were placed on tissue paper for the purpose of drying. The BSFP bodies were kept in -20 °C overnight and were then freeze-dried using the same setting as in section 2.3 for 4 days. The freeze-dried BSFP sample was kept for further proximate analysis.

2.5 Analysis of BSFP

The analysis of BSFP comprised of analytical determination of crude fat, crude protein, crude fiber, ash, moisture and dry matter. The initial analysis procedure is to determine the total lipid content by using the Soxhlet extraction method [13]. The freeze-dried and ground BSFP sample was continuously extracted with petroleum ether for 6 hours and the remaining defatted residue was dried and weighed. The defatted sample was further used for determining crude protein content while the extracted oil sample was further subjected to Gas Chromatography-Mass Spectrophotometry (GC-MS) analysis for fatty acid methyl ester (FAME) determination. Nitrogen determination of BSFP was conducted using the Kjeldahl method [13] which involves digestion, distillation and titration. Protein extraction was done prior to Kjeldahl analysis by using an alkaline extraction method by utilizing NaOH. Detailed methodology is described in the following subsection. The crude protein content was calculated using a protein-to-nitrogen conversion factor of 6.25 (based on protein containing an average of 16% nitrogen) [14].

Crude fiber analysis was performed by boiling the dried defatted sample with 5% H₂SO₄, followed by alkaline hydrolysis with 25% NaOH, which removes some hemicellulose and lignin [13]. The remaining residue after digestion was oven dried and placed in a muffle furnace at 450 °C and burned until no black spot were observed [13]. Crude fiber was measured as the weight loss on incineration of the residue remaining after digestion of the sample. Crude ash, which comprised of non-combustible minerals was analyzed by heating the ground BSFP in a muffle furnace for 4 hours at 550 °C [13]. The moisture content and dry matter were determined by oven-dry the ground freeze-dried BSFP in an air circulating oven at 105 °C until constant weight was achieved [13].

2.6 Protein Extraction

The ether-defatted BSFP product was used for protein extraction by utilizing NaOH [14]. One g of each defatted BSFP powdered sample was weighed accurately into separate triplicate falcon tube. The powdered samples were then dissolved in 15 ml of 0.25 M NaOH [14]. In order to enhance the extraction process, this steps was carried out in an incubator shaker set at 40 °C for 1 hour at 100 rpm. Next, the suspension was centrifuged at 4 °C for 20 minutes at 3500 g [14]. The second extraction was performed on the remaining suspended pellet collected at the base of the tube. The pH of the supernatant and gel layer collected from both extractions were adjusted to 4.3 - 4.5 with 2 M HCl at room temperature [14]. The precipitated protein was then harvested by centrifugation at 4 °C for 15 minutes at 2500 g [14]. The supernatant and gel layer were discarded while the pellet containing the desired protein precipitate was washed 3 times with distilled water to rinse out HCl traces followed by centrifugation at 4 °C for 10 minutes and 2500 g [14]. The precipitate was frozen at -20 °C overnight (about 18 hours). The final protein extract with moisture content less than 5 % was obtained by freeze-dried the frozen precipitate.

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

Fatty acid analysis consisted of two consecutive steps, preparation of fatty acid methyl ester (FAME) and chromatographic analysis [15]. FAME was synthesized from the lipid sample by heating 0.3 g of the oil sample with 5 mL of methanolic NaOH for esterification. The sample was boiled for 5 to 10 minutes under reflux until the fat droplets disappear. The sample was esterified, and 7 mL of the methanolic boron trifluoride solution was added and boiled for another 2 minutes. The amount of 3 mL of *n*-heptane was added and boiled for 1 minute in order to recover the methyl ester in an organic phase. The mixture was cooled to room temperature and followed by the phase separation of the aqueous and organic layers by adding saturated NaCl solution in the mixture. The upper layer of *n*-heptane phase was pipetted out into the test tube, and a small amount of anhydrous Na₂SO₄ was added to remove the traces of water from organic solutions. The methyl ester solution was transferred into vials and subjected to GC-MS analysis to determine the fatty acids composition.

2.8 Analysis of fatty acid methyl ester (FAME) using gas chromatography-mass spectroscopy (GC-MS)

Fatty acid methyl ester (FAME) analysis 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 where 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 mass spectra with the mass spectral database.

3. RESULTS AND DISCUSSION

The comparison of nutrient compositions of BSFP is shown in table 2. The crude protein content of freeze-dried BSFP fed with bacterial-dried cells was comparably higher with a value of 63.67%, which was the highest as compared to studies reported by Ruhnke et al. [19], Liu et al. [5] and Spranghers et al. [17]. This observation indicated that feeding strategy using lyophilized cells which acts as a single cell protein (SCP) contributes to the higher protein content inside the BSFP body. The SCP refers to protein derived from the cells of microorganisms which are grown on various carbon sources such as bacteria, fungi, algae and yeast [16]. The protein content of *C. necator* can reach up to 74% of the total cellular content compared to yeast, fungi and algae which was around 50% [16]. Furthermore, a study carried out by Murugan et al. [12] on the experimental feeding of mealworms with *C. necator* showed a significant increase of protein in the body compared to mealworms fed with oats (conventional diet) which was parallel with the findings in this study by utilizing BSF as a model organism fed with *C. necator* showed increase in protein content up to 17%.

Table 2. Comparison of nutrient compositional analyses of black soldier fly (BSF) larvae (% in dry matter).

No.	Nutrient content (%)	This study Feeding with bacterial dried cells	References Feeding with organic food waste		
			[19]	[5]	[17]
1.	Crude Protein	63.67 *	46.70	40.20	43.10
2.	Crude Fat	24.30	42.20	28.00	38.60
3.	Crude Fibre	4.22	1.56	-	-
4.	Ash	9.90	5.56	8.80	2.70
5.	Moisture Content	4.23	-	-	-
6.	Dry Matter	95.77	-	-	-

* Relative standard deviation = 8.44 % ($n = 3$)

BSF is an edible insect which contains high amount of protein and fat. The BSF protein composition can be manipulated by using different substrates in a rearing conditions [17]. The BSFL used in this study were fed with a mixture of food waste for survival and development, which latter fed with bacterial dried cells at the stage of early prepupae. In this study, the BSFP were fed with lyophilized cells of *C. necator* cultivated on fructose as a sole of carbon source. The BSFP were fed daily for a period of 3 days with 3.16 g of freeze-dried bacterial cells, which is about 5 wt% of the total BSFP body weight. In a recent report on the biological recovery approach for PHB using mealworm by Murugan et al. [12], on average, mealworm consumed about 5 wt% freeze-dried bacterial cells of their total body weight daily. Therefore, this feeding rate can be applied to BSFP since the basic structure of the internal guts is similar across insects, although they possess a diversity of modification associated with adaptation to different feeding states [18].

The crude fat contents of BSF obtained in this study were rather low compared to the crude fat levels reported by Ruhnke et al. [19], Liu et al. [5], and Spranghers et al. [17]. BSFL reared on restaurant waste

[17] had slightly higher crude fat content (38.6%) compared to this current study (24.3%). For both experimental diets, the BSFL were fed with kitchen waste during the larvae development and the nutrient was incorporated into their bodies. The food waste used in this study contained a mixture of protein, fat, carbohydrate and fibre. The food waste collected were segregate according to their nutritional content, for example chicken, meat or fish waste for protein, excess oil accumulated from the food waste for fat, rice waste for carbohydrate and vegetables or fruit pulp waste for fibre. By comparison, the nutritional content in the food waste obtained might be varied at a different location. Hence, this will directly affect the accumulation of nutrient as well as the lipid content inside the BSFP body. In addition, BSFL reared on brewery spent grain [19] had the highest crude fat content (42.2%) compared to those reared on other substrates. Generally, a decreasing trend in fat content is observed with an increase in the protein content. In terms of application of BSF for animal feed, high levels of lipids in the fish diet can lead to problems such as excessive fat deposition in the liver, which may decrease the fish health, quality and shelf life of the final product [20]. Hence, our study demonstrated a favorable property to be applied in animal feed industries.

The effects of the feeding strategy on the FAME composition derived from the BSFP biomass was determined and the obtained results for GC-MS analysis of BSFP FAME was illustrated in figure 1. The first peak representing capric acid methyl ester (C10:0) (1.57%) appeared after 2.487 minutes while the other major peaks, representing lauric acid methyl ester (C12:0) (48.70%) appeared at 3.702 minutes, myristic acid methyl ester (C14:0) (14.07%) at 5.334 minutes, palmitic acid methyl ester (C16:0) (9.78%) at 7.166 minutes, palmitoleic acid methyl ester (C16:1) (4.17%) at 7.418 minutes, stearic acid methyl ester (C18:0) (1.19%) at 8.969 minutes, oleic acid methyl ester (C18:1) (13.49%) at 9.205 minutes and linoleic acid methyl ester (C18:2) (5.17%) at 9.672 minutes.

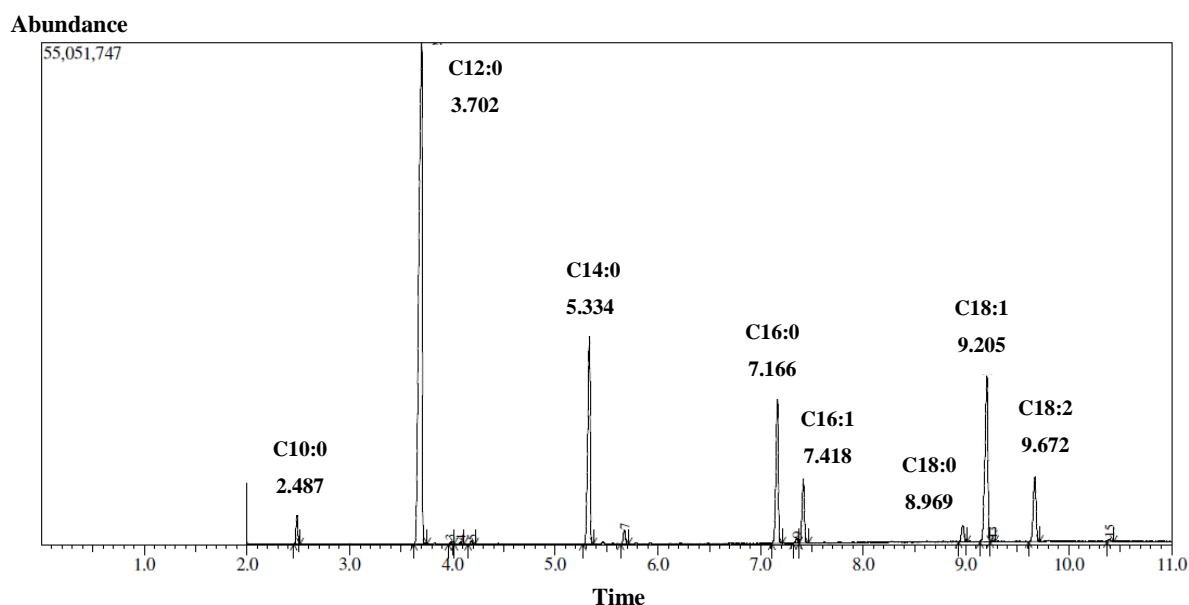


Figure 1. GC-MS analysis results of BSFP FAME.

Referring to table 3, the FAME of BSFP content are comparatively the same with other reported study, with little changes in the relative content. The BSFP FAME composition in this study was found to contain higher saturated fatty acid as compared to unsaturated fatty acid composition, which were in reasonable agreement with the results reported by [21]. In detail, the highest composition of FAME produced by BSFP after three times feeding in this study was lauric acid methyl ester (C12:0) with 48.70%. This notable trend of fatty acid composition corresponds to the previous study [21], despite undergoing different feeding strategy, the highest composition of lauric acid (27.80%) was observed. Overall, the results revealed that even though the current study produces lower total crude fat content;

24.30% as compared to 42.20% [19], the compositions of FAME synthesized from BSFP fed with bacterial dried cells were parallel to the fatty acid profile from other reported finding [21].

Table 3. FAME composition (%) derived from BSF fed with various feeding strategies

FAME Composition	Value (%)		
	This study		[21]
	Control normal feeding with food waste	3 times feeding with bacterial cell	Feeding with pre-consumer waste
Capric acid methyl ester (C10:0)	1.02	1.57	0.6
Lauric acid methyl ester (C12:0)	36.96	48.70	27.8
Myristic acid methyl ester (C14:0)	7.98	14.07	5.9
Palmitic acid methyl ester (C16:0)	15.30	9.78	20.4
Palmitoleic acid methyl ester (C16:1)	2.56	4.17	4.0
Stearic acid methyl ester (C18:0)	1.59	1.19	3.0
Oleic acid methyl ester (C18:1)	20.62	13.49	24.8
Linoleic acid methyl ester (C18:2)	10.46	5.17	13.2
Linolenic acid methyl ester (18:3)	0.43	Nil	Nil

Nil = zero

4. CONCLUSION

The issue of the increasing global demand for protein in future can be prevented by replacing the conventional protein-based food with BSFP as an alternative protein source. This entomophagy, however, is still new not only in Malaysia but also in most parts of the world. Protein derived from this edible insect can be extracted using an alkaline extraction method by utilizing NaOH. In this study, by feeding the BSFP with bacterial dried cells have successfully increased the protein content up to 17% as compared to the previous reported studies of BSFL reared on brewery spent grain by Ruhnke et al. [19]. This feeding strategy has also proved that BSF could digest the lyophilized cells of *C. necator* and the protein content in the BSF body was enriched. Furthermore, this feeding strategy has successfully extract the intracellular PHB of *C. necator* in a fecal pellets forms by utilizing the guts of BSFL. The purity of biologically extracted PHB granules washed with distilled water is 82.95%.

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ACKNOWLEDGEMENTS

The authors greatly acknowledge RUI Grant, Universiti Sains Malaysia (1001/PTEKIND/8011022) for the financial support. Lydia Mohamad expresses her in-depth gratitude towards Universiti Sains Malaysia for supporting her MSc studies through Kuok Foundation MSM Fellowship Award.