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In-Situ Yeast Fermentation to Enhance Bioconversion of Coconut Endosperm Waste into Larval Biomass of *Hermetia illucens*: Statistical Augmentation of Larval Lipid Content

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Received: 12 November 2019; Accepted: 8 January 2020; Published: 19 February 2020



Abstract: The aim of this study was to spur the lipid accumulation by larvae of *Hermetia illucens* or black soldier fly (BSFL) via feeding with yeast fermented medium. The *Saccharomyces cerevisiae*, a single cell yeast, was introduced at different concentrations (0.02, 0.1, 0.5, 1.0, 2.5 wt %) to execute an in-situ fermentation on coconut endosperm waste. The rearing of BSFL was started simultaneously and the rearing was stopped once the BSFL reached the fifth instar. With the increasing of yeast concentration, the rearing duration of BSFL was shortened from 15.5 to 13.5 days. Moreover, it was found that at 0.5 to 1.0 wt % yeast concentration, the lipid yield and lipid productivity of BSFL were statistically enhanced to their highest peaks, namely, at 49.4% and 0.53 g/day, respectively. With regard to biodiesel composition, BSFL-derived biodiesel contained mainly C12:0, C14:0, C16:0 and

C18:1. The higher amount of saturated fatty acids could strengthen the oxidative stability biodiesel produced as compared with non-edible oils or microalgal lipid. At last, the addition of yeast was also found to improve the waste reduction index of coconut endosperm waste (CEW) from 0.31 to 0.40 g/day, heralding the capability of BSFL to valorize organic waste via bioconversion into its biomass to serve as a feedstock for biodiesel production.

Keywords: black soldier fly; lipid; biodiesel; yeast; fermentation; organic waste

1. Introduction

The employment of microalgae to produce biofuels has gained increasing attention recently due to its high biomass productivities as opposed to edible and non-edible oil crops [1]. Several types of biofuel can be derived from microalgal biomass, namely, biodiesel, biogas, bioethanol and hydrogen fuel, through various reactive chemical conversions [2]. However, the main challenges encountered by the microalgal industry are the operating costs and convoluted technicalities in handling the cultivation processes that will directly affect its production rate [3]. On another note, insect biomass-based biofuel has advanced rapidly in the past five years, owing to its high biomass gained, low carbon footprint and the less land required for growing [4]. Moreover, fat yields from insects have been widely studied and the reported values vary across different species from 1.5% to 77%. Even within the same insect species, a variation of fat yields is still likely to be observed due to the dissimilar types of feed ingested by the insects [5]. Out of all the species, *Hermetia illucens*, also known as black soldier fly, shows most promising potential for biodiesel production. The larval feeding stage of this species only takes up to 3 to 4 weeks and its self-harvesting nature, pupating away from the medium, has eased the harvesting process [5]. Black soldier fly larvae (BSFL) are indeed native to America and the larvae stages propagate from the first to the sixth instars, then the larvae pupate before emerging into adult flies [6]. Several studies have shown the feasibility of BSFL biomass for biodiesel production [7–9]. Under different circumstances, there are also studies investigating the impact of various feeding mediums on the growth of BSFL that showed that changes in feeding mediums could substantially alter BSFL growth to a certain degree [10,11]. Moreover, it was also found that fermentation or the addition of microbes into feeding medium could impinge on the growth of BSFL [12,13]. Fermentation is generally a process that involves microorganisms catalyzing the breakdown of large biocompounds and transforming them into a myriad of value-added secondary metabolites under anaerobic conditions. With the progress of fermentation, the desired secondary metabolites produced by microorganisms accumulate and wait for harvest at the end of fermentation. The yield of desired products is largely controlled by the strain of microorganisms and fermentation environments [14]. Yeast, a single-celled microorganism, is broadly studied and applied in many industries for the production of different end products [15–17]. The typical commercial yeast species of *Saccharomyces cerevisiae* was selected in this study to execute the fermentation process on coconut endosperm waste (CEW). Thus, the prime aim was to investigate the bioconversion potential of in-situ fermented CEW by BSFL for eventual larval biodiesel production. The aim included varying the concentrations of yeast added into the CEW and identifying an optimum mixture between yeast and CEW to attain the targeted output, i.e., quality biodiesel. In addition, the treatment of organic waste, CEW in this case, was vindicated via bioreduction to convert it into larval biomass feedstock for producing BSFL-based biodiesel.

2. Materials and Methods

2.1. Coconut Endosperm Waste

Grated CEW was initially acquired from a local stall selling coconut milk and kept within 2 °C to 4 °C in a refrigerator. The moisture content of CEW was determined through the gravimetric method [18]

and adjusted to 70% by homogenizing with sterile distilled water as calculated using Equation (1) prior to use in the experiment.

$$V_{H_2O} = \frac{(\%_{H_2O})(M_S)}{1 - (\%_{H_2O})} - M_{H_2O} \quad (1)$$

where V_{H_2O} represents the total volume of sterile distilled water to be added, $\%_{H_2O}$ represents the percentage of desired moisture, which was 70% (0.7 was inserted into the equation) in this study, M_S represents the total dry weight of the CEW and M_{H_2O} represents the initial moisture content of the CEW.

2.2. Black Soldier Fly Larvae (BSFL)

An amount of 200 g of acquired CEW was weighed and transferred into a plastic container with dimensions of 35 cm × 25 cm (height × diameter) and left in a ventilated and sun-shaded area, serving as a bait to lure the female BSF. Several pieces of paper box cardboards with dimensions of 8 cm × 3 cm (length × width) were attached at the inner wall of the plastic container about 3 to 5 cm above the CEW medium, acting as a platform for female BSF to oviposit eggs. These cardboards were checked daily for the availability of BSF eggs. Then, the attained eggs were transferred into sterile Petri dishes and incubated until the larvae emerged. The new BSFL (neonate) were reared on CEW until they were 6 days old prior to use in the experiments.

2.3. Rearing of BSFL Using CEW Inoculated with Yeast

Different quantities of dry yeast powder (commercial brand: Bunga Raya; Manufactured by Kasihku Marketing Sdn. Bhd., Kedah, Malaysia) of 0.02, 0.1, 0.5, 1.0 and 2.5 wt % were separately homogenized with CEW, serving as an initial inoculum for in-situ fermentation to take place each. An amount of 10 g, dry weight basis, of each CEW inoculated with yeast medium was then immediately administered to 20 6-day-old BSFL. The larval rearing using each CEW medium inoculated with different percentages of yeast was stopped once the BSFL had reached its 5th instar as determined by the head size and body color [6,19]. Each batch of harvested BSFL was then dried at 105 °C until reaching a constant weight and grinded into powder before being stored at −20 °C prior to the chemical analyses [20]. All the CEW residues were separately collected and dried at 105 °C until reaching a constant weight before being grinded and stored in a desiccator for later chemical analyses. All the setups were at least duplicated for the statistical reliability verification using T test and quadratic regression analysis.

2.4. Growth Performance of BSFL

Upon completion of the experiments, the growth of BSFL was evaluated by several parameters, including Equation (2) waste to biomass conversion, (3) BSFL growth rate (4) efficiency of conversion of digested feed (ECD_F) and (5) metabolic cost [21]. The formulations for each parameter are listed below, together with other relevant parameters:

$$\text{Waste to biomass conversion (\%)} = \text{BSFL}_{DM} \text{ (g)} / \text{CEW}_{DM} \text{ (g)} \times 100\% \quad (2)$$

where BSFL_{DM} and CEW_{DM} are the total dry matter in the BSFL biomass, and CEW was introduced as feed (10 g) correspondingly.

$$\text{BSFL growth rate (g/day)} = \text{BSFL biomass gained (g)} / \text{Rearing duration (day)} \quad (3)$$

$$ECD_F \text{ (\%)} = \text{BSFL biomass gained (g)} / \text{Mass of total feed consumed (g)} \times 100\% \quad (4)$$

$$\text{Metabolic cost (\%)} = 100\% - ECD_F \text{ (\%)} \quad (5)$$

2.5. Treatment of CEW Via Reduction by BSFL

In order to determine the degree of valorizations of CEW, two parameters were measured, overall degradation and waste reduction index (WRI) [22], as shown below:

$$\text{Overall degradation (OD)} = \text{Total feed consumed (g)}/\text{Total feed offered (g)} \quad (6)$$

$$\text{WRI (g/day)} = \text{Total feed consumed (g)}/\text{Rearing duration (day)} \quad (7)$$

2.6. Biochemical Analysis

2.6.1. Lipid Solvent Extraction

An amount of 0.1 g of grounded BSFL powder was transferred into a pre-weighed 45 mL glass vial containing 20 mL of petroleum ether. The mixture was stirred for 24 h using a magnetic stirrer. Then, the mixture was filtered through a Whatman No.1 filter paper with a diameter 42.5 mm. The filtrate was collected and the residual BSFL powder was again mixed with 20 mL of petroleum ether twice and all the solvent filtrates were combined and dried using a rotary evaporator (Heidolph R-215; Manufactured by Heidolph Instruments GmbH & CO. KG, Schwabach, Germany) [9]. The retrieved BSFL lipid was again dried in an oven at 105 °C for 1 h to remove the remnant water content [23]. The dried BSFL lipid was cooled down in a desiccator and the total dry weight of BSFL lipid was recorded.

$$\text{Lipid content (\%)} = \text{Mass}_{\text{BSFL lipid}} \text{ (g)}/\text{Mass}_{\text{BSFL biomass}} \text{ (g)} \times 100\% \quad (8)$$

$$\text{Lipid productivity} = \text{Mass of lipid (g)}/\text{Rearing duration (day)} \quad (9)$$

where $\text{Mass}_{\text{BSFL lipid}}$ is the total dry mass of BSFL lipid and $\text{Mass}_{\text{BSFL biomass}}$ is the total initial biomass of BSFL (0.1 g) used for lipid extraction.

2.6.2. Transesterification and FAME Analysis

First, 100 mg of dried BSFL lipid was mixed with methanol containing 1 wt % sulphuric acid as the catalyst at the weight ratio of 1:8 followed by 1 mL of chloroform to improve the dissolution of the lipid. The mixture was heated up to 75 °C with agitation at 200 rpm for 1 h. Upon completion, the esters and unesterified-BSFL lipid were retrieved by adding 2 mL of hexane. Then, all the recovered portions of hexane were combined and dried under a compressed air blow. Base-catalysed transesterification was carried out by mixing the esters and unesterified lipid with the methanol containing 0.8 wt % sodium hydroxide as the catalyst at the weight ratio of 1:8 with 1 mL of chloroform and heated to 65 °C with agitation at 200 rpm for 30 min. The solvent layers containing methanol and chloroform were removed under the compressed air blow and the fatty acid methyl esters (FAMEs) mixture was washed with a 2 mL of 10 wt % sodium chloride solution and recovered with a 2 mL of hexane 3 times each. Again, all the portions were combined, transferred into a pre-weighed glass vial and dried under the compressed air blow. Further drying was carried out in the oven at 105 °C for 1 h to remove residual moisture present [24]. The glass vial was cooled down in a desiccator until ambient temperature before measuring the weight of FAMEs.

The FAME profile of BSFL-derived biodiesel was revealed by using the Shimadzu GC-2010 plus (Manufactured by Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector and a polyethylene glycol capillary column BPX-BD20 (30 m × 0.32 mm × 0.25 μm). The helium was used as a carrier gas and the inlet was operated in a split mode (1:50) at a temperature of 250 °C. The column temperature was programmed at the ramping mode. For sample peak determination, methyl heptadecanoate (C17:0) was prepared at a concentration of 1.00 mg/mL and served as an internal standard. The content of every FAME species in the biodiesel was finally calculated using Equation (10) [25]:

$$\text{FAME in biodiesel (\%)} = \frac{A_{\text{FAME}}}{A_{\text{ISTD}}} \times \frac{C_{\text{ISTD}} \times V_{\text{ISTD}}}{m} \times 100\% \quad (10)$$

where A_{FAME} represents the peak area of a specific FAME species, A_{ISTD} represents the peak area of internal standard, C_{ISTD} represents the concentration of internal standard (1.00 mg/mL), V_{ISTD} represents the volume of internal standard (1 mL) and m represents the dry weight of biodiesel mixed with the internal standard (mg).

3. Results and Discussion

3.1. BSFL Growth through Rearing at Different Yeast Concentrations

Looking at the waste to biomass conversion (WBC), BSFL was able to convert around 10% of CEW into its own body mass and the value was maintained when 0.02 wt % of yeast was introduced. When the yeast concentration increased from 0.1 to 1.0 wt %, the WBC value upsurged to around 11% and reached its plateau of 11.5% at 2.5 wt % yeast concentration. On the other hand, the growth rate of BSFL was maintained at around 0.065 g/day at 0 wt % and 0.02 wt %. The value increased to 0.77 g/day at 0.1 wt % and further increased with the increment of yeast concentrations, attaining its highest growth rate of 0.85 g/day at 2.5 wt %, as shown in Figure 1. This observation could be due to the introduction of yeast that favored the larval digestibility of carbohydrate compounds [26], thus, improving the assimilation of nutrients into BSFL body mass. Moreover, Yoon, et al. [27] reported that yeast was capable of breaking down carbohydrates by fermentation, especially in common monosaccharides, including D-glucose, D-fructose, D-mannose and D-galactose. On the other hand, it was proven that BSFL is able to convert additional glucose into lipid upon excess availability [28].

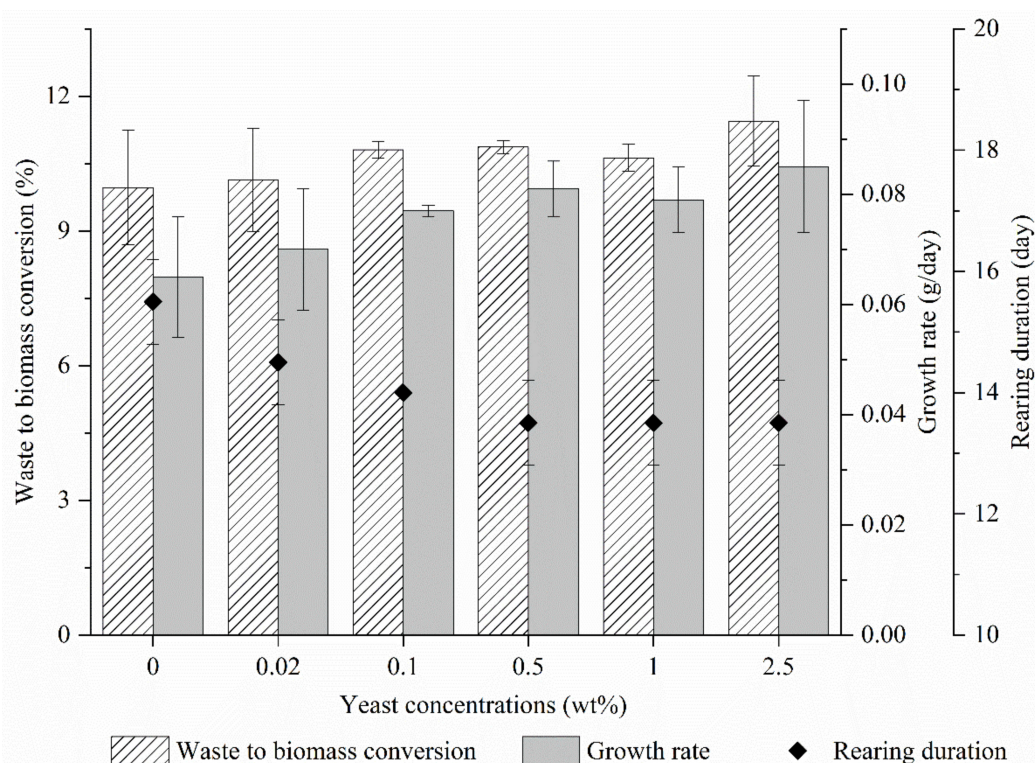


Figure 1. Growth performance of black soldier fly (BSFL) under different yeast concentrations.

Moving on to the feed conversion by BSFL when fed with CEW at different yeast concentrations (Figure 2), the feed to biomass percentage fluctuated between 9.5% and 11.5%. Meanwhile, the percentage of feed metabolised was substantially increased with the increasing yeast concentrations from 38% at 0 wt % yeast concentration to 42% at 2.5 wt % yeast concentration. However, the feed

residue percentage was reduced from 52% to 46% at yeast concentrations of 0 wt % (control) and 2.5 wt %, respectively. These results suggest that the addition of yeast in the feeding medium helped to reduce the solid matter of the waste after passing through the BSFL gut. Next, regardless of yeast concentration, the ECD_F of BSFL was maintained at around 20% to 22%. In this study, it was found that by feeding BSFL with CEW, the BSFL tended to have a high metabolic cost which averaged between around 78% and 80%. In future studies, it is suggested that the inclusion of other types of organic waste serve as BSFL feeding medium. With a lower metabolic cost, BSFL is able to consume and transform more organic waste into its body mass and hence, increase its body mass and biochemical compounds.

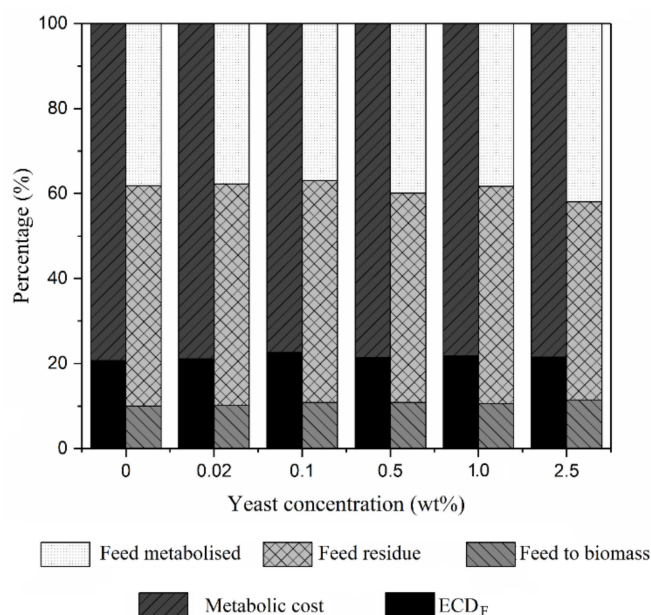


Figure 2. Conversion of coconut endosperm waste into BSFL biomasses.

3.2. Lipid and Biodiesel from BSFL

After solvent extraction, the lipid yield of BSFL was determined gravimetrically (Figure 3). The lipid yield of BSFL rose from 42.5% under control to 45.4% with the addition of only 0.02 wt % yeast in the CEW and this value maintained at 0.1 wt % and 0.5 wt % yeast concentrations. At 1.0 wt % yeast concentration, the lipid yield of BSFL reached its plateau at 49.4% and dropped to 44.4% when the maximum yeast concentration was applied in this study. Considering the lipid productivity, it can be seen that the value gradually increased from 0.027 g/day under control to 0.037 g/day at 0.5 wt % yeast concentration and it was maintained after that. In terms of absolute lipid content, the BSFL lipid increased from 0.42 g under control to 0.46 g at 0.02 wt % yeast concentration for 20 BSFL. This value again increased to 0.49 g and 0.53 g at 0.1 wt % and 1.0 wt % yeast concentrations, respectively. It was suggested that with the introduction of yeast, the yeast cells were able to uptake the lipids from remnant lipids belonging to CEW (about 26%) through simple diffusion when growing aerobically [29]. Subsequently, the BSFL were able to ingest the yeast cells as part of the nutrients and later, metabolize the yeasts' lipids into their own.

In terms of statistical analysis, a T-test was exploited to verify the reliability of duplicated replications for every setup individually containing a predetermined concentration of yeast. The analyses show that the T calculated was always lower than the T critical for both larval lipid yields and productivities; namely, $T_{\text{calculated}} = -1.6950 < T_{\text{critical}} = 2.0150$ and $T_{\text{calculated}} = 0.5547 < T_{\text{critical}} = 2.0150$, respectively. This signifies that all the duplicated replications were not significantly different and their means could be used for quadratic regression analysis via employing the curve fitting tool program (MATLAB 2019b). The quadratic equations derived for dependent variables of lipid yield are shown in Equation (11) and the productivity in Equation (12) against the independent

variable of yeast concentration is presented below where y represents the dependent variable and x represents the independent variable:

$$y = -2.9929x^2 + 7.694x + 43.965 \quad (11)$$

$$y = -0.0042x^2 + 0.0134x + 0.0308 \quad (12)$$

The values of R^2 for dependent variables lipid yield and productivity obtained were 0.7304 and 0.7534, respectively, indicating goodness of fit each for non-linear fitting data. By using the respective statistical models, the best lipid yields and productivities were found to transpire significantly in the range from 0.5 to 1.0 wt % of yeast concentrations.

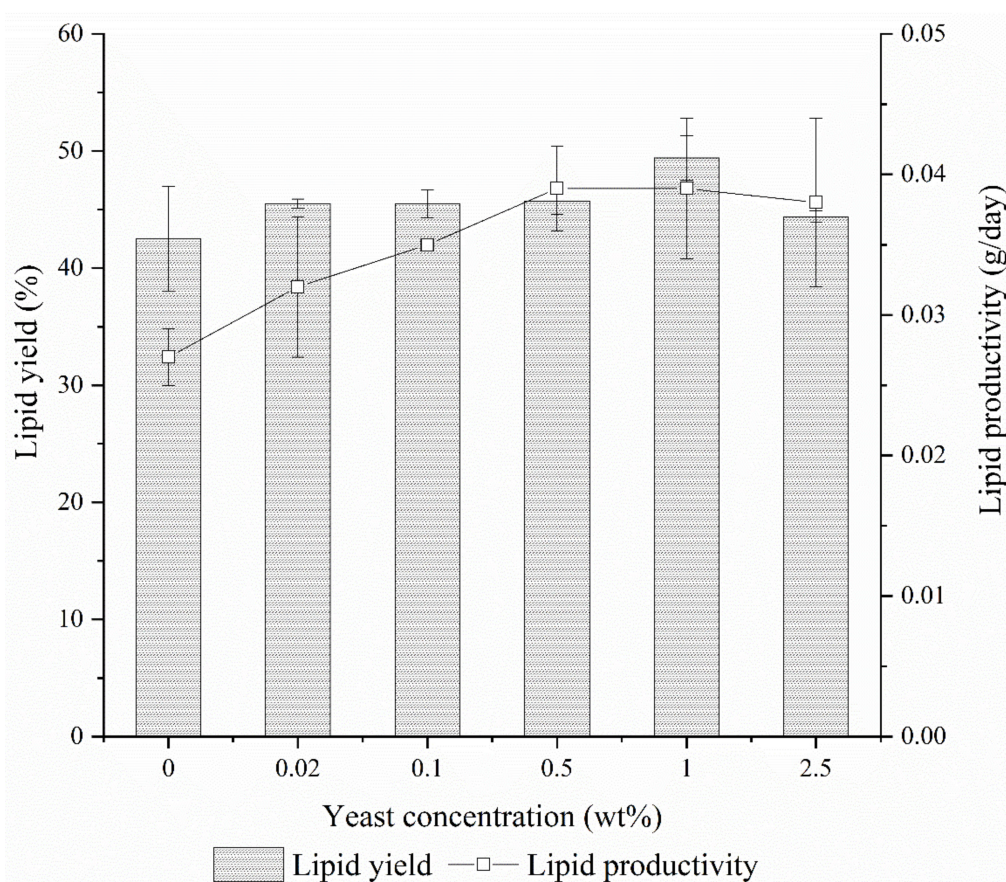


Figure 3. Lipid yield and lipid productivity of BSFL under different yeast concentrations.

BSFL lipids were able to be transesterified into biodiesel and used as diesel fuel to replace the common fossil diesel fuel and reduce the carbon footprint. The FAME composition of BSFL-derived biodiesel is summarized in Table 1. It can be seen that BSFL-derived biodiesel possessed a higher amount of C12:0, i.e., 66%, which was about 21% more than the BSFL fed with food waste. Moreover, C12:0 only could be found in insect-derived biodiesel and it was absent in the microalgal oil, or edible and non-edible oils. In addition, BSFL-derived-biodiesel accounted for 14.4% of C14:0, followed by 6.6% of C16:0, 5.6% of C18:1 and low amounts of C10:0, C14:1, C16:1 and C18:2. In comparison with microalgal oil and jatropha derived biodiesel, this feedstock contains mostly polyunsaturated fatty acids such as C18:1, C18:2 and C18:3. However, palm oil-derived biodiesel was mainly found to contain higher C16:0. Owing to the higher saturated fatty acids in BSFL-derived biodiesel, it possessed a higher oxidative stability and was stable as compared with other type of feedstock [30].

Table 1. Fatty acid methyl esters (FAME) compositions from BSFL-derived biodiesel and other types of feedstock.

Fatty Acid Methyl Ester	This Study	BSFL Reared with Food Waste [8]	<i>Chlorella vulgaris</i> [31]	<i>Jatropha</i> [31]	Palm [31]
C10:0	1.3 ± 0.06	n/a	n/a	n/a	n/a
C12:0	66.0 ± 1.44	44.9	n/a	n/a	n/a
C14:0	14.4 ± 0.12	8.3	2.3	0.2	1.22
C14:1	1.4 ± 0.01	n/a	n/a	n/a	n/a
C15:0	n/a	n/a	n/a	n/a	n/a
C16:0	6.6 ± 0.57	13.5	6	13.4	47.9
C16:1	2.2 ± 0.15	2.4	16.4	0.8	0.04
C17:0	n/a	n/a	n/a	n/a	n/a
C18:0	0.4 ± 0.09	2.1	10.3	6.4	4.23
C18:1	5.6 ± 0.45	12.0	26.5	36.5	37
C18:2	2.3 ± 0.22	9.9	n/a	42.1	9.07
C18:3	n/a	0.1	20.8	0.3	0.26

3.3. Waste Reduction of CEW by BSFL

In addition to the ability of BSFL biomass to be used as biodiesel feedstock, BSFL also shows the potential to reduce solid organic waste during the rearing process. In this study, it was found that the BSFL was able to degrade about 48% to 53% of CEW upon completion of bioconversion activities (Table 2). With the introduction of yeast at different concentrations in feeding medium, it showed that the waste reduction index increased from 0.31 g/day under control to 0.33 g/day at 0.02 wt % of yeast to 0.38% at 0.5 wt % yeast concentration. At last, it reached its highest point of 0.40 g/day at 2.5 wt % yeast concentration, as shown in Table 2. This could plausibly be owed to the addition of yeast in easing the digestion of CEW by BSFL [32]. For the case of CHN elementary analysis, although the contents of H and N in all the CEW residues did not vary significantly as opposed to the fresh CEW, namely about 12.6 ± 0.7% and 1.0 ± 0.1%, respectively, the initial C content of fresh CEW (53.7 ± 1.2%) generally decreased by approximately 10% while reaching the end of the larval rearing period. This decrease was possibly due to the synergistic larval and microbial oxidations in the increasing O content in addition to the trace amount of ashes (<2.0%), thereby, facilitating the nutrients' absorption by plants whilst employing the CEW residues as the fertilizer or soil conditioner.

Table 2. Overall degradation and waste reduction index of coconut endosperm waste mediated by BSFL at different yeast concentrations.

Yeast Concentration (wt %)	Overall Degradation (%)	Waste Reduction Index (g/day)
0	48.10 ± 0.39	0.31 ± 0.02
0.02	47.87 ± 0.95	0.33 ± 0.02
0.1	47.79 ± 1.27	0.34 ± 0.01
0.5	50.80 ± 0.73	0.38 ± 0.01
1.0	48.94 ± 0.07	0.36 ± 0.02
2.5	53.43 ± 1.62	0.40 ± 0.01

4. Conclusions

In conclusion, the addition of yeast at different concentrations impacted the lipid yield, lipid productivity and waste reduction index to certain degree. For higher lipid yield and lipid productivity, it was suggested that addition of 1.0 wt % of yeast into BSFL feeding medium may result in an improved outcome. Moreover, addition of a higher yeast concentration of 2.5 wt % was able to shorten the rearing duration and improve the waste reduction index. Finally, the BSFL-derived biodiesel was

found to contain higher saturated fatty acids that are more stable from being oxidized as opposed to the microalgal, jatropha and palm oils.

Author Contributions: Conceptualization, C.Y.W. and J.W.L.; methodology, C.Y.W.; software, C.Y.W. and M.N.M.A.; validation, M.K.L. and C.S.Y.; formal analysis, C.Y.W.; resources, H.A.H. and S.C.; data curation, P.L.S.; writing—original draft preparation, C.Y.W.; writing—review and editing, J.W.L. and O.D.H.; visualization, Y.C.H.; supervision, J.W.L.; funding acquisition, J.W.L., P.S.G., H.D., H.K. and G.-T.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by International Grant from Universitas Muhammadiyah Purwokerto with the cost center of 015ME0-094, Ministry of Education Malaysia under HICoE with the cost center of 015MA0-052 and International Grant from Universitas Islam Riau with the cost center of 015ME0-012.

Acknowledgments: The administrative and technical supports provided by the members from Centre for Biofuel and Biochemical Research, Universiti Teknologi PETRONAS are greatly acknowledged.

Conflicts of Interest: The authors declare no conflict of interest.

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