

## Article

# *Rhizopus oligosporus*-Assisted Valorization of Coconut Endosperm Waste by Black Soldier Fly Larvae for Simultaneous Protein and Lipid to Biodiesel Production

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**Abstract:** Coconut endosperm waste (CEW) was treated by *Rhizopus oligosporus* via in situ and ex situ fermentations together with bioconversion into valuable black soldier fly larval biomass. The ex situ fermentation could overall enrich the nutritional compositions of CEW by hydrolyzing its complex organic polymers and exuding assimilable nutrients to enhance the black soldier fly larvae (BSFL) growth. Nevertheless, the larval gut bacteria were competing with *Rhizopus oligosporus* in in situ fermentation, derailing the hydrolysis processes and larval growth. Accordingly, the highest growth rates achieved were around 0.095 g/day, as opposed to only 0.065 g/day whilst using 0.5 wt% of *Rhizopus oligosporus* to perform ex situ and in situ fermentations, respectively. These were also underpinned by the greater amount of total CEW consumed when employing ex situ fermentation, with comparable metabolic costs to feeding on in situ-fermented CEW. The mature BSFL were subsequently harvested and the amounts of protein and lipid produced were assessed in terms of their feasibility for biodiesel production. While the statistical analyses showed that the larval protein yields derived from both fermentation modes were insignificant, the BSFL could attain higher lipid and protein productivities upon feeding with ex situ- rather than in situ-fermented CEW mediums. Better yet, the larval biodiesel quality measured in terms of the fatty acid methyl ester composition were not varied significantly by *Rhizopus oligosporus* through the fermentation process. Thereby, the presence of 1.0 wt% *Rhizopus oligosporus* was considered optimum to perform ex situ fermentation, giving rise to the acceptable growth of BSFL loaded with the highest lipid yield and productivity for producing biodiesel and protein simultaneously.

**Keywords:** black soldier fly; fungal fermentation; *Rhizopus oligosporus*; protein; lipid; biodiesel

## 1. Introduction

Presently, the black soldier fly larval biomass has gained tremendous attention worldwide, especially in the aquaculture and livestock farming industries, due to its potential use as a protein alimentation in animal feed [1,2]. According to Schiavone et al. (2018) [3], it was found that the inclusion of black soldier fly larvae (BSFL) fat as a soybean oil replacement at 50% to 100% in broiler diets had no negative impact on broilers' growth and blood analyses. On the other hand, Fisher et al. (2020) [4] reported that a 200 g/kg BSFL meal serving as a protein replacement in low fish meal diets could be included for juvenile Atlantic salmon without impacting their growth performance. Apart from these, BSFL biomass has also been exploited as a feedstock for biodiesel production through a transesterification process, since the larval biomass possesses a high lipid content, in addition to other feedstock such as waste cooking oil or non-edible oil, including rubber seed oil [5–7]. On the other hand, low-cost organic waste including animal manure and restaurant waste as well as lignocellulose waste could be used for BSFL rearing [8–10]. In comparison with the third-generation biodiesel feedstocks such as yeast, the BSFL are preferable, as they can consume various types of organic waste, are low cost, and the leftover biomass can serve as a protein source for animal feeding purposes, whereas yeast oil produces a low lipid yield, has a high cost for its technology, and requires large water volumes for industry-scale application [11–13].

Moreover, BSFL have a short lifecycle and high growth rate, which enable the continuous and rapid production of larval biomass feedstock. The transesterification of larval lipid resulted in the highest biodiesel yield attained, 94.14%, via the following optimum reaction condition: 12 mL of hexane and methanol as the extraction solvents at a volume ratio of 1:2 (*v/v*), 1.2 mL of sulphuric acid catalyst, and reaction temperature of 120 °C for 90 min [14]. Additionally, the BSFL biomass residue generated after the lipid extraction could possibly be used in anaerobic digestion to produce methane gas. However, real applications of this technique still require inclusive validation [15]. The performance of BSFL-based biodiesel at 10% to 20% blending ratios was lately compared with that of conventional diesel fuel. The results showed that the inclusion of larval diesel could overall boost fuel consumption, with the equivalent fuel consumption rate demonstrating an increment with the rise in fuel injection pressure and time [16].

Nonetheless, the growth of BSFL is directly influenced by the type of feeding medium used, and there are not many reported works investigating the fundamental nutritional compositions of feeding mediums associated with BSFL growth at present. Hasnol et al. (2020) [17] demonstrated that the type of feeding medium used could affect the growth of BSFL, as well as the moisture content and substrate aeration during rearing. The type of sample processing method used during larvae inactivation, lipid extraction, and biodiesel transesterification also plays a role in determining the lipid yield and biodiesel qualities [17,18]. The type of larval feed used can undoubtedly affect the growth of BSFL; for instance, as reported by Pamintuan et al. (2019) [19], the BSFL grew faster when introduced with a chick mash in which the larvae took 23 days to reach maturity, with a survival rate of 76%. However, the BSFL took a longer time to grow—i.e., 26 days for both milkfish offal and vegetable waste administrations, with higher survival rates of 90% and 86%, respectively, as opposed to the chick mash. The difference in feeding medium employments to grow BSFL also causes a change in the proximate composition of produced larval biomass. The BSFL fed with chick mash possessed satisfactory amounts of larval protein and carbohydrate—namely, 35% and 36%, respectively—while containing merely 17% larval lipid and 12% larval ash. On the other hand, the BSFL fed with milkfish offal were loaded with the highest amount of larval carbohydrates (44%) and protein (39%), followed by merely 10% larval lipid and 7% larval ash. Lastly, the BSFL fed with vegetable waste led

to the highest larval lipid of 41%, followed by 32% larval protein, 15% larval ash, and only 12% larval carbohydrate.

Furthermore, a few studies have reported that through the fermentation of feeding mediums, the growth of BSFL could be enhanced by the presence of nutritional by-products exuded by fermenting bacteria [20–25]. The bacteria and enzymes were also inoculated in the larval feeding mediums to improve the digestibility of feed as well as enhance the degradation of fibres into sugars that would be assimilated easily by BSFL. Additionally, spontaneous fermentation by the microbes that were originally presenting in the medium could be a way to fortify the nutritional content and later improve the growth of BSFL [25]. Unfortunately, these reported studies employed a mixture or unknown species of bacteria to carry out the unconditioned fermentation process. Hence, the reaction pathways and end by-products are unfathomable and hard to identify. These approaches may have limited feasibility for industry-scale applications, as there are not known bacteria species and metabolism pathways for employment. Knowing this, the introduction of *Rhizopus oligosporus* as a fermentation bioagent was initially suggested, as this fungus is well-known for its use in soybean fermentation to produce tempeh and its metabolism pathway is also well established.

*Rhizopus oligosporus*, a fungus that is safe for food consumption, is generally used to enhance soybeans via fermentation to create a delicacy, tempeh, which is popular in Southeast Asia. This fungus is also capable of reducing anti-nutritional factors such as pyrimidine glucosides, phytic acid, tannins, saponins, lectins, and trypsin inhibitors, whilst producing beneficial active compounds to promote gut health [26]. According to Handoyo and Morita (2006) [27], the formation of free fatty acids from soybean after fermentation has increased between three- and ten-fold as compared with the control. In addition, after the fermentation of soybean by *Rhizopus oligosporus*, the essential, semi-essential, and non-essential amino acids compositions were also increased substantially, except for threonine, which was stable before and after the fermentation process. Interestingly, in addition to producing nutrition by-products to fortify the fermented substrate, the macro-proteins, including albumin, globulin, alkaline soluble protein, and alcohol soluble protein, were also hydrolyzed during the fermentation into intermediate sizes of protein to ease digestion upon uptake. Indeed, the function of *Rhizopus oligosporus* is to improve protein digestibility and ease peptide formation in tempeh; this idea was adopted and later applied in the BSFL rearing process. It was hoped that the inoculation of *Rhizopus oligosporus* into larval feeding mediums could fortify the amino acids content and break the existing macro-protein into smaller sizes to enable the BSFL to digest and assimilate fermented feeding mediums easily into their biomass. Another hypothesis to be confirmed in the current study was that the ease in digestion by BSFL would lessen the metabolism cost, thereby producing mature larvae loaded with high body lipids and proteins. In this regard, our primary aim was to investigate the impact of exposing BSFL to different concentrations of *Rhizopus oligosporus* inoculum on their growth. The changes in biochemical components derived from harvested BSFL biomasses—namely, lipids and proteins, and, later, fatty acid methyl ester compositions—were also studied statistically in association with the different *Rhizopus oligosporus* inoculum sizes.

## 2. Materials and Methods

### 2.1. Preparation of *Rhizopus Oligosporus* Spore Suspension for Inoculation

A total of 20 g of dried inactive culture of *Rhizopus oligosporus*, also known as tempeh starter (Raprima Brand), was initially activated by culturing it in 250 mL of sterile Potato Dextrose Broth within an incubator shaker operating at 180 rpm and 30 °C for 48 h. The sterile Potato Dextrose Broth (Sigma-Aldrich) was prepared by adding 6 g of dehydrated medium to 250 mL of distilled water and autoclaved at 121 °C for 15 min. The sterile Potato Dextrose Agar (Merck) was also prepared by adding 39 g of dehydrated medium to 1 L of distilled water and autoclaved at 121 °C for 15 min. Then, 1 mL of activated *R. oligosporus* culture was transferred to sterile Potato Dextrose Agar, spread, and incubated

at 30 °C for about 7 days until the presence of black spores could be observed. The sterile distilled water was slowly added into the agar medium and the spores were separated using a sterile inoculating loop. The final concentration of spore suspension was adjusted to approximately  $1.0 \times 10^6$  spores per mL, as determined from the cell counting plate [28]. All the steps were carried out in aseptic conditions to avoid the contamination of the biological samples. This spore suspension was subsequently used for inoculation in coconut endosperm waste to execute the fermentation process.

## 2.2. Procurement of BSFL

Fresh BSF eggs were purchased from MLF Ingredient Sdn Bhd located in Johor, Malaysia. The eggs were immediately transported into the laboratory and transferred into sterile Petri dishes, with the surrounding moisture being controlled by wet filter paper. After that, the eggs were left to undergo eclosion while being incubated at 27 °C. The newly hatched BSFL or neonates were collected and reared using fresh coconut endosperm waste until they were 6 days old prior to their use in the experiments.

## 2.3. Experimental Setups to Grow BSFL in the Presence of Various *R. oligosporus* Inoculum Sizes

Fresh coconut endosperm waste (CEW) was purchased from a local coconut milk store located in Seri Iskandar, Perak, Malaysia. The initial moisture content of CEW was determined as outlined by Wong et al. (2019) [24] and later adjusted to 70% prior to the inoculation by *R. oligosporus*. The prepared *R. oligosporus* spore suspension loaded with  $1.0 \times 10^6$  spores per mL was homogenized to separately inoculate CEW at different inoculum sizes—namely, 0.02, 0.1, 0.5, 1.0, 1.5, 2.0, and 2.5 wt%. Subsequently, these fungal fermentations were performed in both in situ and ex situ modes for comparative study. For in situ fungal fermentations, every inoculum size of *R. oligosporus* spore suspension contained 10 g of CEW (dry weight basis) and 20 BSFL that were 6 days old. In the case of ex situ fungal fermentations, every 10 g of CEW (dry weight basis) was initially inoculated with a predetermined inoculum size of *R. oligosporus* spore suspension and permitted to undergo the fermentation process for 72 h. Thereafter, 20 BSFL that were 6 days old were added to grow in every pre-fermented CEW medium of various *R. oligosporus* inoculum size. A controlled experiment free from *R. oligosporus* spore suspension was also set up and loaded with only 10 g of CEW (dry weight basis) and 20 BSFL that were 6 days old. Every setup employed a ventilated polyethylene container to incarcerate the growing BSFL with an inner diameter of 6 cm and a height of 8 cm. The moisture content of the CEW mediums was maintained between 60 wt% and 70 wt% throughout the larval rearing duration. The rearing was discontinued when the BSFL in each setup were spotted to reach the late 5th instar, as determined from their body color (greyish cream) and head size (0.9 mm) [29,30]. The larvae were harvested from each setup via manual separation from CEW medium and then washed with distilled water, deactivated at  $-20$  °C for 5 min, and finally dried under 60 °C until a constant dry weight was obtained [31]. The dried BSFL biomasses were separately stored in air-tight containers at  $-20$  °C prior to the biochemical analyses. At least three setups for each evaluated CEW medium were completed to statistically verify the reproducibility of the experimental results.

## 2.4. Larval Growth Analyses

The BSFL biomass gained was measured to signify the total larval weight gained throughout the rearing duration from consuming the CEW medium. The growth of the BSFL was also measured in terms of growth rate—i.e., the average daily weight growth of the larval biomass. Moreover, the efficiency of the conversion of digested feed ( $ECD_F$ ) was recorded to represent the efficacy of the assimilation of the ingested CEW medium into the larval biomass. Subsequently, the BSFL metabolic cost was calculated to estimate the total loss of ingested CEW medium to the larval metabolism throughout the growing period until harvesting. The respective equations were conducted as follows:

$$\text{Biomass gained (g)} = \text{Final total BSFL dry weight (g)} - \text{Initial total BSFL dry weight (g)}, \quad (1)$$

$$\text{Growth rate (g/day)} = \text{Biomass gained (g)} / \text{Rearing duration (day)}, \quad (2)$$

$$\text{ECD}_F (\%) = \text{Biomass gained (g)} / \text{Total feed consumed (g)} \times 100\%, \quad (3)$$

$$\text{Metabolic cost (\%)} = 100\% - \text{ECD}_F (\%). \quad (4)$$

## 2.5. Larval Biochemical Analyses

### 2.5.1. Lipid

Lipids from the BSFL biomass were extracted using petroleum ether as a solvent via the immersing method. A total of 100 mg of ground larval biomass was initially mixed with 20 mL of petroleum ether and the mixture was stirred for 24 h. Next, the mixture was filtered through a filter paper and the filtrate was dried in a rotary evaporator to separate the larval lipid from petroleum ether. The weight of the extracted lipid was finally recorded and utilized to calculate the BSFL lipid yield and lipid productivity as follows:

$$\text{Lipid yield (\%)} = \text{Weight of dry extracted lipid (g)} / \text{Weight of dry BSFL biomass used (g)} \times 100\%, \quad (5)$$

$$\text{Lipid productivity (g/larvae)} = \text{Total weight of dry extracted lipid (g)} / \text{Total number of BSFL}. \quad (6)$$

### 2.5.2. Protein

The protein content from BSFL biomass was estimated by the multiplication of the larval nitrogen content with a factor of 6.25 [32]. The larval nitrogen content was determined through the Dumas combustion method using the Perkin Elmer CHNS/O Elemental Analyser 2400 Series II. Around 1 mg of ground BSFL biomass was initially wrapped in tin foil. The sample was then burned to oxidize it at 965 °C in the combustion chamber and later reduced in the reduction chamber at 640 °C. The BSFL protein yield and protein productivity are calculated as follows:

$$\text{Protein yield (\%)} = \text{Nitrogen content from BSFL biomass (\%)} \times 6.25, \quad (7)$$

$$\text{Protein productivity (g/larvae)} = \text{Total protein content (g)} / \text{Total number of BSFL}. \quad (8)$$

### 2.5.3. Fatty Acid Methyl Esters

The mixture of fatty acid methyl esters (FAMES) was derived from extracted BSFL lipid through a two-step reaction with methanol—namely, the acid catalyzed-esterification followed by the base-catalyzed transesterification. The reactions were accomplished following the procedures detailed by Wong et al. (2020) [23]. The BSFL FAME mixture was then analyzed using the Shimadzu GC-2010 equipped with the flame ionization detector and polyethylene glycol capillary column BPX-BD20 (30 m × 0.32 mm × 0.25 μm). The attained FAME profile from the extracted BSFL lipid was determined as reported by Lim et al. (2019) [33]. The larval FAME yield was also calculated as follows:

$$\text{FAME in biodiesel (\%)} = \frac{A_{\text{FAME}}}{A_{\text{ISTD}}} \times \frac{C_{\text{ISTD}} \times V_{\text{ISTD}}}{m} \times 100\%, \quad (9)$$

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

## 2.6. Statistical Verification

All the parameters were triplicated for each inoculum size studied in in situ and ex situ fermentations. The collected data were initially analyzed using the Anderson Darling Normality test. Subsequently, a one-way ANOVA and Tukey's post-hoc test with ( $p > 0.05$ )

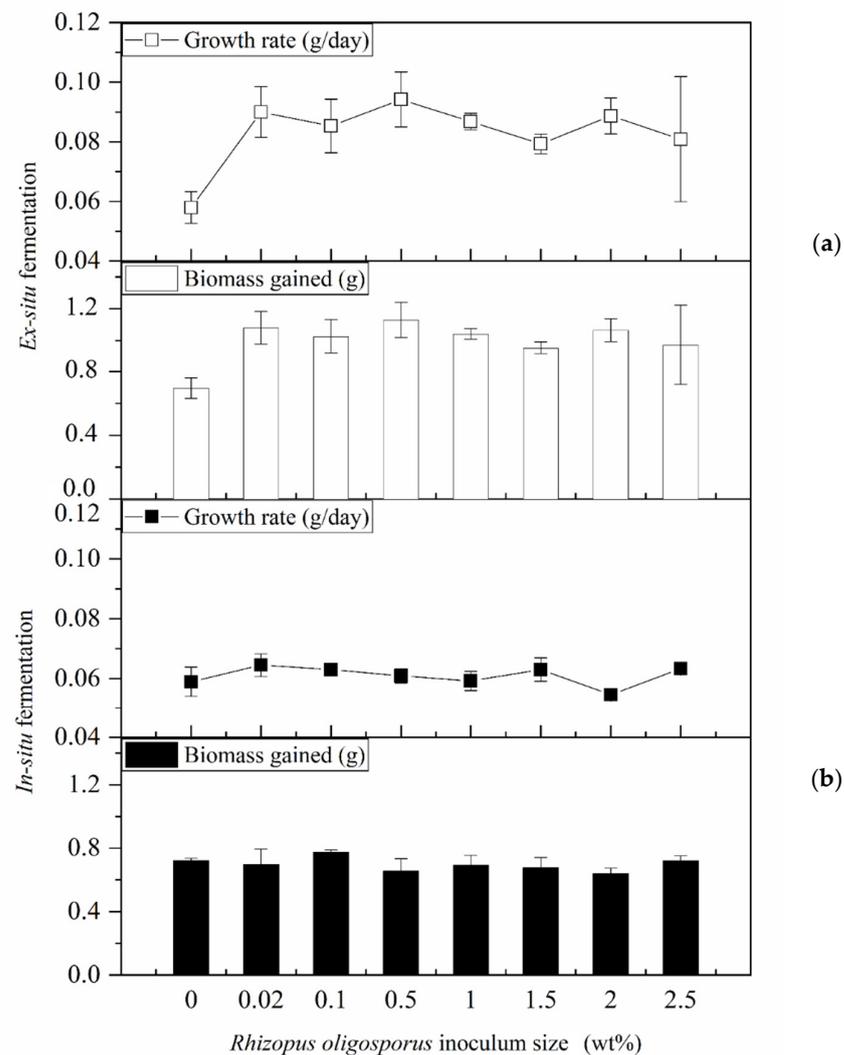
were employed with the Minitab 17 Statistical Software (Minitab Pty Ltd., Sydney, NSW, Australia) to verify the effect of different inoculum sizes of *R. oligosporus* on BSFL lipid and protein productions. Then, a multivariate principal component analysis (PCA) with the Paleontological Statistics Software Package (PAST) (version 3.12) software [34] was exploited to statistically describe the various correlations between *R. oligosporus* inoculum sizes and their impacts on BSFL growth when separately feeding on in situ- and ex situ-fermented CEW.

### 3. Results and Discussion

#### 3.1. Impacts of In Situ and Ex Situ Fermentations on BSFL Growth Performances

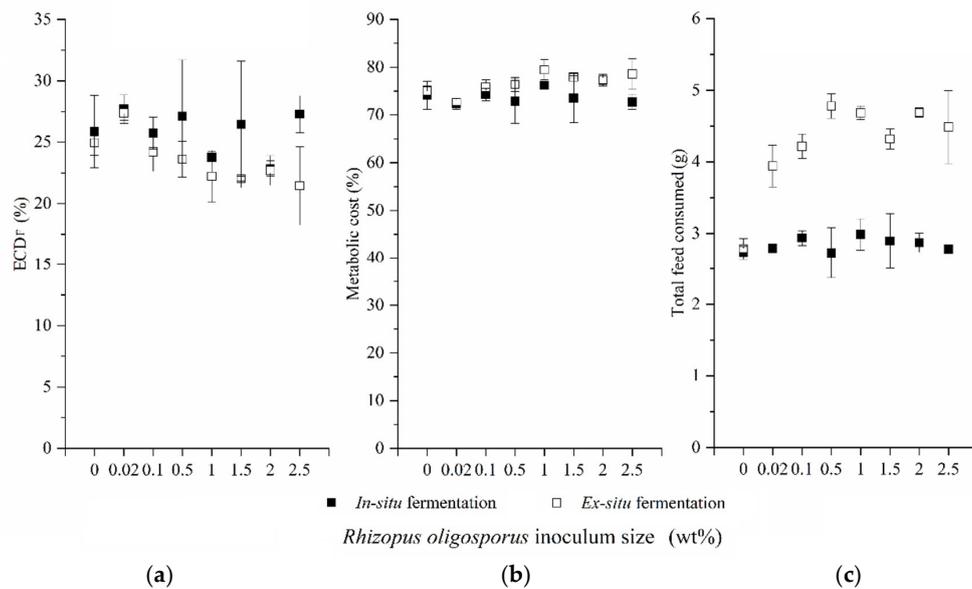
Upon the harvesting, the total biomass values gained from 20 BSFL reared using various inoculum sizes of *R. oligosporus* are presented in Figure 1. The impacts of in situ and ex situ fermentation were compared in terms of growth rate, as calculated from the biomass gained following from Equations (1) and (2), respectively. The results showed that under the control setups for both fermentation modes the biomass gained was merely recorded at about 0.7 g. Even with the inoculation of *R. oligosporus* into CEW, the in situ fermentation demonstrated a negligible impact on the growth of BSFL across all inoculum sizes from 0.02 to 2.5 wt%, with the biomass gained values fluctuating within the 0.7–0.8 g range. Moreover, the growth rate of BSFL fed with in situ-fermented CEW was enhanced slightly from 0.06 g/day under the control to its highest value of 0.65 g/day for both the 0.02 and 1.5 wt% inoculum sizes.

On the other hand, upon the completion of fermentation the BSFL fed with ex situ fermented CEW showed an over 50% better growth performance, as indicated by the rise in gained biomass values from 0.7 g under the control to around 1.1 g with even a small introduction of *R. oligosporus* (0.02 to 0.5 wt%). When a higher inoculum size of *R. oligosporus* was introduced, the growth of BSFL fluctuated between 0.95 and 1.0 g at 1.0 and 2.5 wt%, respectively. In addition, the growth rate of BSFL fed with ex situ-fermented CEW was enhanced from 0.06 g/day under the control to 0.09 g/day at 0.02 wt% and to its highest rate of 0.095 g/day at 0.5 wt%. Thereafter, the further increase in *R. oligosporus* inoculum size slightly slowed the growth rates, fluctuating at around 0.08 to 0.085 g/day. The conspicuous differences among the gained biomass values and growth rates between the in situ and ex situ fermentations were plausibly due to the development of *R. oligosporus* being retarded during the progress of the in situ fermentation. Under this fermentation environment, the presence of indigenous bacteria from the gut of BSFL through excretion competed with *R. oligosporus* and subsequently derailed the enzyme production from *R. oligosporus* to hydrolyze protein, lipid, and starch in CEW. Thereby, the immature in situ-fermented CEW with impoverished nutrients would finally not be able to enhance the BSFL growth, measured in terms of biomass gained and growth rate [35]. Apart from that, in the case of ex situ fermentation, as carried out by *R. oligosporus*, competition with larval gut bacteria could be circumvented. The mature ex situ-fermented CEW, plausibly enriched with free amino acids, less insoluble dietary fibre, more soluble dietary fibre, small peptides, and monosaccharides exuded from the hydrolysis reactions, may have enhanced the BSFL assimilation for growth upon feeding [27,36]. However, for the inoculum size above 0.5 wt% when executing ex situ fermentation, the growth of BSFL was found to be negatively affected. This may have been caused by the presence of concentrated volatile compounds—namely, ethanol and acetone—released during the intensive fermentation rising from the overpopulated *R. oligosporus* to hydrolyze CEW, which was detrimental to the BSFL growth [37].



**Figure 1.** Biomass gained values and growth rates of BSFL fed with in situ—(a) and ex situ—(b) fermented CEW, each with increasing inoculum sizes of *R. oligosporus*.

The efficiency of the conversion of digested feed ( $ECD_F$ ) and the metabolic cost determined from BSFL growth while feeding on in situ- and ex situ-fermented CEW were measured as shown in Figure 2. The  $ECD_F$  indicates the amount of ingested feed that has been converted into larval body biomass. Both of the control setups for in situ- and ex situ-fermented CEW attained around 25% to 26%  $ECD_F$ , respectively. Through in situ fermentation, the presence of *R. oligosporus* at merely 0.02 wt% improved the  $ECD_F$  to its highest value of 28%, and the values started fluctuating between 25% and 28% across the inoculum sizes of 0.1 wt% to 2.5 wt%. When the BSFL was fed with ex situ-fermented CEW, the  $ECD_F$  showed its highest point of 27% at 0.02 wt% and the values dropped to only 22% for other inoculum sizes of 0.05 wt% to 2.5 wt%. Cautiously, a false-positive result for  $ECD_F$  revealed that the in situ fermentation executed by *R. oligosporus* performed better than ex situ fermentation. Nevertheless, considering the total feed consumed by the BSFL, the values were always higher for ex situ fermentation than in situ fermentation. This signified that the BSFL consumed more ex situ-fermented CEW as opposed to in situ-fermented CEW for every inoculum size. This resulted in the ex situ fermentation treatment possessing lower  $ECD_F$  values than the in situ fermentation treatment. Apart from that, the metabolic costs were found to fluctuate in the range of 70% to 80% for both fermentation treatments, indicating similar energy losses even when more ex situ-fermented CEW was consumed by BSFL.

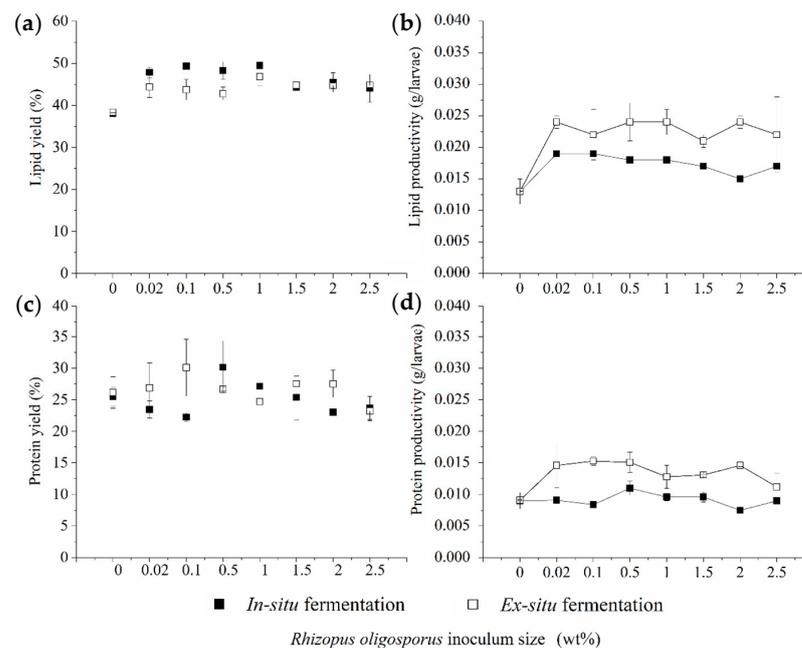


**Figure 2.** Conversion of in situ- and ex situ-fermented CEW into BSFL biomasses measured in terms of ECD<sub>F</sub> (a), metabolic cost (b), and total feed consumed (c).

### 3.2. Lipid and Protein Derived from BSFL Feeding on In Situ and Ex Situ Fermented CEW

Figure 3 unveils the BSFL lipid and protein productions upon feeding with in situ- and ex situ-fermented CEW. From the control setups, both treatment groups achieved around 38% lipid yields, and these were slowly increased to 49% with the increase in inoculum sizes to 1.0 wt% under the in situ fermentation condition. The yield then dropped to around 45% with the further increase in inoculum size from 1.5 wt% to 2.5 wt%. Generally, there was no significant difference ( $p > 0.05$ ) in the lipid yield from BSFL fed with in situ-fermented medium. In the ex situ treatment group, the lipid yields from BSFL were found to be maintained at around 42% to 44% across the different inoculum sizes of *R. oligosporus*. Through Tukey's post-hoc test, it was shown that the lipid yields had similar impacts when fed with CEW inoculated with 0.02 wt% to 2.5 wt%, with the exception of 1.0 wt%. The 1.0 wt% inoculum size was able to enhance the larval lipid yield and achieve its highest value at 47%.

In the case of the larval lipid productivities derived from in situ fermentation, there were significant differences ( $p < 0.05$ ) among various inoculum sizes—namely, 0.02 wt% and 0.1 wt% as well as 0.5 wt% to 2.5 wt% had similar impacts. Nevertheless, the ex situ fermentation was later noticed to perform better than the in situ fermentation in terms of lipid productivity. In terms of statistics, the significant difference was found to be inconspicuous ( $p > 0.05$ ) among the various inoculum sizes of *R. oligosporus* for lipid productivities derived from ex situ fermentation. Thereby, for any instance, the use of ex situ-fermented CEW could give rise to at least 20–60% higher lipid productivities than the in situ-fermented CEW for the similar inoculum sizes studied. The higher lipid productivities were primarily attributed to the higher biomass values gained and the growth rates of BSFL (Figure 1) administered with various inoculum sizes of *R. oligosporus* to carry out ex situ fermentation. In order to attain lipid-rich BSFL, it was suggested that 1.0 wt% of *R. oligosporus* should be introduced into CEW and treated by means of ex situ fermentation. As compared with other exo-microbial fermentations, it was found that the highest lipid yield (49%) was also obtained with 1.0 wt% yeast via in situ fermentation (Wong et al., 2020) [38]. For bacterial ex situ fermentation, the employment of 0.02 to 2.5 wt% inoculum sizes resulted in lipid yields falling within the range of 35% to 40% [23]. More studies on in situ and ex situ fermentation while inoculating with various microorganisms could open the window for opportunities to explore modifying the larval feeding medium via fermentation.



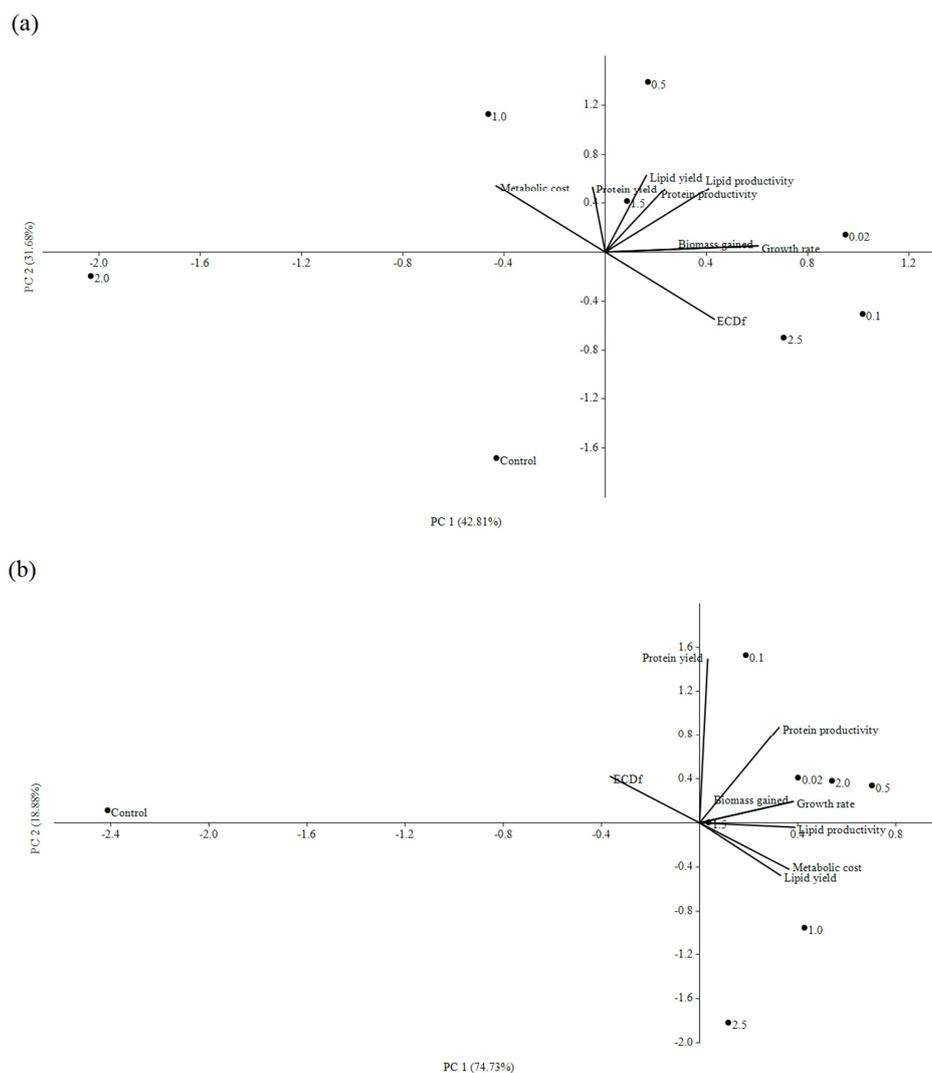
**Figure 3.** Yields and productivities for each lipid ((a,b), respectively) and protein ((c,d), respectively) derived from BSFL upon feeding with in situ- and ex situ-fermented CEW.

For BSFL protein yield, under the control setups both the in situ and ex situ treatment groups recorded yields at 25% and 26%, respectively. For in situ fermentation, the yields reduced to 23% at 0.02 wt%, followed by 21% at 0.1 wt%. Then, the values rose to 30% at 0.5 wt% and slowly decreased to 23% from 1.0 wt% to 2.5 wt%. However, under the ex situ fermentation condition, the protein yields of BSFL increased to 30% at 0.1 wt% and then it fluctuated between 23% and 27% across the inoculum sizes of 0.5 wt% to 2.5 wt%. Despite employing the in situ or ex situ fermentations, there were no significant differences observed from the effect of different inoculum sizes on larval protein yields. For comparison, the introduction of yeast fermentation had resulted in the enhancement of larval protein yields to 30% to 35%, whilst 33% to 39% for bacterial fermentation [23,39]. These results varied among bacteria, yeast and fungi may be plausibly due to the different reaction pathways between eukaryote and prokaryote cells.

Compared with the BSFL lipid productivities, similar trends were observed for the BSFL protein productivities for both the in situ and ex situ fermentation treatment groups. The protein productivity of BSFL under the in situ fermentation showed 0.009 g/larvae for control and later being maintained for 0.02 wt% and 0.1 wt%. The values arose to 0.011 g/larvae at 0.5 wt% and fluctuated between 0.007 and 0.009 g/larvae across 1.0 wt% to 2.5 wt% of inoculation sizes. The Tukey post-hoc test evidenced that the 0.5 wt% had positive impact on BSFL protein productivity. However, when the inoculum size was increased to 2.0 wt%, the protein productivity was noticed deteriorating significantly according to the similar statistical test. Nonetheless, the protein productivities of BSFL under the ex situ fermentation shared a same value for control and it was later raised to 0.015 g/larvae at 0.02 wt%, 0.1 wt% and 0.5 wt%. Thereafter the inoculum sizes, the values were fluctuating between 0.011 and 0.014 g/larvae.

It can be seen from Figure 3 that the in situ fermentation—i.e., the BSFL fed with 0.5 wt% inoculum size of *R. oligosporus*—was able to produce the highest larval protein yield. The BSFL administered with in situ-fermented CEW also showed a slightly higher lipid yield at about 49% as compared with 47% while using ex situ-fermented CEW; however, the difference between these two mediums was insignificant in terms of protein yield. Yet, the lipid and protein productivities derived from the harvested BSFL were much higher when using ex situ-fermented CEW over the in situ-fermented CEW. This was due to the superior growth when the BSFL was administered with the formal medium.

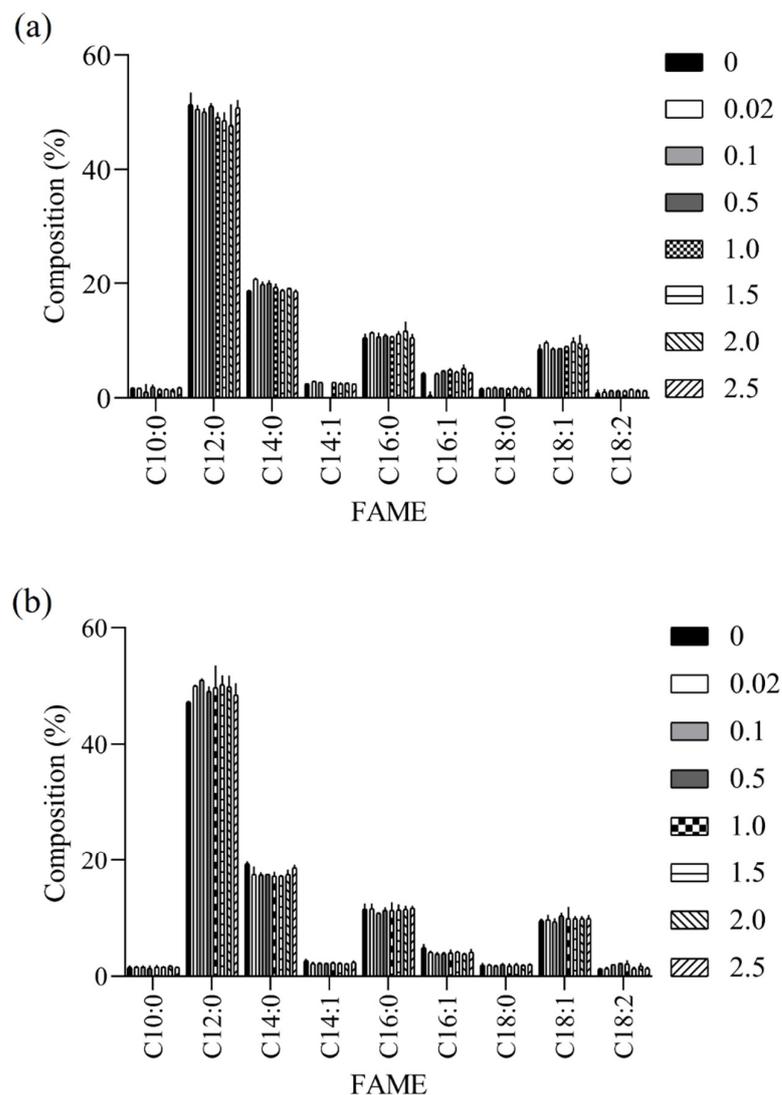
The statistical correlations between BSFL growth as well as its biochemical products with respect to the in situ and ex situ fermentations performed at various inoculum sizes of *R. oligosporus* on CEW are presented in Figure 4. The PCA analyses were capable of describing 75% of the data correlation under the in situ fermentation. It could be perceived that the introduction of *R. oligosporus* into the CEW to carry out in situ fermentation did impact the larval growth performance as well as its lipid and protein accumulations. At low inoculum sizes of 0.02 wt% and 0.1 wt%, it favored the growths of BSFL. With the increase in inoculum sizes from 0.5 to 1.5 wt%, high lipid and protein yields as well as productivities could be obtained from BSFL biomass. Thus, in order to achieve a lipid- and protein-rich larval biomass simultaneously, the inoculum sizes of *R. oligosporus* should range from 0.5 wt% to 1.5 wt% to execute the in situ fermentation of CEW and gain optimum outputs. On the other hand, around 94% of the data variability under ex situ fermentation could be described by PCA. The results showed that there was no correlation found for all the measured variables at the highest inoculum size of 2.5 wt%. At low inoculum sizes of 0.02 wt% and 0.1 wt%, the protein yield and productivity of BSFL were both beneficial. Nevertheless, the increase in inoculum sizes at 1.0 and 1.5 wt% favored BSFL lipid yield and productivity. Considering that the larval growth performance was prominent from 0.5 wt% to 2.0 wt% *R. oligosporus* inoculation ratios, it was more convincing to conclude that lipid-rich BSFL biomass could be plausibly attained at 1.0 wt%.



**Figure 4.** PCA analyses demonstrating the correlations between BSFL growth together with its biochemical products and each in situ (a) and ex situ (b) fermentation executed by *R. oligosporus* of various inoculum sizes on CEW.

### 3.3. Quality of BSFL-Based Biodiesels

In accounting the quality of biodiesels derived from BSFL biomasses feeding on in situ fermented CEW, the major FAMES consisting of C12:0 (45–51%), C14:0 (18–20%), C16:0 (10–12%), C18:1 (8–10%), and C16:1 (4–5%), followed by small amounts of C10:0, C14:1, C18:0, and C18:2 (Figure 5). The compositions showed that the biodiesels derived from BSFL biomasses could be potentially used as diesel sources, as the total amount of linolenic acid methyl ester (C18:2) was lower than 12 wt% according to the European Standard EN14103. The total amount of polyunsaturated methyl esters was also low (approximately 1 wt%), following the similar standard requirements. There was a negligible impact exerted by different *R. oligosporus* inoculum sizes on the FAME compositions, as the changes were not significantly different.



**Figure 5.** Fatty acid methyl ester compositions from harvested BSFL biomass feeding on in situ—(a) and ex situ—(b) fermented CEW with *R. oligosporus* of various inoculum sizes.

Moving on to ex situ fermentation, a similar trend in FAME compositions was also observed. The major FAMES were C12:0 (47–52%), C14:0 (16–19%), C16:0 (10–12%), C18:1 (8–10%), and C16:1 (3–5%), followed by tiny amounts of C10:0, C14:1, C18:0, and C18:2. These results showed that the FAME compositions derived from the BSFL biomasses were marginally impacted by the in situ or ex situ fermentation treatments on CEW executed by *R. oligosporus* at various inoculum sizes. Likewise, Wong et al. (2019) [24] observed

a similar occurrence in their study concerning biodiesel production from BSFL biomass feedstock. Thereby, as discussed in the previous section, it was suggested that 1.0 wt% of *R. oligosporus* should be introduced into CEW and subsequently treated by means of ex situ fermentation in order to achieve the highest lipid-rich BSFL biomass for producing an acceptable-quality larval biodiesel. In comparison with other feedstock, BSFL fed with CEW obtained higher values in C12:0 than the BSFL fed with rice straw and restaurant waste, which were rich in cellulose, hemicellulose, and soluble sugar (rice straw) and protein, grease, and starch (restaurant waste). This could plausibly be due to the impacts of the feeding mediums, as the FAME composition of the BSFL fed with CEW was highly similar to FAME composition of coconut oil that was extracted from wet white coconut kernels. In addition, the yeast oil contained around 2/3 saturated fatty acids, including C16:0 and C18:0 and 1/3 polyunsaturated fatty acids. The detailed comparisons are available in Table 1.

**Table 1.** Comparison of FAME compositions between BSFL with other feedstock.

Feedstock FAME	BSFL	BSFL [20]	Wet White Coconut Kernel [40]	Yeast [41]
C8:0	-	-	8.1%	-
C10:0	1.6%	3.8%	7.8%	-
C12:0	49.5%	27.8%	50.5%	-
C14:0	17.8%	8.1%	16.1%	-
C14:1	2.3%	-	-	-
C16:0	11.4%	14.2%	6.8%	26.2%
C16:1	4.1%	4.5%	-	-
C18:0	1.9%	7.6%	2.3%	37.3%
C18:1	9.8%	22.5%	5.6%	22.3%
C18:2	1.6%	1.8%	1.8%	6.5%
C18:3	-	2.1%	-	-
Others	-	7.6%	1%	2.8%

#### 4. Conclusions

The comparative investigation showed that ex situ fermentation executed by *R. oligosporus* could fortify the nutritional composition of CEW in enhancing BSFL growth as opposed to the in situ fermentation treatment mode. The employment of ex situ fermentation could offer more time for *R. oligosporus* to hydrolyze complex organic polymers from CEW to easily assimilable compounds by BSFL. In the case of in situ fermentation, the competition between symbionts from the gut of BSFL with *R. oligosporus* could possibly derail the hydrolysis of CEW, thereby limiting the available nutrients that could be ingested by BSFL and slowing the larval growth rate. Although the difference between these two treatment modes was insignificant in terms of the larval protein yields, the BSFL administered with ex situ-fermented CEW could attain higher lipid and protein productivities than the in situ medium. Looking at the biodiesel quality, the FAME mixtures derived from all the BSFL biomasses did not vary conspicuously. Accordingly, 1.0 wt% of *R. oligosporus* was considered optimum to execute ex situ fermentation and attain the highest lipid yield and productivity to satisfy the biodiesel quality requirement.

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