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**Sabrina Hasnol, Jun Wei Lim, Chung
Yiin Wong, Man Kee Lam & Seteno
K. O. Ntwampe**

**Environmental Science and Pollution
Research**

ISSN 0944-1344

Environ Sci Pollut Res
DOI 10.1007/s11356-020-09034-2



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Liminal presence of exo-microbes inoculating coconut endosperm waste to enhance black soldier fly larval protein and lipid

Sabrina Hasnol¹ · Jun Wei Lim¹ · Chung Yiin Wong¹ · Man Kee Lam² · Seteno K. O. Ntwampe³

Received: 6 March 2020 / Accepted: 22 April 2020
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Abstract

The anaerobic decomposition of coconut endosperm waste (CEW), residue derived from cooking, has been insidiously spewing greenhouse gasses. Thus, the bioconversion of CEW via in situ fermentation by exo-microbes from commercial Rid-X and subsequent valorization by black soldier fly larvae (BSFL) was the primary objective of the current study to gain sustainable larval lipid and protein. Accordingly, various concentrations of exo-microbes were separately homogenized with CEW to perform fermentation amidst feeding to BSFL. It was found that 2.50% of exo-microbes was the threshold amount entailed to assuage competition between exo-microbes and BSFL for common nutrients. The presence of remnant nutrients exuded from the fermentation using 2.50% of exo-microbes was confirmed to promote BSFL growth measured as maximum larval weight gained and growth rate. Although the BSFL could accumulate the highest protein (16 mg/larva) upon feeding with CEW containing 2.50% of exo-microbes, more lipid (13 mg/larva) was stored in employing 0.10% of exo-microbes because of minimum loss to metabolic processes while prolonging the BSFL in its 5th instar stage.

Keywords Organic waste · *Hermetia illucens* · Fermentation · Biochemical · Mixed microorganisms · Larval substrate · Entomoremediation

Introduction

Residues that are originated from biodegradable sources such as animals, plants, and microbes are defined by the term organic wastes. Even though the organic waste has a complex structure, it can be systematically valorized (Oliwit et al. 2019; Vakalis et al. 2019) when its decomposition pathways are thoroughly conceived. Organic waste can be categorized into three

types, namely, industrial organic waste (e.g., sludge, slaughterhouse waste, food market waste, hospitals, and manufacturing factory waste), agriculture organic waste (e.g., farming, forest, fruits, vegetables, and livestock processing facilities), and household organic waste (e.g., food wastes from kitchens and restaurants). According to the trend of solid waste management from the World Bank, annually, 2.01 billion tonnes of organic wastes are generated globally, and at least 33% is polluting the environment and degrading ecological system. It has been estimated that by 2050, the waste generation will increase by 19%, especially in high income countries; and it is expected for middle- and low-income countries to experience such an increase by 40%. Regionally, whereby the organic wastes are produced, waste generation growth will rise exponentially, with East Asia and Pacific regions producing the highest amount of waste at 23% as compared with Middle Eastern and North African regions which are estimated to have the lowest (6%) waste generation increases (Kaza et al. 2018). It has become a priority to ensure that proper procedures are taken for managing the enormous organic wastes generated. Since organic wastes are biodegradable, it can be appropriately exploited for composing. The food wastes from grocery stores and restaurants, for example, can become the sources of fertilizers upon

Responsible editor: Ta Yeong Wu

✉ Jun Wei Lim
junwei.lim@utp.edu.my

- ¹ Department of Fundamental and Applied Sciences, HICoE-Centre for Biofuel and Biochemical Research, Institute of Self-Sustainable Building, Universiti Teknologi PETRONAS, 32610 Seri Iskandar, Perak Darul Ridzuan, Malaysia
- ² Department of Chemical Engineering, HICoE-Centre for Biofuel and Biochemical Research, Institute of Self-Sustainable Building, Universiti Teknologi PETRONAS, 32610 Seri Iskandar, Perak Darul Ridzuan, Malaysia
- ³ School of Chemical and Minerals Engineering, North-West University, Private Bag X1290, Potchefstroom 2520, South Africa

composting. Nevertheless, improper procedures during composting can contribute to the emission of carbon dioxide and methane; greenhouse gases that increase the global temperature and result in major negative changes toward the ecosystem.

In Malaysia, household wastes, consisting mainly organics, are the primary source of municipal solid wastes (64%), followed by industrial waste at 25%, commercial waste at 8%, and construction waste at 3% (Moh and Manaf 2014). In the agricultural sector, the main waste containing the largest cellulosic raw materials is from the coconut plantation. As much as 147,000 ha of plantations cover the country's arable land in Malaysia, generating a large amount of organic wastes, mainly coconut endosperm waste (CEW) from cooking (Abdul Khalil et al. 2006). The CEW has created a myriad of complications in coconut replanting operations, negatively affecting the environment. To manage this waste, landfills are used to bury the organic residues and subsequently, emitting methane due to the anaerobic decomposition of such waste within the subsurface. Indeed, Malaysia's waste management sector is contributing toward the total greenhouse gases emission of up to 12%, according to the report from the Ministry of Natural Resources and Environment (NRE) and United Nations Framework Convention on Climate Change (UNFCCC). Methane gas, the main agent causing global warming is being emitted from the landfills and it is 21 times more potent than carbon dioxide in its ability to absorb heat (National Solid Waste Management Department 2018). Thus, it is extremely crucial that proper and alternative ways of organic waste management be developed to reduce methane emission from Malaysia's landfills.

Reduction of the biodegradable organic wastes via valorization by insects has become another alternative and an environmentally friendly bioremediation method that soon will rise as a new niche area of researches (Qi et al. 2019; Cai et al. 2018; Wang et al. 2018; Yin et al. 2018). Indeed, through entomoremediation, black soldier fly larvae (BSFL) were able to treat the phytoextraction-polluted biomass, containing cadmium and zinc metal ions. After 36 days of rearing period, the cadmium was mostly accumulated in the puparia and the zinc was brought further to the adult stage. This method is known not only to reduce the phytotoxicity but also could possibly become a novel metal recovery process in the near future (Bulak et al. 2018). Gao et al. (2017) had also reported that the heavy metals of cadmium and chromium exerted no negative impact on the larval survival and eclosion rates. However, these metals could impact the larval growth duration and pupation rate as the metals were mainly accumulated in larvae, prepupae and pupae stages. Based on the results, the authors suggested that the BSFL could be potential employed to treat biomass that had been contaminated with cadmium as Cd^{2+} could be easily absorbed through Ca^{2+} channel by the larval cells due to the similarity between the two ion species.

Up to date, BSFL had been confirmed could bioaccumulate Ba, Bi, and Ga (non-essential elements); and Cu, Fe, Hg, Mg, Mo, Se, and Zn were found to be bioaccumulating throughout all stages of larval growth and its puparia. Meanwhile, there was no bioaccumulation activity found for Al, As, Co, K, Pb and Si. On the other hand, the Ca, Cd, Ga, Mn, P and S were only able to be found in certain stages of BSFL growth. These findings highlight an important role for BSFL to be use as a bioagent for entomoremediation process (Proc et al. 2020). More on entomoremediation, the BSFL also can treat various ubiquitous organic wastes via valorization-cum-assimilation into its biomass, in which could be exploited for myriad valuable biochemicals production. According to Li et al. (2011), the use of cattle manure to feed BSFL could generate 38.2 g of extracted lipid from 29.9% of larval fat yield. Later, from the transesterification process, 93% of lipid could be converted into biodiesel. Newton et al. (2005) had found that the protein content of BSFL was slightly higher when fed with swine manure (43.2%) than poultry manure (42.1%). The valorization of blended dairy manure and chicken manure at the ratio of 40:60 had also resulted in the highest lipid and protein yields at 47.7% and 53.9%, respectively (Rehman et al. 2017).

In the case for CEW, the bioconversion into BSFL biomass can serve as the feedstock for the production of lipids and proteins for other industrial applications (Lim et al. 2019; Mohd-Noor et al. 2017; Wong et al. 2019). The larval lipid extracted can be used in the production of biodiesel, a renewable energy source. Accordingly, the BSFL or *Hermetia illucens* larva has been demonstrated to be an ideal insect candidate (Leong et al. 2016) since the larvae's breeding process is economical and environmentally sustainable as a large breeding space is unnecessary, thereby, allowing the BSFL breeders to easily scale-up at low cost. Unlike common houseflies, the adult BSFL have missing mouth parts and will not feed during the fly stage; thus, will not transmit disease and parasites toward humans. Organic matters such as kitchen waste and manure can be easily degraded by the BSFL with the presences of appropriate enzymes and symbionts in the gut passage. Absorption of the nutrients from the waste used as a feed has contributed greatly towards the larval growth and development (Gold et al. 2018). The CEW, however, is composed of 56.3% holocellulose (Abdul Khalil et al. 2006) and a high content of lignocellulose fibers which are not easily hydrolyzed upon larval ingestion. The quantity and type of microbes in the larval gut may be insufficient and unsuitable to break down the strong fibers and the matrix of the holocellulose in the CEW feed while simultaneously trying to maintain a positive larval development to support the intrinsic eclosion process for survivability. Therefore, the main objective of this study was to enhance the palatability of BSFL via an in situ fermentation of CEW executed by exo-microbes, targeting high larval lipid and protein harvests. In this regard, various concentrations of exo-microbes in the form of commercial Rid-X were used to

inoculate CEW separately along with the subsequent ingestion of the resultant products by BSFL until it reached the 6th instar stage.

Materials and methods

Black soldier fly larvae (BSFL) growth set-up

The raw coconut endosperm waste (CEW) was collected from the local stall distributing coconut milk as the cooking ingredient for local delicacies. The collected raw CEW was already in a grated form, having a size range of 1.5–6.5 mm. The proximate analysis results to represent the composition of CEW were available in our early documented study (Wong et al. 2020). The source of commercial exo-microbes used in this project was a branded product known as Rid-X (Reckitt Benckiser Inc., New Jersey, USA) in which it contains a patented formulation of numerous bacteria as well as enzymes, e.g., cellulase, lipase, and amylase for lignocellulose biomass digestion (MSDS: D0193955). The cylindrical plastic cups (diameter of 5 cm and height of 11 cm each) were utilized as the feeding containers to hold the feeding medium for BSFL. The BSFL were incarcerated by capping the cylindrical plastic cups each with a ventilated lid. The BSFL were obtained from the local breeder—EnviProtein Sdn. Bhd. located in Penang, Malaysia in the eggs form. Upon the eclosion from eggs, the neonates of black soldier fly were initially reared in a fresh CEW medium for 6 days prior to the use in the experiments.

The raw CEW was then separately homogenized with different concentrations of Rid-X, namely, 0%, 0.02%, 0.10%, 0.50%, and 2.50% (control) via manual mixing. The Rid-X was used as it had been received without any prior modification/preparation following the previously reported researches (Wong et al. 2020; Zheng et al. 2012). The moisture content for each concentration was adjusted from 60–65% prior to the introduction into the feeding containers. Each feeding container had 10 g of raw CEW (dry weight) inoculated with a predetermined concentration of Rid-X containing exo-microbes. Six-day-old of BSFL ($n = 20$) was then immediately introduced into all the feeding containers, capped with a ventilation lid to prevent the escaping of larvae, followed by its rearing until about 50% of the BSFL had achieved the 6th instar stage. The moisture content of CEW was maintained throughout the larval rearing process via spraying with distilled water. Upon reaching maturity, every batch of harvested BSFL from the separate feeding container was inactivated in boiling water for 5 s and dried at 60 °C to a constant weight. Similarly, each of the residual CEW in feeding containers was also dried to a constant weight at 60 °C. All the set-ups were at least triplicated to confirm the reproducibility of the collected data. All the data was subsequently verified via the Tukey post hoc pairwise comparison test to confirm the significance of

means. The following equations were used in the calculations to confirm the performances of BSFL in valorizing CEW inoculated with various concentrations of Rid-X:

$$\text{Biomass gained per larva} = \frac{\text{Total larval weight (mg)}}{\text{Number of larvae}} \quad (1)$$

$$\begin{aligned} \text{Growth rate of per larva} \\ = \frac{\text{Biomass gained per larva (mg)}}{\text{Rearing duration (day)}} \end{aligned} \quad (2)$$

$$\begin{aligned} \text{Converted CEW per larva} \\ = \frac{\text{Initial CEW (g)} - \text{Final CEW (g)}}{\text{Initial CEW (g)} \times \text{Number of larvae}} \times 100\% \end{aligned} \quad (3)$$

Lipid extraction from harvested BSFL

The harvested dried BSFL biomass was initially grounded by using a mortar and pestle; thereafter, 200 mg of pulverized larval biomass was inserted into a glass vial. A volume (15 mL) of petroleum ether solvent was introduced into similar glass vial and mixed with a vortex mixer at 250 rpm for 24 h. The biomass residue from the solvent lipid extraction process was then separated by filtration using a filter paper (Whatman 1) and the residue was further washed with petroleum ether solvent thrice using 10 mL for each rinse cycle. All the filtered solvents containing larval lipids were combined and later subjected to aeration (blow-down technique) until all the petroleum ether solvent had evaporated. The remaining larval lipids were further dried in an oven at the temperature of 105 °C for 10 min and cooled in a desiccator at ambient temperature. The weight of petroleum ether extracted larval lipids was determined and used for assessing the capability of BSFL to accumulate lipid via equations below.

$$\text{Lipid yield} = \frac{\text{Extracted BSFL lipid dry weight (mg)}}{\text{Larval biomass dry weight (mg)}} \times 100\% \quad (4)$$

$$\begin{aligned} \text{Larval body lipid} \\ = \frac{\text{Lipid yield}/100\% \times \text{Total larval weight (mg)}}{\text{Number of larvae}} \end{aligned} \quad (5)$$

Protein from harvested BSFL

The harvested dried BSFL biomass was analyzed for its nitrogen content using elementary analysis method, i.e., Dumas combustion method (Perkin Elmer, CHNS/O 2400). The larval biomass was weighed in the range of 1 to 1.5 mg and transferred into a tin capsule, wrapped, and combusted at the temperature of 925 °C. The nitrogen compounds were then converted into NO_x, and were further reduced to nitrogen gas at the temperature of 640 °C and finally detected by thermal

conductivity detector. The harvested BSFL protein content was calculated using a conversion factor of 6.25 on the nitrogen content obtained from the elementary analysis after subtracting the nitrogen content belonged to the larval chitin (Giannetto et al. 2020; Finke 2013). The equations below were employed to ascertain the capability of BSFL to accumulate protein.

$$\text{Protein yield} = \text{Nitrogen content (\%)} \times 6.25 \quad (6)$$

Larval body protein

$$= \frac{\text{Protein yield}/100\% \times \text{Total larval weight (mg)}}{\text{Number of larvae}} \quad (7)$$

Results and discussion

Development of BSFL

The numbers of BSFL which emerged in the 5th and 6th instar stages during harvesting, together with their respective average weights of biomass gained per larva, are all presented in Fig. 1. In comparing with the control CEW medium experiments, the presence of Rid-X at any concentration lower than 0.50% would overall retard the growth of BSFL, quantified as larval growth weight and larval eclosion from the 5th to 6th instar stages. At the low Rid-X concentration of 0.02%, a decrease in biomass gained per larva could be seen, plausibly arising from the competition between exo-bacteria from Rid-X introduced to carry out an in situ fermentation and the BSFL for common nutrients. A similar instance was also previously reported by Mohd-Noor et al. (2017) in which the growth of various microorganisms had impoverished the dissolved

organic nutrients intended for larval growth. Moreover, the BSFL could not facilely digest and hydrolyze the microorganisms as the cells were shielded by extracellular polymeric substances and cell wall that protecting the microorganisms from predators (Leong et al. 2016). However, the BSFL growing in CEW homogenized with Rid-X concentration of 0.02% still could garner enough nutrients to undergo eclosion from the 5th to 6th instar stages. Thereby, more 6th instar BSFL were recorded at this Rid-X concentration as opposed to the control experiment containing the CEW medium, although smaller larvae were also harvested. The competitions were intensified with the introduction of 0.10% and 0.50% of Rid-X into CEW mediums separately. At the Rid-X concentration of 0.50%, the smallest larvae were eventually harvested as reflected by the lowest weight of biomass gained per larva. Moreover, during the employment of these two separate concentrations of Rid-X, the BSFL growths were individually inhibited since most larvae could not garner adequate nutrients to undergo eclosion from the 5th to 6th instar stages as shown by the lower numbers of 6th instar larvae each for 0.10% and 0.50% concentrations of Rid-X in comparing with the 0.02% of Rid-X concentration. Nevertheless, when 2.50% of Rid-X concentration was homogenized with the CEW medium, a sudden increase of biomass gained per larva was measured, demonstrated by bigger size of BSFL both in the 5th to 6th instar stages as opposed to lower concentrations of Rid-X as well as the control experiment medium. Therefore, 2.50% of Rid-X concentration was considered as the threshold amount of exo-bacteria required to execute an in situ fermentation of CEW in which progressing concurrently with the growth of BSFL.

The main function of adding exo-microbes into the larval feeding medium was to simulate the role of the microbes found in the larval gut used to decompose the carbohydrates (Zhao et al. 2017), proteins (Storelli et al. 2011), etc., prior to larval ingestion. Some specifically selected exo-microbes can effectively hydrolyze fibers from the larval feeding medium which are typically difficult to be digested by the larvae. In the investigation carried out by Zheng et al. (2012) using Rid-X mixed with rice straw and restaurant waste which was later being fed to the BSFL, it was found that the threshold amount of microbes had contributed greatly in the hydrolysis of fibers. This had resulted in an increase in the reduction of cellulose (37%) and hemicellulose (23%) that largely influenced the composition of hydrolyzed products available for both the BSFL and exo-microbes. The inoculation of the threshold amount of exo-microbes using Rid-X for instance would enhance the nutrients availability for the intrinsic development of the larvae into higher instar stages. The presence of an insufficient quantity of exo-microbes could not conspicuously hydrolyze the stable larval feed medium such as CEW and produce hydrolyzed products faster than what was being ingested by the BSFL for the case whereby in-situ

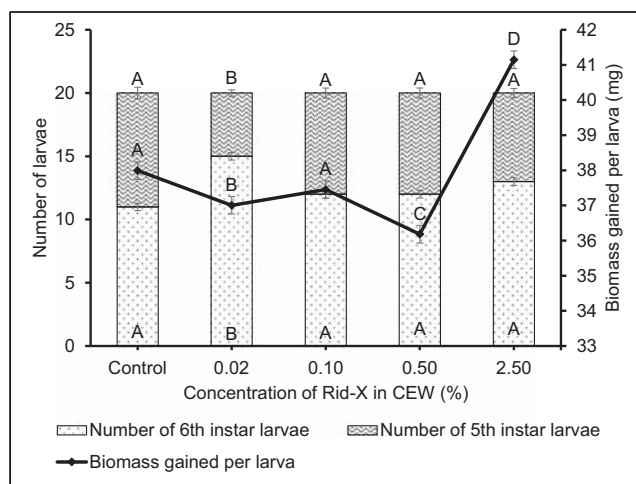


Fig. 1 Numbers of 5th and 6th instar larvae and its respective average weights of biomass gained per larva after rearing in CEW separately homogenized with predetermined concentration of Rid-X. Mean values of similar parameter indicated by the different characters are significant in variation ($p < 0.05$) by Tukey post hoc pairwise comparison test

fermentation was used. Also, the exo-microbes generally need nutrients before initiating their activities, leading to the nutrients competition with the BSFL. It was found from the study conducted by Albuquerque and Zurek (2014) that the development of BSFL was inhibited due to the decomposition and conversion carried out by the insufficient exo-microbes, resulting in low value residual nutrients for larval ingestion. Notably, other mechanisms that could explain the contribution of exo-microbes on the quality of the feeding medium was the selective inactivation of microbes as demonstrated when *Salmonella enterica* and UX174 were reduced in the gut passage of the BSFL in the studies by Nordentoft et al. (2017). This study had shown that the exo-microbes would undergo inactivation depending on the microbial dose and nutrient availability. When the larvae were able to exceed a certain size and density, they were able to control the exo-microbes in the feed that simultaneously competing with the larvae for common nutrients. The microbes that survived the mechanism would then contribute into the development of BSFL by symbiosis (Douglas 2010). In this case, the surviving exo-microbes seemed serving as secondary nutrient source for the BSFL. Therefore, in the case of an in situ fermented CEW, the 2.5% was confirmed be the minimum threshold of Rid-X concentration required, as it was sufficient in ensuring the decomposition of stable biopolymers such as carbohydrates, proteins, lipids, and fibers to produce microbially hydrolyzed products that contributed to the biomass gained by each larva. A sufficient amount of exo-microbes could also decrease the competition between the exo-microbes and the BSFL, as well as to permit the inactivation mechanism of some microbes.

BSFL growth against conversion of CEW

A comparison of the growth rate per larva which was the weight of biomass gained for each larva throughout the rearing duration using a predetermined concentration of Rid-X is presented in Fig. 2 along with the respective amounts of CEW converted by every larva prior to harvesting. As more 6th instar larvae were harvested from the use of 0.02% Rid-X concentration, the metabolic loss was conspicuous as opposed to the control; thereby, leading to higher converted CEW and a lower growth rate per larva for in situ fermentation with 0.02% of Rid-X as compared with the control experiment. During the eclosion, most of the larval reserved energy was lost to the development from the 5th to 6th instar stages. Also, the competition between exo-microbes from Rid-X and BSFL for common nutrients as discussed in Fig. 1 would accelerate the development of the larvae towards the 6th instar stage, an intrinsic response for the BSFL nutrient limited conditions as reported by Rodrigues et al. (2015). A slight increment of growth rate per larva was noticed when a 0.10% of Rid-X concentration was homogenized with CEW. By using this larval feeding medium, a lower number of growing larvae

was being transformed from the 5th to 6th instar stages as opposed to when the 0.02% of Rid-X was used (Fig. 1). In this case, stemming from the competition for common nutrients with exo-microbes, the BSFL failed to garner a sufficient quantity of nutrients for the eclosion, thereby, prolonging its 5th instar stage to further accumulate more nutrients for its development in ensuring its survivability at the later phase. This had directly reduced the overall metabolic loss because of less transformation from 5th to 6th instar stages while permitting the accumulation of more larval body lipids which will be discussed in the subsequent section. Nevertheless, the intense competition from the use of 0.50% of Rid-X concentration had greatly retarded the BSFL growth rate in concert with the biomass gained per larva. Although the converted CEW per larva had increased slightly when comparing with the 0.10% Rid-X cultures, the energy loss amidst the competition had resulted in the lowest growth rate per larva while using the 0.50% of Rid-X concentration. Finally, the competition for common nutrients subsided with the use of a threshold amount of Rid-X concentration (2.50%). At this concentration, the highest converted CEW per larva had inevitably led to the highest growth rate per larva. The exo-microbes were able to produce more microbial hydrolyzed products from the in situ fermentation of CEW than for its assimilation, with the remnants served as the additional nutrients to fortify the growth of BSFL.

Accumulated lipids and proteins from harvested BSFL biomass

The yields of lipids and protein from the harvested BSFL biomass were compared (Fig. 3) with the respective contents of lipids and protein produced by each larva. The use of a 0.10% Rid-X concentration had culminated in the highest larval lipid accumulation for both the yield and body content per larva. This was primarily arising from the failure of most larvae to transform from 5th to 6th instar stages by virtue of the limited nutrient contents in their feeding medium; having been impoverished by the exo-microbes from the Rid-X. The BSFL would spend more time mustering nutrients that would be eventually stored as the larval body fat in which was not subsequently exploited for the natural eclosion process. In comparison with lower concentration of Rid-X, namely, 0.02%, the more occurrences of eclosion from 5th to 6th instar stages and higher concentration of Rid-X, namely, 0.50%, the intensive competition with introduced exo-microbes, had both resulted in lower accumulations of stored larval lipid due to the extensive metabolic loss. Thus, the employment of a 0.10% concentration of Rid-X which was regarded as an optimum exo-microbes inoculation in CEW in giving rise to the efficient usage of metabolic energy from the stored larval lipid for its development and growth.

Fig. 2 Growth rate and converted CEW per larva determined after rearing in CEW separately homogenized with predetermined concentration of Rid-X. Mean values of similar parameter indicated by the different characters are significant in variation ($p < 0.05$) by Tukey post hoc pairwise comparison test

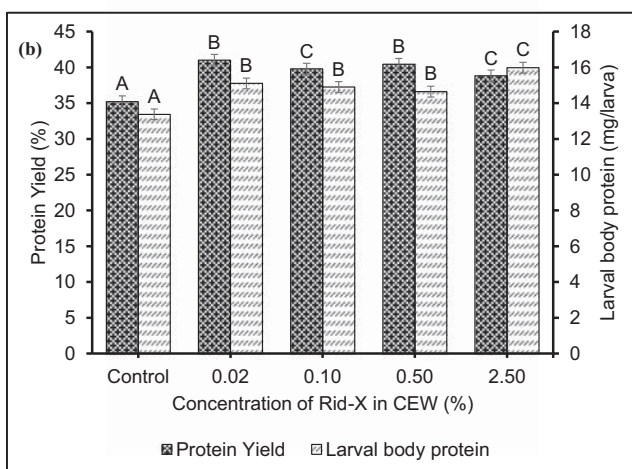
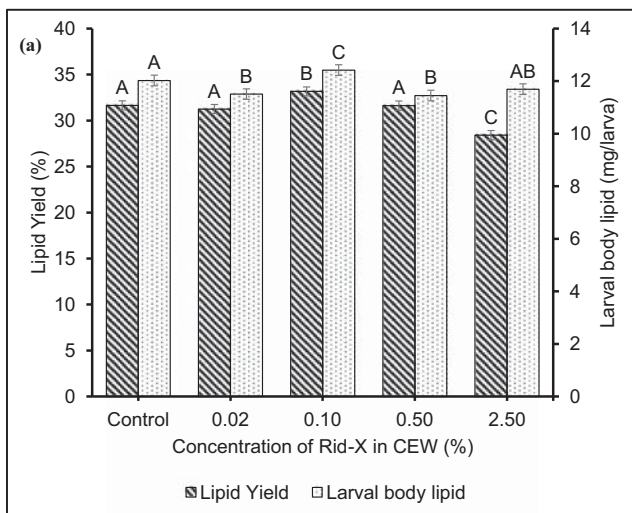
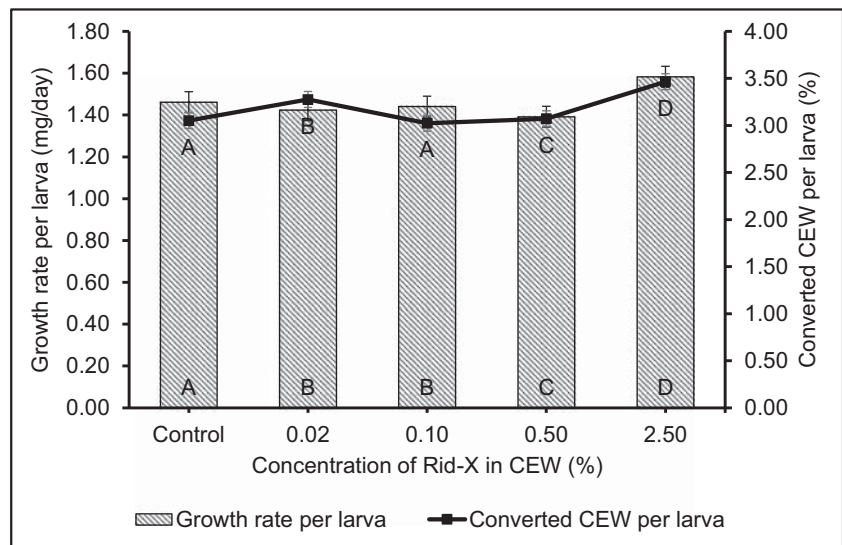


Fig. 3 Lipid (a) and protein (b) sources from harvested BSFL after rearing in CEW separately homogenized with predetermined concentration of Rid-X. Mean values of similar parameter indicated by the different characters are significant in variation ($p < 0.05$) by Tukey post hoc pairwise comparison test

In the case of protein source from the harvested BSFL biomass, albeit the use of 0.02% of Rid-X concentration could permit the highest accumulation of larval protein yield, the maximum larval body protein was attained when the BSFL were fed with the 2.50% of Rid-X performing in situ fermentation in CEW medium. This was because the harvested BSFL from the introduction of 2.50% of Rid-X possessed heavier biomass gained per larva than the 0.02% of Rid-X (Fig. 1). Indeed, the presence of exo-microbes from Rid-X had overall improved the larval protein accumulation during harvesting as observed from the higher larval body proteins for all in-situ fermented mediums than the control. Among the fermented mediums, the occurrence of an intensive competition between exo-microbes and BSFL for common nutrients had led to the extensive loss of larval stored lipid via metabolic loss. Indirectly, this had concentrated the remaining larval stored protein along its development in which higher protein yields could be seen for 0.02% and 0.50% than the 0.10% Rid-X concentrations. According to Chapman (2013), the nutrients that were digested within the BSFL body would be stored and accumulated in the larval fat body as lipids to mainly support the metabolism processes. The protein intake is also controlled and regulated by BSFL via an intrinsic sensible need. The presence of excess protein could result in the production of nitrogenous wastes that are toxic, which could negatively affect larval metabolism. The limiting protein intake, on the other hand, would retard the larval growth, bearing the development cost (Almeida de Carvalho and Mirth 2017). When the presence of amino acids from the protein was detected by the larval body, insulin-like hormones could be released to initiate the metabolic process that allowed for the larval growth (Arquier et al. 2008; Colombani et al. 2003; Géminard et al. 2009; Okamoto and Yamanaka 2015). With the assistance of exo-microbes in the feeding medium as discussed by Shin et al. (2011) and Storelli et al. (2011), the

influence of exo-microbes in decomposing larval diets affected the development of larvae as it triggered and influenced the growth signaling pathways.

Conclusions

The 2.50% concentration of commercial Rid-X was identified as the threshold amount to enhance the BSFL growth by reducing the competition between exo-microbes and BSFL for common nutrients derived from CEW. Using this concentration of Rid-X to execute an in situ fermentation in CEW while feeding the BSFL, a maximum larval body protein could be harvested because of the highest biomass gained per larva. Nevertheless, the maximum larval body lipid was attained when the BSFL were fed with CEW containing 0.10% of Rid-X due to the lowest metabolic loss when the BSFL did not transform into 6th instar stage.

Funding information Financial supports from Yayasan Universiti Teknologi PETRONAS via YUTP-FRG with the cost center of 015LC0-126, Ministry of Education Malaysia under HICoE with the cost center of 015MA0-052, and The Murata Science Foundation with the cost center of 015ME0-104 are gratefully acknowledged.

Compliance with ethical standards

Conflict interests The authors declare that they have no conflict of interests.

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