EVALUATION OF CELLULOLYTIC ACTIVITY OF BLACK SOLDIER FLY [*Hermetia illucens* (Linnaeus)] LARVAE FED DIFFERENT COMBINATIONS OF RICE BRAN AND MARKET VEGETABLE WASTE FROM LOS BAÑOS, LAGUNA, PHILIPPINES

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ABSTRACT

Utilization of black soldier fly (BSF) larvae as biological decomposers shows potential in developing market vegetable waste (MW) management strategies alongside the production of natural fertilizers and animal feeds. BSF larvae's ability to degrade lignocellulosic materials remains elusive due to various factors such as their digestive enzymes' capacity and the rearing substrate composition. This study evaluated the cellulolytic activity and growth rate of BSF larvae fed different combinations of rice bran (RB) and MW. BSF larvae were reared up to 5th instar using an in-house standard procedure. The cellulolytic activity was assayed on larvae crude extract, the growth rate was estimated via larvae weight monitoring, and protein content was analyzed using the Kjeldahl method and Bradford assay. Results showed that BSF larvae fed with 25:75 %RB:%MW yielded the highest cellulolytic activity while those fed with 50:50 %RB:%MW had the fastest growth rate. Cellulolytic activity was found to increase with larvae soluble protein content, but neither did correlate with growth rate. Protein content of rearing substrate correlated positively with that of the larvae and negatively with total days of rearing, indicating that protein levels of substrates may affect the rearing duration and protein content of BSF larvae intended for fertilizer and animal feed use.

Keywords: black soldier fly larvae, cellulolytic activity, feed ingredient, natural fertilizer, protein content

INTRODUCTION

One of the potential sustainable ways being investigated nowadays, geared towards helping address biowaste-related environmental problems, is the utilization of black soldier fly (BSF) larvae as biological decomposing agents. BSF larvae are voracious eaters which have the capability of consuming biowaste weighing twice their own body mass, starting from the 1st instar up to 5th instar stage (Ewusie *et al.*, 2019). Unlike ordinary houseflies, *i.e.*, *Musca domestica*, BSF are not considered insect pests as they do not transmit human diseases and due to their shorter lifespan and unique characteristic of not consuming food during their adult stage. As such, BSF larvae decompose organic products to obtain food and other

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nutrients necessary for their growth, development, and survival during their entire adult stage (Sheppard *et al.*, 2002). The black soldier fly has played big roles not just in solving waste problems but also in addressing some present agriculture concerns, especially in organic farming. The frass that the BSF larvae produce, as a byproduct of larval growth, can be used as an alternative natural fertilizer to complement the costly synthetic fertilizers. Furthermore, BSF larvae can also be employed as an alternative source of nutrients, specifically protein, in formulating feeds for poultry, aquatic, and livestock animals (Tomberlin *et al.*, 2002). In the Philippines, the mass production of BSF larvae has already been lucrative, especially for various feed manufacturers, aquaculture farmers, and backyard livestock growers (Brosas, 2022). However, one of the major challenges in BSF larvae rearing industries is the variability of the nutrient composition of the available biowaste for decomposition by the BSF larvae. Since the waste generated highly depends upon the consumption, activities, and geographical location of the population in a community, it becomes difficult to keep its physiochemical characteristics and composition at a relatively constant level, causing the BSF larvae to adapt for quite some time to the available rearing substrate (Bonelli *et al.*, 2020; Samrot *et al.*, 2022), hence, making the standardization of BSF larvae's rearing substrate impeded, using biowaste alone.

A common feed concentrate that exhibits high protein digestibility and is commonly used in feed formulation for farm animals is rice bran (Yang *et al.*, 2021). Rice bran is the external covering of the rice endosperm, just beneath the hull, produced as a byproduct during milling or polishing (Han *et al.*, 2015). In the Philippines, the use of rice bran in rearing BSF larvae is still quite limited despite its abundance in several rice producing areas. Moreover, the capability of the BSF larvae to decompose cellulose, an abundant polysaccharide in most biowaste, has yet to be explored, and the mechanism be fully elucidated. Although it has been frequently studied how some other insects produce cellulases and form symbiotic relationships with cellulolytic microorganisms, this area of research is relatively new to the biowaste degradation function of the BSF larvae. Particularly, there are still limited reports geared towards understanding the different biochemical characteristics of the BSF larvae in relation to their utilization as animal feeds and/or fertilizers. Further, baseline biochemical information about the BSF larvae extract (either crude, fractionated, or purified) such as cellulolytic activity and soluble protein level may help in the standardization of protocols for BSF larvae rearing. These may also be useful in crafting strategies for biowaste treatment and management and in animal feed and/or natural fertilizers development and processing. The present study investigated the growth rate of BSF larvae along with the protein content and cellulolytic activity of 5th instar BSF larvae (*i.e.*, the usual stage of harvesting larvae for feed use), reared and fed five different combinations (% w/w) of rice bran and market vegetable waste collected from the Municipality of Los Baños, Laguna.

MATERIALS AND METHODS

Preparation and Analysis of the Rearing Substrates

The rice bran (RB) used in this study was of class D2 and purchased from Gintong Buhay Rice Mill in Bay, Laguna in March 2023 while the market vegetable waste (MW) was collected from various public markets in Los Baños, Laguna in March–April 2023, through the Municipal Government of Los Baños. In preparing the different rearing substrates/ treatment groups (Treatments A to E), the MW, which mainly consisted of food scraps such

as damaged vegetables, fruits, and peelings, was ground into smaller pieces using a grinder machine. Portions of RB and MW were weighed accordingly depending on the total amount of substrates needed per replicate – RB:MW (% w/w) for Treatments A, B, C, D, and E were 100:0, 75:25, 50:50, 25:75, and 0:100, respectively. These were placed in a plastic basin, then, mixed thoroughly using a hand trowel. The prepared substrates were stored in tightly capped 20-L pails for one week or until ready for use in BSF larvae rearing. Each rearing treatment group was replicated five times with equal initial weights of BSF eggs and hatching substrate (*i.e.*, 100% RB).

The moisture contents (MC) of the rearing substrates and 5th instar BSF larvae were determined based on AOAC (2005). The oven was pre-heated at 105°C and duplicate samples were analyzed, each having 20-g sample using a clean, pre-weighed and tared 250-mL tin can, partially covered with aluminum foil. The samples were oven-dried for 5 h at 105°C and allowed to cool in a desiccator for 30 min, then, weighed. Semi-micro Kjeldahl analysis was employed to assess crude protein contents of the substrates and the 5th instar BSF larvae following the AOAC (2005) procedure and using 5.95 as conversion factor. The Wendee method was employed to evaluate the crude fiber content of the samples (AOAC, 2005).

Rearing and Homogenization of BSF Larvae

The BSF larvae used in the study were reared at Insiklo PH Farm, Brgy. Putho-Tuntungin, Los Baños, Laguna using in-house standard rearing procedure. BSF eggs were purchased from BSF Nueva Ecija Farm, Sta. Rosa, Nueva Ecija. The eggs were laid on 09 April 2023, collected the following day, and were in transit to Los Baños, Laguna for around 24 h, then, transferred into the hatching substrate composed of 100% RB. Approximately 25 g of BSF eggs were incubated at room temperature for 72 h or until hatching. After the three-day egg-laying period, the majority of the BSF eggs were observed to have hatched and were incubated at room temperature for five days to promote larvae development and growth. Five days after hatching, neonatal BSF larvae, along with the hatching substrate, were reared using five different combinations of RB and MW. The weight of both the BSF larvae and the substrate on which they hatched was measured, resulting in a combined weight of 3.25 kg, then, subsequently divided into five treatment groups with five replicates each. The appearance of the larvae at 5th instar was evaluated following the in-house procedure of the Insiklo PH Farm in order to ascertain larval age at harvest. The rearing conditions were maintained and monitored during the experiment and ranged from 28–32°C environmental temperature and 60–70% relative humidity.

The BSF larvae were weighed once the rearing substrate was observed to be fully consumed, in order to monitor their growth rate, prior to adding certain amount of the appropriate rearing substrate. Pooled larvae weight was recorded for each replication per treatment group and weighing of the 100-pc BSF larvae was done at four time points from the day of egg hatching until the 5th instar stage, then, rearing substrates were replenished afterwards and the weighed larvae were returned to the rearing pans. This cycle was repeated until the BSF larvae were harvested at the 5th instar stage for use in further analysis.

The BSF larvae homogenization and extraction methods were based on the study of Bonelli *et al.* (2020), with minor modifications. Exactly 100 g of the cleaned live 5th instar BSF larvae were weighed in a 250-mL beaker and homogenized for 3 min at maximum speed using an osterizer containing 200 mL of cold lysis buffer (0.10 M phosphate buffer pH

7.2 with 1% (v/v) Triton X-100). The resulting homogenate was filtered and transferred into 50-mL conical tubes followed by incubation in an ice bath for 10 min, and centrifuged at 4°C and 9500 rpm for 20 min using Beckman Coulter Allegra-30R refrigerated centrifuge. Afterwards, the supernatant was collected, transferred into new 50-mL conical tubes, and kept at 4°C for subsequent soluble protein and cellulolytic activity assays.

Soluble Protein Content Determination and Cellulolytic Activity Assay

The Bradford method (Bradford, 1976) was utilized to measure the soluble protein content of the crude extract obtained from the BSF larvae homogenate. Exactly 50 uL each of the bovine serum albumin standards and samples (four replicates per treatment group) were mixed with 2 mL Bradford reagent in 10-mL test tubes. The solutions were then placed in the dark at room temperature for 15 min and the absorbance was read at 595 nm using Shimadzu UV-Mini 1240 UV-Vis spectrophotometer.

The method of Gusakov *et al.* (2011) for cellulolytic activity determination was followed, with some modifications, following reducing sugar content (*i.e.*, free glucose in µmol) analysis via Nelson-Somogyi method, after incubation of 1% (w/v) carboxymethylcellulose. The assay was initiated by transferring 0.50 mL of the reaction buffer (*i.e.*, 0.1 M phosphate buffer pH 7.2) into a 10-mL test tube, followed by the addition of 0.50 mL of freshly prepared 1% (w/v) carboxymethyl cellulose. Subsequently, 0.25 mL of the BSF larvae crude extract was added, mixed using a vortex mixer, and incubated for 10 min at 37°C using a water bath. Then, 1 mL of freshly prepared Nelson reagent was added into the test tube, placed in a boiling water for around 20 min, and allowed to cool at room temperature for 5 min. Afterwards, 0.50 mL arsenomolybdate reagent was added into the test tube, mixed using a vortex mixer, and incubated for about 10 min at room temperature. Then, 3.75 mL of distilled water was pipetted into the tube, mixed, and centrifuged using a table-top centrifuge at 4000 rpm for 10 min. The supernatant was transferred into a new test tube, and absorbance was determined at 510 nm using Shimadzu UV-Mini 1240 UV-Vis spectrophotometer. All determinations were done on four replicates per treatment group along with corresponding blank and control solutions. The amount of glucose released relative to the volume of the BSF larvae crude extract used (in µmol/mL) was determined and the cellulolytic activity of each crude extract was calculated following Gusakov *et al.* (2011).

Statistical Analysis

All the collected data were subjected to analysis of variance (ANOVA) via the Statistical Tool for Agricultural Research (STAR) Version 2.0.1, using a confidence level of 95% and completely randomized design (CRD). Data were presented as mean ± standard deviation, and mean comparisons were conducted using post-Hoc Tukey's Honest Significant Difference (HSD) test at 95% confidence level.

RESULTS AND DISCUSSION

Rearing Substrates Characteristics

Table 1 shows the mean moisture, crude protein, and crude fiber contents of the five combinations of substrates used in this study. Moisture content (MC) showed a wide

range with 100% RB (Treatment A) having the lowest MC (39.02%) while 100% MW (Treatment E) had the highest MC (84.21%). Conversely, the substrate composed entirely of RB exhibited the highest crude protein content at 4.86%, whereas the substrate composed entirely of MW had the lowest at 1.96%. Similarly, the highest percentage of crude fiber was noted for 100% RB while the lowest was noted for 100% MW. A decreasing trend, both in crude protein and fiber of the substrates, was observed as the percentage of MW increased (Table 1). Since 100% MW was mainly composed of damaged vegetables, fruits, and peels collected in bulk from public wet markets in Los Baños, Laguna, it was expected to have low protein content but high fiber content, however, the latter was not observed in this study. The low fiber content obtained for this substrate may be due to the effect of fermentation and decomposition of the fiber components, which might have increased the lignocellulosic fragments and free sugars, however, not determined here. On a dry matter basis, the crude fiber content of Treatment A (100% RB) was 25.58%, which was relatively similar to the crude fiber content of Treatment E (100% MW) at 25.55%. However, the crude protein content of Treatment A was generally lower at 7.98% compared to the crude protein content of Treatment E at 12.39%, on a dry matter basis. Varma *et al.* (2017) reported that lignocellulosic components of biowaste, specifically hemicellulose and cellulose, typically degrade within a timeframe of 12–20 days after collection. During the degradation process, these components yield aliphatic compounds such as free sugars as well as other degradation products including an increase in the amount of humic acids.

Table 1. Mean moisture, crude protein, and crude fiber contents (wet and as fed basis) of the rearing substrates used based on different combinations of rice bran (RB) and market vegetable waste (MW)*.

Treatment	Substrate proportions	Moisture, %	Crude Protein, %	Crude Fiber, %
A	100% RB: 0% MW	39.02^{e}	4.86^{a}	15.60^{a}
B	75% RB: 25% MW	50.32^{d}	4.14^{b}	12.71^{b}
C	50% RB: 50% MW	61.62^{c}	3.41^{c}	9.81^{c}
D	25% RB: 75% MW	72.92^{b}	2.68^{d}	6.92^{d}
E	0% RB: 100% MW	84.21^{a}	1.96^{e}	4.03^{e}

Means within a column followed by the same superscript letters are not significantly different via Tukey's Honest Significant Difference (HSD) test at 95% confidence level. *MW was collected from Los Baños, Laguna public markets through the Municipal Government of Los Baños in March–April 2023.

Pooled Larvae Weight and Approximate Growth Rate

Figure 1 illustrates the mean weight of 100 pieces BSF larvae relative to the number of rearing days after egg hatching. Among the treatments, the BSF larvae fed with 50:50 %RB:%MW (Treatment C) exhibited the highest mean weight of pooled larvae (12.8 g), while those fed with 100% RB (Treatment A) showed the lowest mean weight (8.7 g). The BSF larvae in Treatment A (100% RB) also reached the 5th instar stage the fastest, *i.e.*, after 13 days after egg hatching similar to the study of Bekker *et al.* (2021). Meanwhile, the BSF larvae in Treatments B (75:25 %RB:%MW), C (50:50 %RB:%MW), and D (25:75 %RB:%MW), reached the 5th instar stage at 15 days after egg hatching. Lastly, the BSF larvae fed with 100% MW (Treatment E) took the longest time to reach the 5th instar stage,

showing the dominant dark color of their bodies at 21 days after egg hatching (Figures 1 and 2). A sigmoidal curve was observed in Treatment C, indicating a gradual larval growth from day 5 to day 8, followed by a rapid development phase between day 8 and day 12 (all after egg hatching), and finally reaching a plateau-forming phase from day 12 to day 15 (Figure 1). This rapid development phase was also observed in the other treatment groups. Among treatments B, C, and D, which included both rice bran and market vegetable waste, Treatment C also showed the highest mean pooled larvae weight at day 12, measuring 9.9 g, followed by Treatment D and Treatment B with mean pooled larvae weights of 7.4 g and 6.5 g, respectively. This trend continued until day 15, with Treatment C having the highest mean weight at 12.8 g, followed closely by Treatment D at 12.4 g, and Treatment B at 10.5 g. Between Treatments A (100% RB) and E (100% MW), the highest increase in pooled larvae weight was observed in Treatment E (10.1 g), while Treatment A showed an increase of around 8.3 g towards the harvest stage relative to day 5 after egg hatching.

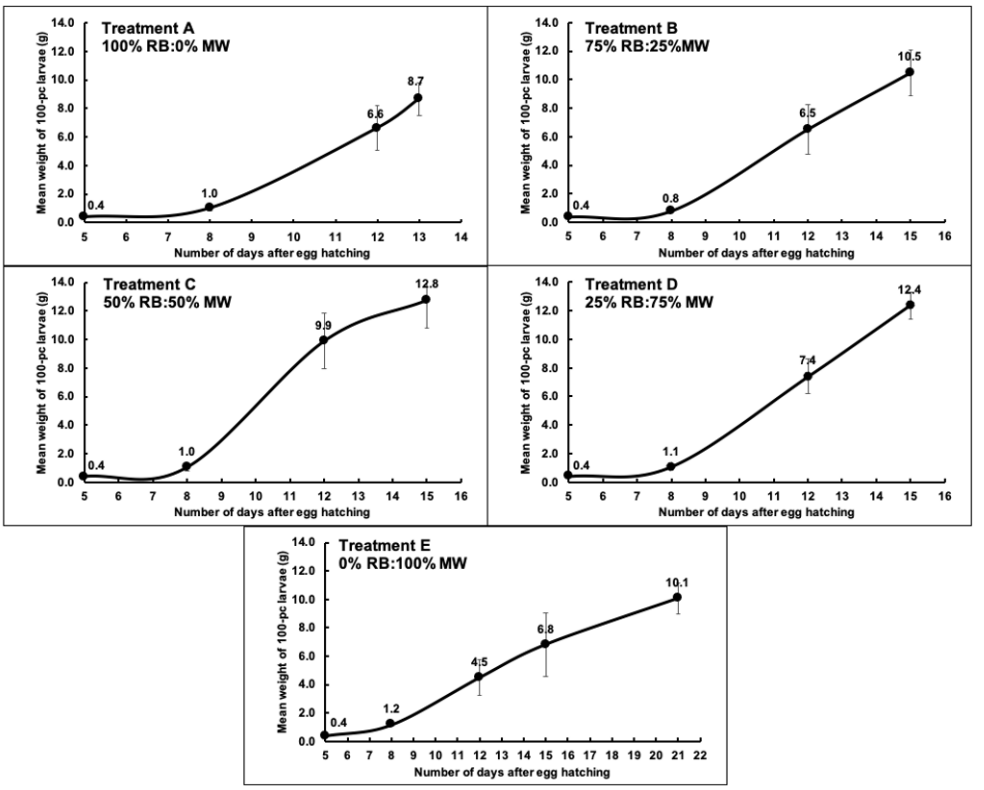

Figure 1. Mean weight of pooled (100-pc) black soldier fly larvae, measured at different days after egg hatching, fed different combinations of rice bran (RB) and market vegetable waste (MW) collected from Los Baños, Laguna public markets in March–April 2023.

As the BSF larvae mature, their digestive accessories also develop, allowing them to efficiently utilize nutrients. This improved nutrient utilization is reflected in the rapid accumulation of biomass observed during subsequent stages of their development (Sun *et*

al., 2021). This has been observed in the present study (Figure 3), particularly between days 9–12 after egg hatching or stage 2 in this study. At this stage, the BSF larvae in Treatment C (50:50 %RB:%MW) exhibited the fastest approximate growth rate at 0.830 g/day, higher than that reported by Bekker *et al.* (2021) using substrate at 45% moisture. On the other hand, BSF larvae in Treatment E (100% MW) showed the slowest growth rate at 0.380 g/day. During the final stage of monitoring (*i.e.*, leading towards the harvest stage), the BSF larvae in Treatment C had the highest growth rate at 0.850 g/day, followed closely by the BSF larvae in Treatment D (25:75 %RB:%MW) at 0.830 g/day. The BSF larvae in these two treatments showed no significant difference in their approximate growth rates. However, they were both significantly different from the BSF larvae in Treatment E, which exhibited the slowest growth rate at 0.590 g/day (Figure 3). A significant negative correlation (r = –0.888) was observed between the total days of rearing and the substrate's crude protein content (Angeles, 2023). This suggests that protein-rich substrates may shorten the rearing period of BSF larvae until the prepupa stage, though having relatively lower biomass weight at harvest.

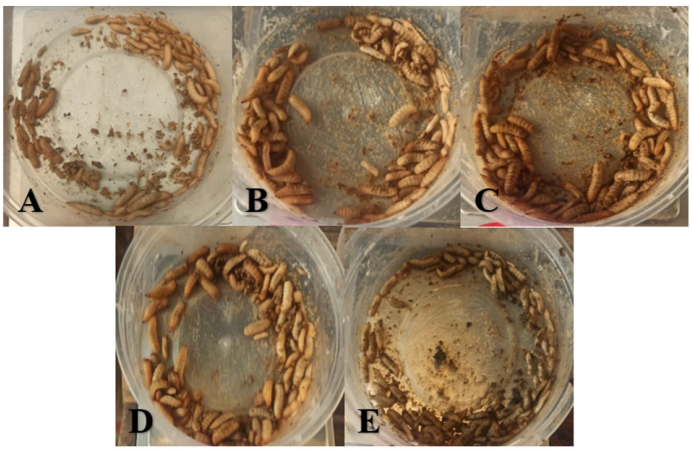

Figure 2. Black soldier fly larvae at 5th instar stage, harvested from treatment groups A to E (A. 100% rice bran [RB]; B. 75% RB: 25% market vegetable waste [MW]; C. 50% RB: 50% MW; D. 25% RB: 75% MW; and E. 100% MW).

The harvest stage of the BSF larvae has been commonly indicated by their grayish-yellow coloration (Almeida *et al.*, 2020). Figure 2 shows these physical characteristics of the BSF larvae across the five treatment groups at 5th instar stage. It was also observed that the BSF larvae in Treatments A and E exhibited relatively smaller sizes compared to the BSF larvae in the other treatment groups (Figure 2), as indexed by the mean weight of pooled larvae at 15 days after hatching (Figure 1). Meanwhile, the BSF larvae fed with 100% RB (Treatment A) did not last until 15 days since they reached the prepupa (5th instar) stage at 13 days after egg hatching, hence, they were collected at this stage.

Crude Protein and Soluble Protein Contents of Black Soldier Fly Larvae

A decreasing trend in larval crude protein content was noted with decreasing

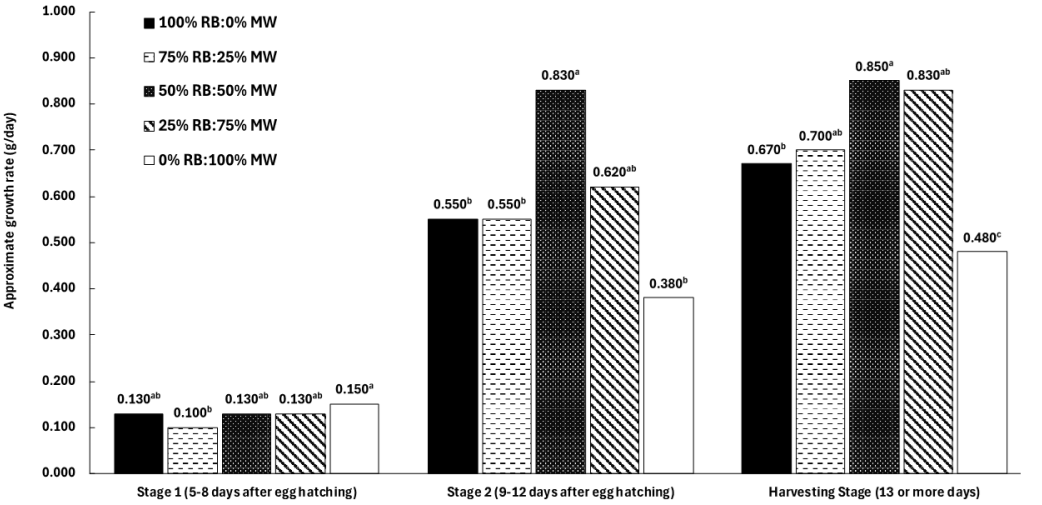

Figure 3. Approximate growth rates of pooled (100-pc) black soldier fly larvae, at different days after egg hatching, fed different combinations of rice bran (RB) and market vegetable waste (MW) collected from Los Baños, Laguna public markets in March–April 2023. Values having the same superscript letters are not significantly different via Tukey's Honest Significant Difference (HSD) test at 95% confidence level.

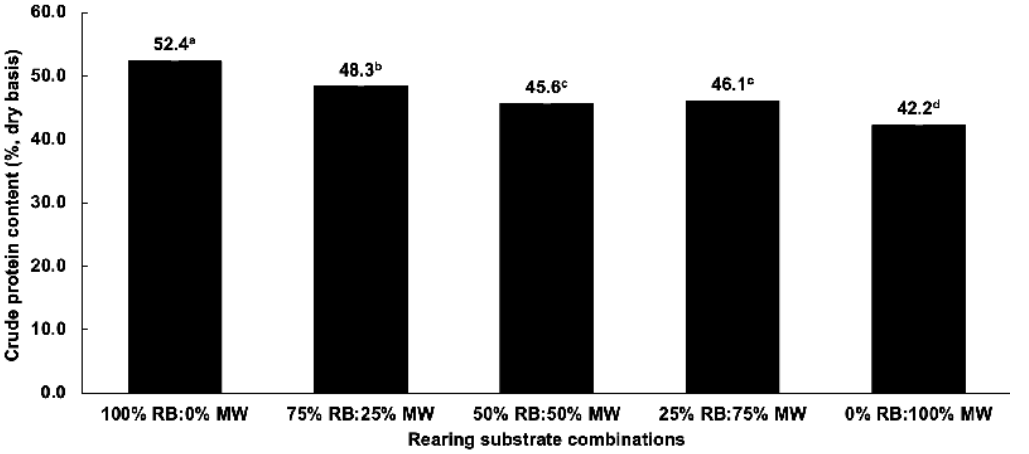

Figure 4. Crude protein contents (dry basis) of harvested 5th instar black soldier fly larvae fed different combinations of rice bran (RB) and market vegetable waste (MW) collected from Los Baños, Laguna public markets in March–April 2023. Values having the same superscript letters are not significantly different via Tukey's Honest Significant Difference (HSD) test at 95% confidence level.

proportion of rice bran (Figure 4). A significant positive (r = 0.956) correlation exists between the crude protein content of the BSF larvae (Figure 4) and the crude protein content of the rearing substrate (Table 1) across Treatments A–E (Angeles, 2023) which were all significantly different from each other. It was also observed that BSF larvae growth parameters such as approximate growth rate 1 (APGR 1) and total number of rearing days (Figures 1 and 3) tend to correlate negatively with BSF larvae crude protein level, though not statistically significant (Angeles, 2023). The crude extract from the BSF larvae harvested from Treatment D (25:75 %RB:%MW) exhibited the highest concentration of soluble proteins at 1 mg/mL, significantly higher than Treatment A at 0.840 mg/mL, and both were significantly higher than those of Treatments B, C, and E (Figure 5). The latter yielded statistically similar soluble protein levels indicating that addition of 25% rice bran in the rearing substrate, mainly composed of market vegetable waste, may increase the soluble enzymes production in the BSF larvae gut. With increasing larval soluble protein levels, APGR 3 tended to increase but was found not statistically significant (Angeles, 2023).

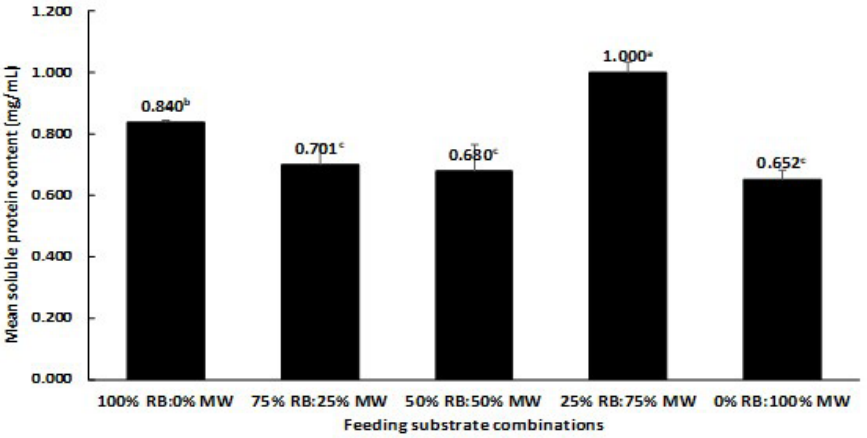

Figure 5. Soluble protein contents of crude extracts from the homogenates of 5th instar black soldier fly larvae fed different combinations of rice bran (RB) and market vegetable waste (MW) collected from Los Baños, Laguna public markets in March–April 2023. Values having the same superscript letters are not significantly different via Tukey's Honest Significant Difference (HSD) test at 95% confidence level.

Cellulolytic Activity of Black Soldier Fly Larvae Crude Extract

The crude extract from the BSF larvae in Treatment D (25:75 %RB:%MW) yielded the highest cellulolytic activity (0.465 µmol/min-mL), followed by Treatment B (75:25 %RB:%MW) at 0.340 µmol/min-mL. The lowest activity was observed for Treatment E (100% MW) (Figure 6). A significant positive correlation (r = 0.968) was observed between the cellulolytic activity and the amount of soluble proteins in the 5th instar BSF larvae crude extract, specifically when only considering Treatments B–D, *i.e.*, the treatments having proportions of rice bran and market vegetable waste. When considering all the five treatment groups, these two parameters tended to correlate positively but did not reach statistical significance (Angeles, 2023). Among these three treatments, it was further observed that the

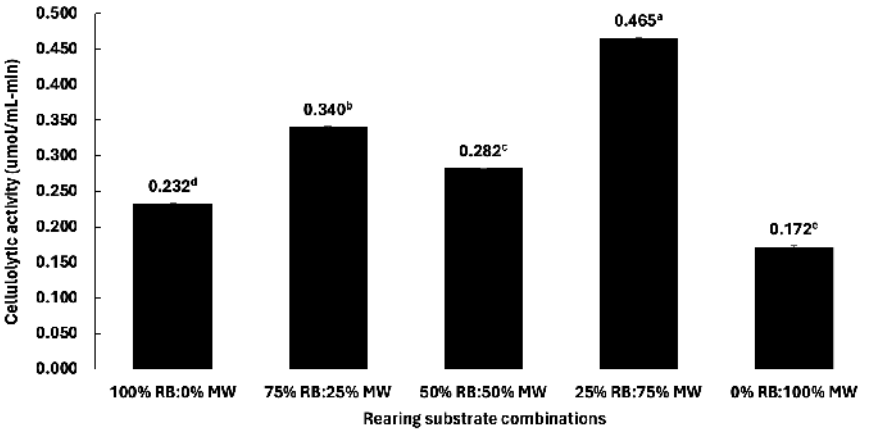

Figure 6. Cellulolytic activities of crude extracts from the homogenates of 5th instar black soldier fly larvae fed with different combinations of rice bran (RB) and market vegetable waste (MW) collected from Los Baños, Laguna public markets in March–April 2023. Values having the same superscript letters are not significantly different via Tukey's Honest Significant Difference (HSD) test at 95% confidence level.

one with the highest amount of soluble protein also exhibited the highest cellulolytic activity, *i.e.*, Treatment D, and similar trend was obtained for Treatments B and C, respectively (Figure 6). Kim *et al.* (2011) reported that BSF larvae midgut had high activities of leucine arylamidase, alpha–galactosidase, amylase, and lipase and low activity of salivary extracts. On the other hand, Supriyatna and Ukit (2016) showed that the total cellulolytic activity of BSF larvae may be due to three most abundant cellulose-degrading bacteria, namely *Bacillus sp.*, *Ruminococcus sp.*, and *Proteus sp.*, which they have isolated from BSF larvae gut. Present results suggest that cellulolytic activity of BSF larvae may be assessed using larval crude extract, even without gut enzymes fractionation or midgut microbiota isolation and characterization, which can be used in evaluating digestive capacity of BSF larvae fed with varying amounts of market vegetable waste. Furthermore, mixing of even a small proportion of nutritive feed concentrates, such as rice bran, for use as rearing substrates for BSF larvae in combination with MW, could be beneficial in enhancing the cellulolytic activity of the BSF larvae as well as their growth rate until the 5th instar stage, compared with using market vegetable waste alone.

CONCLUSION

The rearing substrate with 25% rice bran exhibited the highest cellulolytic activity and no significant correlation was found between the cellulolytic activity of the 5th instar BSF larvae crude extract and the crude protein content of the rearing substrate. Interestingly, a high positive correlation was observed between the cellulolytic activity and amount of soluble proteins in the crude extract of BSF larvae fed with substrates containing rice bran and market vegetable waste, and not the uncombined ones. Further studies on rearing BSF larvae with rice bran as additive to market vegetable waste, specifically utilizing lower proportions of rice bran (*i.e.*, less than 25% of the total rearing substrate) is recommended

in order to determine the minimum amount of rice bran required to for optimum growth rate and cellulolytic activity of BSF larvae. Profiling of BSF larvae midgut proteins may also be conducted in order to shed light on the levels and activities of other digestive enzymes that may help explain the mechanism underlying rapid growth and maximum biomass accumulation of this decomposition agent with potential as natural feed/fertilizer source.

ACKNOWLEDGMENTS

The authors would like to thank the Lifebank Microfinance Foundation, Inc.(LBMFI) for partially funding this research through its BS Thesis Grant for Organic Agriculture facilitated by the University of the Philippines Los Baños (UPLB) Interdisciplinary Studies Center for Organic Agriculture (IdSC-OA). The technicians of Insiklo PH Farm, Antonieta Peñaloza and Angeline Malayas of PhilRice Los Baños Rice Chemistry and Quality Laboratory (RCQL), and the members of the Chemistry in Agriculture, Nutrition, and Cereals Research Laboratory (CHANCE Lab) of UPLB Institute of Chemistry are gratefully acknowledged for their support and assistance. This paper is part of the BS Agricultural Chemistry thesis of GT Angeles at UPLB and has been presented and won Best Oral Paper Award (Undergraduate Thesis Category) during the 60th Philippine Society of Animal Science (PSAS) Scientific Seminar and Annual Convention and 7th International Livestock Biotechnology Symposium at Tagbilaran City, Bohol last October 17-20, 2023.

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