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IN VIVO EVALUATION OF ECOENZYME AS A NATURAL BIOINSECTICIDE AGAINST TENEBRIO MOLITOR LARVAE

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Abstract

Introduction: *Organic kitchen waste fermented into eco-enzyme contains organic acids and secondary metabolites with insecticidal potential. We hypothesized that intermediate dilutions would yield higher larval mortality than very low or undiluted solutions by maximizing uptake while limiting rapid deterrence or detoxification responses.* ***Methods:*** *Eco-enzyme was produced from kitchen residues and brown sugar through 90-day fermentation and confirmed to contain acetic acid, lactic acid, flavonoids, and alkaloids (pH 3.0). A randomized design tested five dilutions (0%, 25%, 50%, 75%, 100%) and a chemical insecticide control (Curacron 500 EC). Each treatment had three replicates of 10 Tenebrio molitor larvae (n=180). Solutions were sprayed twice daily for seven days, and mortality recorded before each application. Data were analyzed using Shapiro–Wilk tests and one-way ANOVA (α=0.05).* ***Results and Discussion:*** *Phytochemical screening verified organic acids and bioactive metabolites. By Day 7, cumulative mortality was: control 23.33%, 25% eco-enzyme 46.67%, 50% 13.33%, 75% 30.00%, 100% 33.33%, and chemical insecticide 86.67%. The 25% group showed normal distribution and significantly higher mortality than several other eco-enzyme treatments (p<0.05). This intermediate concentration permitted ingestion and disruption of gut pH, digestive enzymes, and neural pathways, whereas higher concentrations provoked irritation and avoidance, reducing toxin uptake.* ***Conclusion:*** *Eco-enzyme at 25% provides optimal larvicidal activity, highlighting its potential as a biodegradable, low-cost alternative. Its application could support integrated pest management, lowering environmental residues, and promoting sustainable agriculture. Future work should include field validation, non-target assessments, and formulation strategies to enhance stability and scalability.*

INTRODUCTION

Millions of tons of waste accumulate daily across Indonesia, posing a severe environmental challenge that demands immediate intervention. Data from the BPS-Statistics DKI Jakarta Province (2023) indicates that DKI Jakarta generated approximately 7,543.42 tons of waste in 2022, of which 3,761.90 tons were organic (1). Although organic waste is often valorized through composting or biogas conversion, inadequate infrastructure and low community participation significantly hinder these processes. A substantial portion of organic refuse remains untreated and is discarded in open dumpsites. Under anaerobic conditions in landfills, organic matter decomposes to release ethane, a greenhouse gas more potent than carbon dioxide (2). This process also emits CO_2, thereby exacerbating climate change.

Globally, improper management of organic waste contributes significantly to greenhouse gas emissions

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and poses persistent challenges for sustainable agriculture, highlighting the urgency of developing scalable bioconversion approaches. Moreover, persistent organic residues can generate leachate that infiltrates soil and water sources, threatening ecosystems and public health (3). Eco-enzyme offers a dual benefit as both a waste valorization product and a potential biopesticide; however, its relative efficacy and ecological footprint compared with other natural pesticides, such as essential oils or microbial formulations, require further investigation.

Recent innovations have focused on eco-enzyme production as a sustainable method for managing organic waste. Eco-enzyme is obtained by fermenting kitchen residues; primarily fruit peels with brown sugar and water over approximately three months (4). The resulting dark-brown solution contains a complex mixture of organic acids (e.g., acetic and lactic acids) and hydrolytic enzymes such as protease, amylase, and lipase (5). These bioactive compounds synergistically catalyze the breakdown of insoluble biomolecules into soluble, low-molecular-weight substances, facilitating downstream applications in waste treatment. Beyond its role in waste valorization, eco-enzyme has demonstrated antimicrobial, bioremediation, and potential bioinsecticidal properties (6). Previous studies have shown that eco-enzyme derived from onion peel waste was effective as a bio-insecticide against Spodoptera litura, achieving mortality rates of 82.5–95% depending on the concentration applied (7). However, these studies also noted limitations, particularly the relatively slow action of eco-enzyme compared to chemical insecticides and the lack of significant differences between some concentration treatments, which suggests that the optimization of dosage and target pest selection remains unresolved. Building on these findings, our study seeks to enhance the application of eco-enzyme by testing different concentrations against Tenebrio molitor larvae, thereby expanding its potential utility for stored-grain pest management and improving its comparative effectiveness with chemical controls. Its low-cost production and biodegradability further underscore its appeal for large-scale environmental use. Continued research is needed to optimize fermentation parameters and validate eco-enzyme efficacy under field conditions.

Conventional synthetic insecticides, including organochlorines and organophosphates, have long been employed for pest control but carry significant ecological and health risks. Organochlorines such as dichlorodiphenyltrichloroethane (DDT) persist in the environment for years, leading to bioaccumulation and non-target toxicity. Organophosphates, while less persistent, have been linked to widespread ecological pollution and acute poisoning, accounting for up to 25 percent of pesticide-related fatalities globally (7-8). These concerns have driven demand for renewable, eco-friendly alternatives. Eco-enzyme metabolites, including flavonoids, alkaloids, quinones, and saponins exhibit multiple modes of insecticidal action, such as acetylcholinesterase inhibition, disruption of nervous transmission, and degradation of the insect cuticle (9-10). Additionally, organic acids in eco-enzyme compromise larval development and gut microbiota, enhancing its bioinsecticidal potential (11-12). Thus, eco-enzyme represents a promising natural biopesticide deserving rigorous *in vivo* evaluation. *Tenebrio molitor*, was selected as the *in vivo* model to assess eco-enzyme efficacy against stored-product pests. This beetle larva infests grains, flour, bran, and other stored commodities, causing significant economic losses and product contamination through frass and exuviae (13). Beyond its pest status, fresh larvae pose food-safety concerns as potential vectors of foodborne pathogens and allergens (14). Workers in mealworm rearing facilities have reported severe allergic reactions, including anaphylaxis and respiratory impairment, upon chronic exposure. Evaluating eco-enzyme bioinsecticidal activity against *Tenebrio molitor* thus addresses both agricultural and public health imperatives. The results will inform the development of scalable, eco-friendly pest management strategies. In this study, we hypothesized that intermediate eco-enzyme concentrations, particularly 25%, would yield higher larval mortality by maximizing ingestion and physiological disruption, while very low or undiluted concentrations would be less effective due to insufficient bioactivity or rapid deterrence. Accordingly, each concentration tested was expected to produce distinct larvicidal outcomes, allowing direct comparison with conventional chemical insecticides not only in terms of mortality but also regarding their potential environmental sustainability.

METHODS

Eco-Enzyme Preparation

Kitchen waste substrates: turmeric (100 g), curcuma (100 g), ginger (100 g), orange peel (150 g), and melon peel (150 g) were combined with 200 g of brown sugar dissolved in 2 L of distilled water. Prior to fermentation, all organic substrates were washed and coarsely chopped to ~1–2 cm pieces using a clean knife. Chopping increases surface area sufficiently to support microbial access and fermentation while keeping handling simple and avoiding excessive free liquid. Large seeds, woody cores and spoiled portions were removed to prevent unwanted microbial contamination. The mixture was transferred into an airtight, food-grade fermentation vessel and allowed to ferment at ambient temperature (25 ± 2 °C) for 90 days. After fermentation, the crude eco-enzyme was filtered through two layers of muslin cloth to remove particulate matter. The filtrate was stored at 4 °C until further use (15).

Characterization of Eco-Enzyme

Prior to bioassays, the eco-enzyme was characterized for pH using a calibrated digital pH meter. After the fermentation, the eco-enzymes solutions should be acidic with a pH range of 4.1-5.24, indicate the precence of organic acids. Qualitative assays confirmed the presence of acetic acid (1 N $AgNO_3$ precipitation test), lactic acid ($FeCl_3$ colorimetric test), flavonoids (Mg–HCl coloration test), and alkaloids (Bouchardat's iodine test) following standard phytochemical protocols (16-17).

Insect Rearing and Controlled Environmental Conditions

Experiments were performed in a controlled environment maintained at 25 ± 2 °C and 60 ± 5% relative humidity, monitored continuously with a digital thermo-hygrometer. Larvae were acclimatized for 48 hours under these same conditions prior to treatment to minimize stress-related variability. *Tenebrio molitor* was chosen due it is a widely accepted model of stored-product pests. It infests grains and processed cereals, is easy to rear consistently in the laboratory, and shares feeding physiology and exposure routes with many real-world storage pests. Therefore, results from *T. molitor* bioassays provide a relevant proxy for predicting eco-enzyme performance in stored-grain pest management, while recognizing that field validation on diverse storage pests is recommended. Any individuals that appeared lethargic, injured, or otherwise unhealthy during acclimatization were removed and excluded from the experiment to ensure uniform sample quality.

Experimental Design

A completely randomized design was employed with five eco-enzyme treatments (0%, 25%, 50%, 75%, 100%) and a positive control (Curacron 500 EC), with three replicates per treatment. Each replicate consisted of 10 larvae housed in a separate box (total n=180). Test concentrations were prepared as volume/volume (v/v) dilutions of the crude filtrate using distilled water as diluent (e.g., 25%=25 mL eco-enzyme+75 mL distilled water; 100%=undiluted; 0%=water only), mixed in calibrated glassware immediately before use. Prepared solutions were stored at 4 °C, equilibrated to room temperature prior to application, and discarded if older than 24 h. The chemical control was prepared following the manufacturer's instructions.

Treatment Application

Eco-enzyme dilutions were prepared by mixing the crude filtrate with distilled water to achieve the target concentrations. Twice daily (06:30 and 16:00), each box was uniformly sprayed from a 15 cm distance with two actuations of a hand-pump sprayer delivering approximately 1 mL per spray. Control boxes received either only water (0%) or the recommended dose of chemical insecticide (16).

Data Collection

Caterpillar mortality was recorded immediately before each spraying schedule, yielding fourteen observations per replicate over the study period. To evaluate treatment effects, comparing mortality in each eco-enzyme group, and the insecticide control against the 0% (water-only), and synthetic pesticide group (15,17).

Statistical Analysis

Mortality percentages for each treatment were initially assessed for normality via the *Shapiro–Wilk* test and for variance equality using Levene's test. Once these parametric assumptions were confirmed, a one-way ANOVA was carried out to compare mean mortality across all treatments, followed by the *Shapiro–Wilk* test to determine significant pairwise differences at α=0.05. All statistical analyses were executed in SPSS version 25 (18-19).

RESULTS

Qualitative phytochemical screening of the fermented eco-enzyme (n=1 sample) yielded positive reactions for four classes of compounds. A dark precipitate formed upon addition of 1 N $AgNO_3$, indicating acetic acid (A). Lactic acid was confirmed by a reddish-brown coloration with $FeCl_3$ (B). Alkaloids yielded a brown precipitate in the Bouchardat reaction (C), and flavonoids produced an orange–yellow hue in the Mg–HCl test (D). Test tube E contains the undiluted 100% eco-enzyme as a visual control. The solution's pH was 3.0, confirming its acidic nature.

Figure 1. Result of Qualitative Phytochemical Tests

Daily mortality percentages report new deaths recorded during each 24-hour period and are expressed as a percentage of the initial replicate size (10 larvae). Daily mortality=(new deaths during that day÷10) × 100. Observations were made twice daily and the daily value is the sum of newly dead individuals from both checks; no individuals were replaced. Cumulative mortality (used for statistical comparisons) is reported separately as the total deaths to date expressed as a percentage of the initial 10 larvae per replicate. A total of 180 larvae (6 treatments×3 replicates×10 larvae) were observed twice daily for seven days (14 observations per replicate; no missing data). Daily mortality percentages for each treatment are plotted in Figure 2. The highest single-day mortality among eco-enzyme treatments occurred at 25% concentration on Day 5 (13.33%), while the chemical insecticide peaked on Day 7 (23.33%). The 50% eco-enzyme treatment never exceeded 6.67% daily mortality.

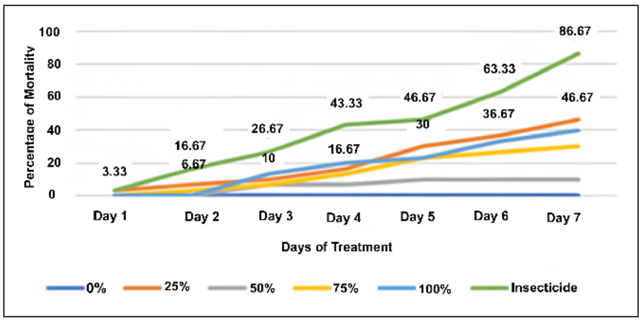

Figure 2. Daily Mortality Percentage of Mealworm Larvae Under Treatments

Cumulative mortality by Day 7 (n=30 larvae per treatment) was 23.33% in the water control, 46.67% in the 25% eco-enzyme group, 13.33% in the 50% eco-enzyme group, 30.00% in the 75% eco-enzyme group, 33.33% in the 100% eco-enzyme group, and 86.67% in the chemical insecticide group; these results are displayed in Figure 3, and no data were excluded or imputed. Each treatment was monitored twice daily, yielding 14 observations per replicate over the seven-day period.

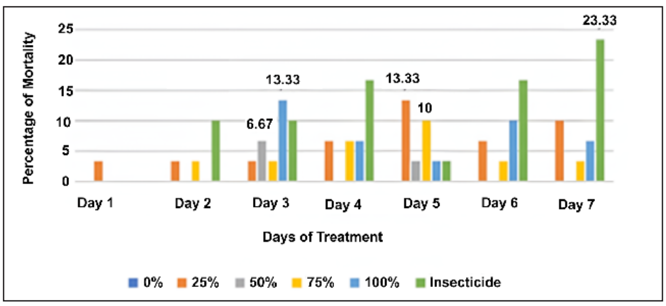

Figure 3. Seven-Day Cumulative Mortality of Mealworm Larvae per Treatment

Shapiro Wilk tests were performed on each treatment's cumulative mortality (three replicate values per group). Results (Table 1) show normal distribution for 25% eco-enzyme (W=0.964, p=0.637), 100% eco-enzyme (W=0.871, p=0.298), and chemical insecticide (W=0.974, p=0.779). The 0%, 50%, and 75% groups did not meet normality (all p<0.001). Sample size per test: n=3; no outliers or missing observations.

Table 1. *Shapiro–Wilk* Normality Test Results for Each Treatment Group

Treatment	n	Mean (%)	SD (%)	*Shapiro W*	p-value	Normality
0%	3	23.33	5.77	0.750	<0.001	No
25%	3	46.67	15.28	0.964	0.637	Yes
50%	3	13.33	5.77	0.750	<0.001	No
75%	3	30.00	17.32	0.750	<0.001	No
100%	3	33.33	32.14	0.871	0.298	Yes
Chemical Insecticide	3	86.67	10.00	0.974	0.779	Yes

DISCUSSION

Eco-enzyme with 25% concentration strikes an optimal balance between potency and exposure time, allowing larvae to ingest sufficient bioactive compounds without immediately deterring contact. At this dilution, organic acids such as acetic and lactic acid disrupt midgut integrity more effectively than at higher concentrations, where rapid cuticular damage can trigger escape behaviors (4,11,20). Flavonoids and alkaloids present in the eco-enzyme inhibit digestive enzymes and neurotransmission, respectively, leading to progressive physiological collapse over several days (15,21-22) Higher eco-enzyme concentrations likely acted as *irritants* rather than efficient toxicants: strong acidity and high surface activity can cause immediate cuticular and antennal irritation, prompting larvae to reduce contact and feeding and therefore lowering toxin uptake. Volatile or highly concentrated components may also volatilize or precipitate at the cuticle interface, reducing internal bioavailability. At the physiological level, excessive doses can trigger inducible detoxification pathways such cytochrome P450s, esterases, glutathione-S-transferases. Antifeedant responses that mitigate mortality, whereas intermediate concentrations favor ingestion and internal disruption of gut pH and enzymatic function (23-26). This contrasts with many synthetic neurotoxic insecticides (pyrethroids and organophosphates) that cause rapid knockdown by directly targeting neural ion channels or AChE, explaining the faster and higher mortality observed with chemical controls (27-30). Moderate concentrations avoid the activation of inducible detoxification pathways such as cytochrome P450 and esterases that are upregulated in response to high toxin loads. Thus, 25% eco-enzyme leverages both chemical potency and behavioral susceptibility to achieve superior lethality. The 25% solution also maintains a stable pH environment, preventing rapid volatilization of volatile compounds and ensuring sustained larvicidal activity Field observations have shown that larvae exposed to this dilution exhibit erratic movement and feeding disruptions over a longer period, maximizing cumulative mortality.

Statistical analyses further support the superior efficacy of the 25% concentration, with mortality data conforming to normality assumptions (*Shapiro–Wilk* $p>0.05$) and showing significant differences in one-way ANOVA ($p<0.05$) compared to other treatments. Post hoc tests (*Duncan's* multiple range) consistently rank the 25% group highest in larval death rates, indicating that its performance is not due to random variation (31-32). Lower dilutions (0–20%) produce sublethal stress responses, while higher dilutions (>50%) induce rapid but partial mortality, reducing overall effectiveness (33). The normal distribution of mortality percentages enables robust parametric testing, confirming that the observed superiority of 25% eco-enzyme is statistically valid and reproducible. Additionally, residual analysis indicates minimal variance heterogeneity in the 25% group, ensuring reliable conclusions (34-35). These findings align with ecological toxin–dose theories, which posit maximum effect at intermediate concentrations where uptake and physiological disruption coincide. Consequently, both mechanistic and statistical evidence converge to highlight 25% eco-enzyme as the most effective larvicidal concentration.

The presence of secondary metabolic content in eco-enzymes in the form of acetic acid, lactic acid, flavonoids, and alkaloids has a direct effect on the death of Hong Kong caterpillar larvae. Acetic acid and lactic acid cause a drastic decrease in pH in the intestinal lumen and affect the permeability of the larval intestine (36). Acidity conditions outside the optimal range of the larval intestine have an impact on the activity of digestive enzymes, thereby inhibiting the process of food digestion and nutrient absorption. This decrease in pH can also damage intestinal epithelial cells, causing leakage of body fluids which leads to larval death. Acid molecules in eco-enzyme can penetrate the larval cuticle, break down the pleated bonds on the surface of the larva's body, thereby increasing water loss through transpiration. The resulting systemic dehydration can trigger cell death in parts of important larval tissues (36-37). Furthermore, the localized acidity may enhance diffusion of other bioactive molecules across the cuticle, amplifying overall toxicity. This multifaceted mode of action underscores the potential of eco-enzyme acids as sustainable biocontrol agents with reduced risk of chemical resistance.

Some flavonoids inhibit the glutathione S-transferase enzyme Noppera-bo (Nobo) which is involved in the synthesis of the hormone ecdysone. This hormone is key to larval molting and metamorphosis; without it, the molting process fails and larvae die before reaching the pupal stage (35-37). Flavonoids can also bind and inhibit acetylcholinesterase (AChE), causing accumulation of acetylcholine in nerve synapses, muscle spasms, and paralysis. Additionally, certain biflavones have been shown to induce oxidative stress in neural tissues, further compromising larval viability (38-39). By targeting multiple enzymatic pathways, flavonoids deliver a compounded lethal effect that may prolong field persistence and reduce application frequency.

Alkaloids act as antagonists on insect neurotransmitter receptors (e.g., nicotinic acetylcholine or octopamine receptors), disrupting normal synaptic transmission and causing rapid paralysis and death (40-41). Many alkaloids also function as anti-feeding agents, prompting larvae to reject treated substrates and suffer malnutrition. Furthermore, alkaloids can interfere with hormonal regulation of development by inhibiting proteins that mediate growth and metamorphosis, leading to stunted growth and failure to pupate (42). The dual action of deterrence and physiological disruption makes alkaloids valuable components in eco-enzyme formulations, contributing synergistically to overall larvicidal efficacy (43-44).

The eco-enzyme's bioactivity likely reflects multi-component, multi-site actions such organic acids (acetic/lactic) acidify the midgut, impair digestive enzymes, disrupt epithelial integrity and gut microbiota flavonoids can inhibit detoxification and hormone-related enzymes (Noppera-bo GST), impair AChE and molting. Alkaloids act as neuroreceptor antagonists and antifeedants. This contrasts with many conventional insecticides (e.g., pyrethroids targeting voltage-gated sodium channels, organophosphates/carbamates inhibiting AChE), which produce rapid, single-site neurotoxic effects associated with faster knockdown, higher non-target risk, and more predictable resistance pathways. In order to substantiate these distinctions, targeted assays such as AChE inhibition, P450/esterase/GST activity measurements, gut histopathology, and microbiome profiling are recommended to empirically separate eco-enzyme's multi-site effects from single-target synthetic modes of action.

This study was conducted under controlled laboratory conditions using a single test species (*T. molitor*) and a seven-day exposure period, which may not capture species-specific responses, seasonal/ environmental variability, or long-term effects such as resistance development. Additionally, eco-enzyme composition can vary with substrate and fermentation conditions, so efficacy observed here may differ across formulations and field settings. Future work should include multi-species bioassays, extended monitoring for resistance and sublethal effects, and field trials to evaluate non-target impacts and operational feasibility.

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CONCLUSION

Eco-enzyme functions effectively as a natural larvicide against *Tenebrio molitor*, with a 25% dilution achieving the highest cumulative mortality among eco-enzyme treatments. Statistical analysis confirmed the data's normal distribution for the 25% group, supporting the validity of observed differences in efficacy. These findings highlight 25% eco-enzyme as an optimized, sustainable alternative to chemical insecticides. It is recommended that future studies assess long-term field performance of this formulation under variable environmental conditions, evaluate non-target impacts on beneficial arthropods, and explore formulation enhancements (e.g., microencapsulation) to improve stability and ease of application.

AUTHORS' CONTRIBUTION

PS: Conceptualization, funding acquisition, project administration, supervision, review and editing draft. RG: Methodology development, investigation, data curation, write original draft preparation. YLT: Formal analysis; statistical testing; visualization of results, review and editing draft. ASID: Laboratory experimentation for eco-enzyme characterization, sample preparation, investigation. AGP: Insect rearing and bioassay execution, data collection, investigation. MAS: Software configuration, validation of analysis scripts, review and editing draft. All authors have read and approved the final manuscript. SR: Data collection, validation of analysis scripts, review and editing draft.

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