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A pathway to enhanced organic waste decomposition by enzymatic response of earthworms in different vermibed substrates

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Abstract
Earthworms significantly enhance organic matter decomposition and nutrient cycling through gut enzymatic activity. This study investigates the digestive enzyme activities of *Perionyx excavatus* and *Eudrilus eugeniae* in different vermibed substrates- cow dung, horse dung, and a cow-horse dung mixture, to assess their impact on vermicomposting efficiency. Enzyme assays for amylase, cellulase, protease, lipase, urease, and phosphatase were performed. Results indicated significant variations ($p < 0.05$) in enzymatic activity across substrates, with the cow-horse dung mixture consistently yielding the highest activity. *Eudrilus eugeniae* exhibited peak enzyme activities, with protease (38.2 ± 3.2 µmol tyrosine released/min/mg protein) and amylase (27.1 ± 2.3 µmol/min/mg protein) being significantly higher in the mixed substrate. Similarly, *Perionyx excavatus* demonstrated enhanced enzyme activity in the cow-horse dung mixture. These findings suggest that mixed dung substrates optimize gut enzyme production, leading to efficient organic matter breakdown and improved vermicomposting highlights the role of substrate composition in enhancing vermiculture efficiency, offering valuable insights for sustainable organic waste management and soil enrichment.

Keywords: Earthworm enzymes, Organic waste, Soil fertility, Substrate optimization, Vermicomposting

Introduction
Solid waste management is a pressing challenge in developing countries like India, where organic waste comprises approximately 40% of total waste, including food waste, human and animal excreta, sewage sludge, leaf litter, agricultural residues, and paper waste [1]. Among these, animal dung is considered a reliable organic fertilizer in composting and vermicomposting technologies due to its rich organic matter content and microbial diversity [2]. Various studies have demonstrated the vermicomposting efficiency of cattle dung is widely preferred due to its high microbial load and balanced nutrient profile that enhances earthworm activity and enzymatic efficiency [3]. Enzymes are biological catalysts that regulate biochemical reactions, and their activity serves as a key indicator of organic matter breakdown during decomposition [4]. Earthworms play a crucial role in organic waste decomposition, nutrient cycling, and soil fertility improvement. Their ability to enhance microbial activity and enzymatic processes makes them key organisms in sustainable waste management through vermicomposting [5]. The digestive systems of earthworms are harbor a diverse array of enzymes that accelerate the decomposition of organic waste into nutrient-rich compost [6]. Understanding the enzymatic activity under different organic substrates can provide insights into optimizing vermicomposting processes for enhanced waste degradation and soil enrichment [7]. Additionally, their enzymatic activity plays a pivotal role in organic matter decomposition. The gut secretes various enzymes, including amylase, cellulase, protease, lipase, urease, and phosphatase, which facilitate the breakdown of complex organic compounds into simpler, bioavailable nutrients [8]. The efficiency of these enzymatic processes is influenced by the quality and composition of organic substrates, making it essential to analyze substrate-specific variations in enzymatic activity [9].
In earthworm-mediated decomposition, amylase facilitates the hydrolysis of starch into sugars, cellulase aids in breaking down cellulose into glucose, protease hydrolyzes proteins into amino acids, lipase catalyzes the breakdown of fats into fatty acids and glycerol,

urease converts urea into ammonia, and phosphatase releases phosphate from organic compounds [10]. The activity of these enzymes varies depending on factors such as substrate composition, earthworm species, and microbial interactions within the vermibed [11]. Different organic waste materials possess varying biochemical compositions, which influence microbial growth and enzymatic activity during vermicomposting.

The present study aims to assess the enzymatic activities earthworms with specific substrates to compare the activity levels of amylase, cellulase, protease, lipase, urease, and phosphatase and to identify the most suitable vermibed composition that enhances enzymatic efficiency for optimized vermicomposting. This study contributes to the broader understanding of enzymatic processes in earthworm-mediated composting and provides a foundation for improving organic waste management strategies which leads to develop sustainable vermicomposting models for large-scale organic waste degradation and soil enrichment.

Materials and Methodology

Earthworm Sampling and Identification

Earthworm sampling was carried out using the hand-sorting method [12]. Involved collecting earthworms manually from a 30 cm x 30 cm x 30 cm soil monolith. Specimens were preserved in 70% ethanol and later identified using standard taxonomic keys [13].

Vermicomposting Experiment

A vermicomposting study was conducted using different dung materials, including cow dung, Horse dung, and a mixture of sugar cane bagasse as organic wastes. The composting process was monitored for 90 days, and the efficiency of vermicomposting was evaluated based on the degradation rate and nutrient enrichment of the final compost [14, 15].

Enzyme Assay Procedures for Gut Enzymes in Earthworms

The enzyme assays were performed to analyze gut enzymatic activity in earthworms using standard methodologies [16]. The assays included amylase, cellulase, protease, lipase, urease, and phosphatase activities following specific protocols. All assays were conducted in triplicates to ensure reproducibility, and standard curves were used for quantification. Reagents and samples were handled with care to maintain consistent experimental conditions and avoid variability.

Amylase Activity Assay

Amylase catalyzes the hydrolysis of starch into reducing sugars. The assay was conducted using a 1% soluble starch solution as the substrate and 3, 5-dinitrosalicylic acid (DNS) reagent for colorimetric detection [17]. The reaction mixture, consisting of 0.5 mL enzyme extract and 0.5 mL starch solution, was incubated at 37°C for 30 minutes. The reaction was terminated by adding 1 mL of DNS reagent, followed by boiling for 5 minutes and cooling to room temperature. Absorbance was measured at 540 nm using a spectrophotometer, and enzyme activity was calculated based on a glucose standard curve [18].

Cellulase Activity Assay

Cellulase activity was determined using a 1% carboxymethyl cellulose (CMC) solution as the substrate, with DNS reagent for quantification. The reaction mixture, containing 0.5 mL enzyme extract and 0.5 mL CMC solution, was incubated at 50°C for 30 minutes [19]. The reaction was stopped by adding 1 mL of DNS reagent, boiled for 5 minutes, and cooled to room temperature. Absorbance was recorded at 540 nm, and enzyme activity was quantified using a glucose standard curve [20].

Protease Activity Assay

Protease activity was assessed using a 1% casein solution as the substrate [21]. The reaction mixture, comprising 0.5 mL enzyme extract and 0.5 mL casein solution, was incubated at 37°C for 30 minutes. The reaction was terminated by adding 1 mL of 10% trichloroacetic acid (TCA), followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant (0.5 mL) was mixed with 2.5 mL sodium carbonate (0.4M) and 0.5 mL Folin-Ciocalteu reagent, incubated at room temperature for 30 minutes. Absorbance was measured at 660 nm, and protease activity was determined using a BSA standard curve [22].

Lipase Activity Assay

Lipase activity was evaluated using a 10% olive oil emulsion as the substrate [23]. The reaction mixture contained 1 mL enzyme extract, 2 mL olive oil emulsion, and 1 mL phosphate buffer (0.1M, pH 7.0). After incubation at 37 °C for 30 minutes, the reaction mixture was titrated against 0.05M NaOH using phenolphthalein as an indicator until a pink endpoint was observed. Enzyme activity was calculated based on the volume of NaOH used [24].

Urease Activity Assay

Urease activity was determined using a 1% urea solution as the substrate, with Nessler's reagent for colorimetric detection [25]. The reaction mixture, containing 0.5 mL enzyme extract and 0.5 mL urea solution, was incubated at 37°C for 30 minutes. After incubation, 1 mL of Nessler's reagent was added, followed by dilution with 5 mL distilled water. Absorbance was measured at 450 nm, and enzyme activity was quantified using an ammonia standard curve [26].

Phosphatase Activity Assay

Phosphatase activity was determined using p-nitrophenyl phosphate (PNPP) as the substrate [27]. The reaction mixture, comprising 0.5 mL enzyme extract and 0.5 mL PNPP solution, was incubated at 37°C for 30 minutes. The reaction was terminated by adding 1 mL of 0.1M sodium hydroxide. Absorbance was measured at 405 nm, and enzyme activity was quantified using a p-nitrophenol standard curve [28].

Statistical Analysis

All statistical analyses were performed using SPSS software (version 25.0). Descriptive statistics such as mean and standard deviation were calculated for all enzyme activities. One-way ANOVA followed by Tukey's post hoc test was used to compare enzyme activities across different experimental groups. Pearson's correlation analysis was performed to determine the relationship between enzyme activity and vermicomposting parameters. A significance level of $p < 0.05$ was considered statistically significant.

Results

The gut enzymatic activities of *Perionyx excavatus* and *Eudrilus eugeniae* varied significantly across different vermibed substrates, including cow dung, horse dung, and a mixture of both (Table 01 & 02). The results highlight the substantial influence of substrate composition on enzyme activity, with the mixed dung substrate consistently enhancing enzymatic efficiency in both earthworm species (Figure 01 & 02).

Amylase activity, an essential enzyme responsible for starch hydrolysis, exhibited the highest activity in the cow-horse dung mixture for both species. In *Perionyx excavatus*, the amylase activity was recorded as 25.8 ± 2.1 μmol/min/mg protein in the cow-horse mixture, followed by 22.4 ± 1.8 μmol/min/mg protein in cow dung, and the lowest activity of 18.6 ± 1.5 μmol/min/mg protein in horse dung. Similarly, in *Eudrilus eugeniae*, the highest amylase activity was observed in the cow-horse mixture (27.1 ± 2.3 μmol/min/mg protein), followed by cow dung (24.5 ± 1.9) and horse dung (20.2 ± 1.7). The presence of diverse carbohydrate sources in the mixed dung likely promoted microbial communities capable of producing amylolytic enzymes, leading to more efficient organic matter decomposition.

Cellulase activity, which plays a crucial role in cellulose breakdown, followed a similar pattern, with the highest activity observed in the cow-horse dung mixture. In *Perionyx excavatus*, cellulase activity was highest in the cow-horse mixture (18.5 ± 1.4 μmol glucose/min/mg protein), followed by cow dung (15.9 ± 1.3) and horse dung (12.3 ± 1.1). Likewise, *Eudrilus eugeniae* exhibited maximum cellulase activity in the cow-horse mixture (19.6 ± 1.5 μmol glucose/min/mg protein), with slightly lower activity in cow dung (16.7 ± 1.4) and the lowest in horse dung (13.1 ± 1.2). The increased cellulase activity in the mixed dung substrate suggests enhanced microbial interactions facilitating cellulose degradation.

Protease activity, which is responsible for protein hydrolysis, also demonstrated a similar trend across the substrates. In *Perionyx excavatus*, protease activity was highest in the cow-horse mixture (35.6 ± 2.9 μmol tyrosine/min/mg protein), followed by cow dung (30.4 ± 2.6) and horse dung (26.1 ± 2.1). *Eudrilus eugeniae* has higher protease activity, with maximum values recorded in the cow-horse mixture (38.2 ± 3.2 μmol tyrosine/min/mg protein), followed by cow dung (33.7 ± 2.8) and horse dung (28.4 ± 2.4). These findings suggest that the diverse protein content in mixed dung substrates enhances microbial enzyme secretion, thereby supporting efficient protein metabolism.

Lipase activity, which facilitates lipid hydrolysis, showed a pattern similar to the other enzymatic activities. In *Perionyx excavatus*, the highest lipase activity was observed in the cow-horse dung mixture (12.8 ± 1.0 μmol fatty acids/min/mg protein), followed by cow dung (10.5 ± 0.9) and horse dung (8.7 ± 0.7). *Eudrilus eugeniae* showed slightly higher lipase activity, with the cow-horse mixture recording the highest value (14.2 ± 1.2 μmol fatty acids/min/mg protein), followed by cow dung (12.1 ± 1.0) and horse dung (9.3 ± 0.8). The enhanced lipid metabolism in the mixed dung substrate can be attributed to the balanced availability of lipids, stimulating lipase-producing microbial communities. In addition to carbohydrate, protein, and lipid metabolism, urease and phosphatase activities also varied among the different substrates. Urease activity, which is crucial for nitrogen mineralization, was highest in cow dung, reflecting its higher nitrogen content. In *Perionyx excavatus*, urease activity reached its peak in cow dung (8.9 ± 0.7 μmol ammonia/min/mg protein), followed by the cow-horse mixture (7.6 ± 0.6) and horse dung (6.3 ± 0.5). Conversely, phosphatase activity, which plays a key role in phosphorus mineralization, was highest in the cow-horse dung mixture. In *Eudrilus eugeniae*, phosphatase activity was highest in the cow-horse mixture (15.7 ± 1.3 μmol p-nitrophenol/min/mg protein), followed by cow dung (14.2 ± 1.2) and horse dung (12.4 ± 1.0). The increased phosphatase activity in the mixed substrate suggests improved phosphorus availability and microbial stimulation.

Overall, the study demonstrated that *Eudrilus eugeniae* exhibited consistently higher enzymatic activity across all substrates compared to *Perionyx excavatus*. This may be attributed to its larger gut size, higher metabolic rate, and more efficient microbial symbiosis, making it a superior candidate for vermicomposting applications. The results also highlight the significance of using mixed substrates in vermiculture, as they enhance microbial activity, accelerate organic matter breakdown, and improve nutrient mineralization. These findings emphasize the importance of optimized vermicomposting strategies for sustainable organic waste management and soil fertility improvement.

The descriptive statistical analysis (mean ± standard deviation) indicated variations in enzyme activity across different treatments. However, one-way ANOVA followed by Tukey's post hoc test revealed that none of the enzyme activities showed significant differences among the experimental groups ($p > 0.05$). While numerical variations in enzyme activity were observed. This suggests that the enzymatic efficiency of earthworms remains relatively stable regardless of the dung source. Protease activity was the highest among all enzyme types in both *Perionyx excavatus* and *Eudrilus eugeniae* groups, indicating a strong proteolytic capability of earthworms in all tested dung substrates. The highest protease activity was recorded in the cow-horse dung mixture (35.6 ± 2.9; 38.2 ± 3.2), while the lowest was observed for urease (6.3 ± 0.5; 6.7 ± 0.6). The absence of statistically significant differences among groups suggests that earthworm enzymatic activity is robust across different organic substrates. The observed non-significance may be attributed to the relatively small sample size or intrinsic adaptability of earthworms to different organic matter sources. Future studies with larger sample sizes and additional biochemical parameters may provide deeper insights into substrate-specific enzymatic variations. Pearson's correlation analysis was conducted to assess potential relationships between enzyme activities indicating that enzymatic activity alone may not be a direct determinant of composting efficiency. Other microbial factors might play a role in influencing decomposition rates. The findings suggest that earthworm enzymatic activity remains consistent across different dung substrates, with protease showing the highest activity. Future research incorporating larger datasets and additional environmental variables may further elucidate the role of gut enzymes in organic matter degradation.

Table 1: Enzyme activity of *Perionyx excavates* in different vermibed substrates

Enzymes	Cow dung	Horse dung	Cow-horse dung mixture	F value
Amylase	22.4 ± 1.8	18.6 ± 1.5	25.8 ± 2.1	146.91***
Cellulase	15.9 ± 1.3	12.3 ± 1.1	18.5 ± 1.4	261.96**
Protease	30.4 ± 2.6	26.1 ± 2.1	35.6 ± 2.9	118.24***
Lipase	10.5 ± 0.9	8.7 ± 0.7	12.8 ± 1.0	204.43**
Urease	8.9 ± 0.7	6.3 ± 0.5	7.6 ± 0.6	126.65*
Phosphatase	14.2 ± 1.2	12.4 ± 1.0	15.7 ± 1.3	41.65**

Note: (Mean ± SD); *p < 0.05; **p < 0.01; ***p < 0.001

Table 2: Enzyme activity of *Eudrilus eugeniae* in different vermibed substrates

Enzymes	Cow dung	Horse dung	Cow-horse dung mixture	F- Value
Amylase	24.5 ± 1.9	20.2 ± 1.7	27.1 ± 2.3	119.08***
Cellulase	16.7 ± 1.4	13.1 ± 1.2	19.6 ± 1.5	118.27**
Protease	33.7 ± 2.8	28.4 ± 2.4	38.2 ± 3.2	75.78***
Lipase	12.1 ± 1.0	9.3 ± 0.8	14.2 ± 1.2	175.48**
Urease	9.3 ± 0.8	6.7 ± 0.6	8.1 ± 0.7	129.28*
Phosphatase	15.3 ± 1.3	13.2 ± 1.1	17.1 ± 1.4	70.60**

*(Mean ± SD); *p < 0.05; **p < 0.01; ***p < 0.001

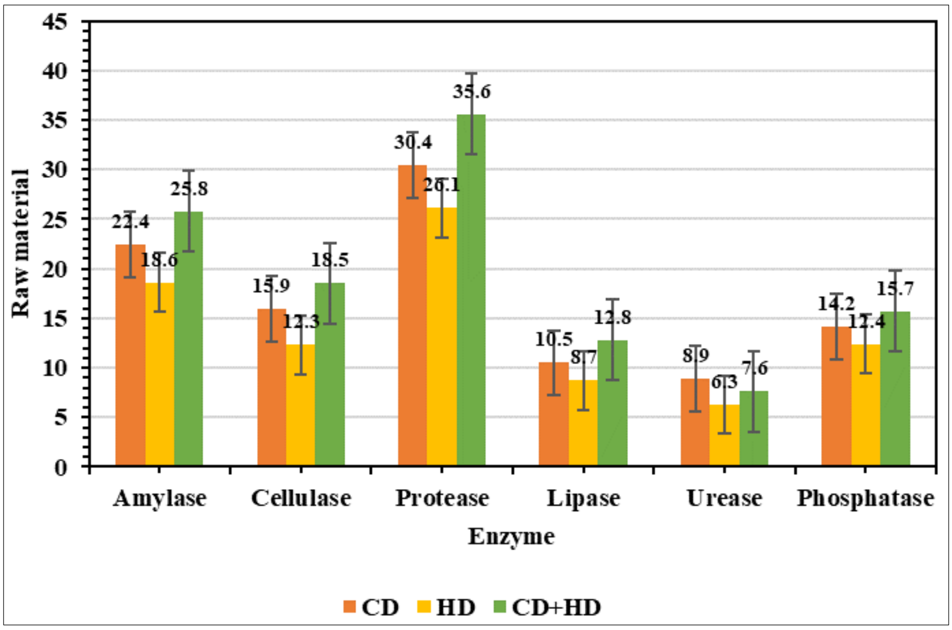

Fig 1: Enzyme activity in *Perionyx excavates* treated with different Vermibed substrates

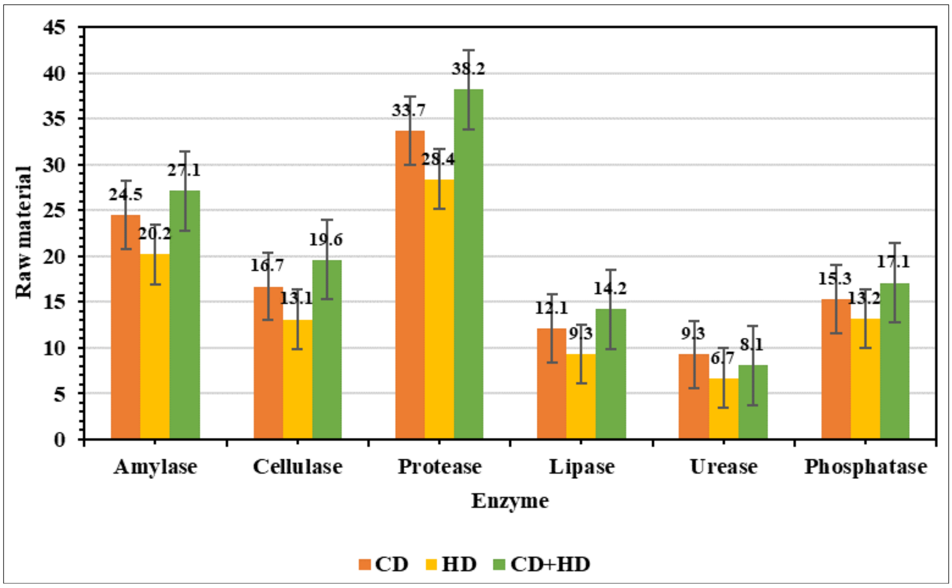

Fig 2: Enzyme activity in *Eudrilus eugeniae* treated with different Vermibed substrates

Discussion

The gut enzymatic activities of *Perionyx excavatus* and *Eudrilus eugeniae* varied significantly across different vermibed substrates (cow dung, horse dung, and cow-horse dung mixture). The results highlight the influence of substrate composition on enzyme activity, with mixed dung substrates enhancing enzymatic efficiency [14]. The findings align with previous studies emphasizing the role of organic matter composition in modulating microbial and enzymatic activity in earthworm gut environments [29, 30].

Amylase activity, crucial for starch hydrolysis, was highest in the cow-horse dung mixture for both species, indicating enhanced microbial interactions within the mixed substrate [31]. The presence of diverse carbohydrate sources in mixed dung likely promoted microbial communities that produce amylolytic enzymes, contributing to efficient organic matter decomposition [32]. Similarly, cellulase activity, essential for cellulose breakdown, showed maximum levels in the cow-horse dung mixture, confirming that fibrous material decomposition is enhanced with balanced nutrient availability [20].

Protease activity followed a similar pattern, with the cow-horse mixture exhibiting the highest values. The role of proteases in protein degradation is critical for nutrient recycling, and the findings indicate that substrate diversity supports microbial enzymatic secretions involved in protein metabolism [33]. Lipase activity, which facilitates lipid hydrolysis, was also highest in the cow-horse mixture, supporting previous research that highlights the importance of substrate richness in stimulating lipid metabolism enzymes [23]. The study further confirmed that urease activity peaked in cow dung due to its high nitrogen content, while phosphatase activity was highest in the cow-horse mixture, suggesting improved phosphorus mineralization in mixed substrates [34].

The comparison between *Perionyx excavatus* and *Eudrilus eugeniae* demonstrated that *Eudrilus eugeniae* exhibited consistently higher enzymatic activity, which may be attributed to its larger gut size, higher metabolic rate, and enhanced microbial symbiosis [30]. These findings suggest that *Eudrilus eugeniae* is a more efficient candidate for vermicomposting applications, as its digestive physiology supports superior organic matter decomposition [29].

The practical implications of these results indicate that using mixed substrates in vermiculture can significantly enhance composting efficiency. Higher enzymatic activity accelerates organic matter breakdown, leading to improved nutrient mineralization and compost quality [31]. This is in line with studies that emphasize the need for optimized vermicomposting strategies to improve soil health and nutrient availability in sustainable agriculture [23, 28]. The study also highlights the ecological relevance of substrate selection in vermiculture, as enhanced enzyme activities in mixed substrates directly contribute to improved soil fertility and organic waste decomposition efficiency [29, 33].

The study revealed that the cow-horse dung mixture consistently promoted the highest enzymatic activity in both *Perionyx excavatus* and *Eudrilus eugeniae*, highlighting its superior substrate composition for vermicomposting. Cow dung supported moderate enzyme activity, whereas horse dung exhibited the lowest levels across all cases. Among the two species, *Eudrilus eugeniae* demonstrated higher enzymatic efficiency across all substrates compared to *Perionyx excavatus*, indicating its greater metabolic potential. Among the different enzymes analyzed, protease and amylase exhibited the highest activity levels, underscoring their crucial roles in organic matter decomposition. In contrast, lipase and urease displayed relatively lower activity, reflecting their secondary roles in substrate breakdown. These findings emphasize the influence of substrate composition and earthworm species on gut enzymatic activity, contributing to a better understanding of their roles in organic waste degradation and nutrient recycling.

Conclusion

The findings of this study demonstrate that gut enzymatic activities in *Perionyx excavatus* and *Eudrilus eugeniae* are significantly influenced by the type of vermibed substrate used. The cow-horse dung mixture consistently supported the highest enzymatic activities, highlighting its potential as an optimal substrate for vermicomposting. Enzymes such as amylase, cellulase, protease, and lipase exhibited maximum activity in the cow-horse mixture, indicating that a balanced nutrient composition enhances microbial interactions and enzymatic efficiency. Urease activity peaked in cow dung due to its high nitrogen content, while phosphatase was most active in the cow-horse mixture, suggesting improved phosphorus mineralization in mixed substrates. The overall trend suggests that *Eudrilus eugeniae* is a more efficient species for vermicomposting applications. This study provides valuable insights into the enzymatic mechanisms of earthworms and their role in efficient organic matter decomposition, reinforcing the ecological and agricultural benefits of vermicomposting.

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