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Biocontrol Potential of Neem Leaf-Based Vermicompost as Indicated by Chitinase, Protease and β-1,3-Glucanase Activity

(Potensi Biokawalan Vermikompos Berasaskan Daun Semambu seperti yang Ditunjukkan oleh Aktiviti Kitinase, Protease dan β-1,3-Glucanase)

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ABSTRACT

The rising concern regarding the negative impact of synthetic pesticides has led to the search for alternative means of pest control. Vermicomposting the mixture of oil palm empty fruit bunch and neem (Azadirachta indica) *leaves, with the latter known to have pesticidal value, is therefore of great interest and significance to be studied. The present study was conducted to evaluate the chitinase, protease and β-1,3-glucanase activity of neem leaf-based vermicompost as an indication of its biocontrol properties. The total microbial population of different composition of the vermicompost was also investigated. The results showed that at 10% neem composition, an increment in microbial population, chitinase and protease activities was observed in the end product. A higher concentration of neem exerted a suppressive effect on the microbial population as well as enzymatic activity. This study suggested that the addition of an appropriate composition of neem leaves as one of the raw materials for vermicomposting would potentially enhance the performance of vermicompost as biofertilizer as well as biopesticide.*

Keywords: Biopesticide; chitinase; neem leaf; protease; β-1,3-glucanase

ABSTRAK

Kebimbangan yang semakin meningkat mengenai kesan negatif racun perosak sintetik telah menyebabkan pencarian kaedah alternatif kawalan perosak. Oleh itu, pengkomposan campuran tandan buah kosong kelapa sawit dan daun semambu (Azadirachta indica) *yang diketahui mempunyai nilai racun perosak telah menarik perhatian dan lebih bermakna untuk dikaji. Kajian ini dilakukan untuk menilai aktiviti kitinase, protease dan β-1,3-glukanase vermikompos yang berasaskan daun semambu sebagai petunjuk sifat biokawalannya. Jumlah populasi mikroorganisma bagi vermikompos yang berbeza daripada segi komposisinya juga telah dikaji. Hasil kajian menunjukkan peningkatan populasi mikroorganisma, aktiviti kitinase dan protease pada produk akhir yang mempunyai 10% daun semambu. Kepekatan semambu yang lebih tinggi memberi kesan penindasan terhadap populasi mikroorganisma dan juga aktiviti enzim. Kajian ini mencadangkan bahawa penambahan komposisi daun semambu yang sesuai sebagai salah satu bahan mentah untuk pengkomposan berpotensi meningkatkan prestasi vermikompos sebagai baja dan racun perosak biologi.*

Kata kunci: Daun semambu; kitinase; protease; racun perosak biologi; β-1,3-glukanase

INTRODUCTION

The rising concern regarding the negative impact of synthetic pesticides has led to an urgent need for a more organic way of pest control. It has been reported that the application of organic matter to soils can reduce arthropod pests' population. Farmers have indicated that plants grown with organic fertilizers or pesticides are more resistant to diseases and pests (Yardim & Edwards 2003). Vermicompost is a nutrient rich biofertilizer obtained in organic matter recycling. It is well known for its rich content of available nutrients, such as nitrates, phosphorus, potassium, calcium and magnesium that is readily absorbed by plants (Mistry et al. 2015). Vermicompost is also rich in beneficial microorganisms and soil enzymes. The action of microorganisms and enzymes is essential for the sustainable release of nutrients, thereby reducing the

negative impacts of synthetic soil amendments to the environment (Uz & Tavali 2014).

According to Pathma and Sakthivel (2012), vermicompost can also be used as biopesticides in suppressing plant pathogens and plant parasitic nematodes. The effects of vermicompost on the suppression of soil-borne diseases are likely due to both biological and chemical factors. Biological factors are mainly attributed to the action of the microbial community inhabiting the composts. The combined action of earthworms and microorganisms favor the increase in microbial population density and biodiversity, and subsequently the enzymatic activities in vermicompost (Macci et al. 2010). During the entire process of vermicomposting, different types of hydrolytic enzymes which aid in the biodegradation of the organic wastes are produced. The microorganisms cast from earthworm's gut can digest a broad range of organic materials, including cellulose, chitin, lignin and polylactic acids (Vivas et al. 2009).

Hydrolytic enzymes that are capable of hydrolyzing fungal cell wall components are known to possess mycoparasitism, in which some examples of important mycoparasitic enzymes are protease, chitinase and β-1,3-glucanase (Jadhav & Sayyed 2016). Fungal cell wall is composed of polymers, such as mannans, glucans and chitin. It is an important barrier protecting the microorganism from the attack of other microorganisms or against the harsh environment. Some well-known biocontrol agents digest phytopathogen cell wall by excreting hydrolases, such as protease, cellulase, β-1,3-glucanase and chitinase (Jadhav & Sayyed 2016). Therefore, utilizing materials known to possess pesticidal value as part of the vermicomposting material on top of the antifungal hydrolytic enzymes present in vermicompost could potentially enhance its function as biofertilizer-cum-biopesticide.

Neem tree *(Azadirachta indica)* belongs to the family of Meliaceae. These trees can be found in subtropical regions and are native to countries like India and Asia. Neem can serve as an excellent fertilizer because it is nutrient rich (Lokanadhan et al. 2012). Previous work showed that the addition of neem leaves as one of the raw materials in vermicomposting enriches the nutrient, such as nitrogen, phosphorus, potassium and calcium content of the end product (Loh et al. 2012). Parts of the neem tree, such as seeds and leaves, contain an active ingredient known as azadirachtin, which has insecticidal, fungicidal, bactericidal, and nematicidal properties (Chaudhary et al. 2017; Gajalakshmi & Abbasi 2004; Gopal et al. 2007). To date, there are limited studies exploring neem leaves as a substrate for vermicomposting despite its nutritional value due to the fact that it might kill the earthworms.

In addition to that, the negative impacts of synthetic fertilizers and pesticides have shifted the interests of researchers towards organic amendments like vermicompost as biofertilizer and biocontrol agents without polluting the environment. To bridge this knowledge gap, this study was conducted to analyze chitinase, protease and β-1,3-glucanase activities as well as to determine the microbial population in neem leaf-based vermicompost.

Materials and Methods

Earthworms and waste materials

Adult *Eudrilus eugeniae* of about the same size (about 1 g) were randomly selected from the vermibed of the Vermiculture Laboratory, Department of Biology, Universiti Putra Malaysia (UPM). Empty fruit bunch was collected from a palm oil mill (Dengkil, Selangor). Neem leaves were collected from UPM campus and was identified by Dr. Mohd. Firdaus Ismail at the Biodiversity Unit, Institute of Bioscience, UPM and a voucher specimen (SK3139/17) was deposited in the herbarium of that institute. Cow manure was collected from Taman Pertanian Universiti, UPM. The neem leaves, empty fruit bunch and cow manure were air dried, ground into small particles and sieved through a 2 mm sieve.

Experimental setup

Round plastic containers (3 L) were used in this experiment. A total of 250 g (dry weight) of raw materials were thoroughly mixed to produce different ratios of vermibeds (Table 1). All mixtures were subjected to 20% cow dung as food supplement to the worms. This is to ensure that vermicomposting is conducted under ambient conditions, as cow dung is needed for optimum worm growth and is essential for microbial decomposition of organic wastes (Palta & Bhatnagar 2007). The substrates were incubated for four weeks at 80% moisture content by periodic sprinkling of distilled water for thermal stabilization, and were turned occasionally to allow aeration. Approximately 6.25 g of adult *Eudrilus eugeniae* was then inoculated into each container. The vermibins were covered with a net and placed in a laboratory with a temperature of 25-27 °C. The substrates were vermicomposted for 60 days. For each treatment, control treatment (compost without worms) was also prepared in a similar setup (n = 4). After 2 months, watering was stopped. Fresh and moist vermicomposts and composts were sampled randomly for enzymatic and microbiological analyses.

TABLE 1. Composition of waste materials used in vermibeds

Treatments	Descriptions
VC1	40% neem leaves : 40% empty fruit bunch : 20% cow dung + earthworms
C1	40% neem leaves : 40% empty fruit bunch : 20% cow dung
VC2	20% neem leaves : 60% empty fruit bunch : 20% cow dung + earthworms
C2	20% neem leaves : 60% empty fruit bunch : 20% cow dung
VC3	10% neem leaves : 70% empty fruit bunch : 20% cow dung + earthworms
C3	10% neem leaves : 70% empty fruit bunch : 20% cow dung
VC4	80% empty fruit bunch : 20% cow dung + earthworms
C4	80% empty fruit bunch : 20% cow dung
VC5	100% empty fruit bunch + earthworms
C5	100% empty fruit bunch

VC: Vermicomposting, C: control

CHITINASE ACTIVITY ANALYSIS

Chitinase activity was determined by using fluorogenic substrate, 4-methylumbeliferyl-*N*-acetyl-β-d-glucosaminide (Sigma), which measures 4-methylumbeliferone (4MU) released because of hydrolysis (Poulsen et al. 2008). The vermicomposts/composts (20 mg/mL) were suspended in 50 mM Tris-malate buffer (pH 5) and vortexed for 10 s. The assay was carried out in replication of four for each treatment. The chitinase activity was measured using a fluorometer (LS 55 Fluorescence Spectrometer, Perkin Elmer) at 377 nm excitation and 446 nm emission. Chitinase activity was determined from a standard curve obtained from 4MU and was expressed as nmole 4MU liberated per g (dry weight) of sample per hour.

PROTEASE ACTIVITY ANALYSIS

Casein (Sigma) was used for the quantification of protease activity (Ladd & Butler 1972). Protease activity was measured at 700 nm using a spectrophotometer (U-1900 Spectrophotometer, Hitachi). The assay was replicated four times and the activity was determined from a standard curve obtained from tyrosine (Merck). Protease activity was expressed as µmole tyrosine liberated per gram (dry weight) of sample per hour.

B-1,3-GLUCANASE ACTIVITY ANALYSIS

β-1,3-glucanase activity was determined using the modified method of Chae et al. (2006), which measures glucose released as the result of laminarin (Sigma) digestion (n = 4). β-1,3-glucanase activity was measured at 550 nm using a spectrophotometer (U-1900 Spectrophotometer, Hitachi) and calculated from a glucose (Aldrich) standard curve. The activity was defined as µmole glucose liberated per gram (dry weight) of sample per hour.

MICROBIAL POPULATION DENSITY DETERMINATION

Drop plate method (Herigstad et al. 2001) was used to determine the microbial population density. Serial dilutions (10^{-1} - 10^{-7}) of the sample suspension were prepared. Dilutions 10^{-2} - 10^{-5} were used for the estimation of fungi population, dilutions 10^{-3} - 10^{-6} were used for the estimation of actinomycetes' population, and dilutions 10^{-4} - 10^{-7} were used for the estimation of bacteria population. Rose Bengal agar (RBA), Nutrient agar (NA) and Actinomycete agar (AA) were used for isolating fungi, bacteria, and actinomycetes, respectively. For drop plating, 10 µL of dilutions were pipetted and expelled onto the plate immediately after vortex. All plates were incubated at room temperature (25-27 °C) for 1-2 days for bacteria, and 5-7 days for fungi and actinomycetes. Viable cell counts are expressed as Log_{10} colony forming unit (cfu) per g of dry compost.

STATISTICAL ANALYSIS

For statistical analysis, SPSS (Statistical Package for Social Sciences), version 17.0 was used. The data obtained was analyzed using One-way Analysis of Variance (ANOVA), Duncan post hoc test and Pearson's correlation at significance level of 0.05.

RESULTS AND DISCUSSION

The present study showed that the addition of 10 and 20% neem enhanced the total microbial population density, but the addition of 40% neem significantly suppressed the microbial population in both VC1 and C1 (Table 2). Previous studies have reported the anti-microbial and anti-fungal properties of azadirachtin in neem (Coventry & Allan 2001; Govindachari et al. 2000). Therefore, the addition of neem leaves at a certain level might suppress the microorganisms or fungi in vermicompost. A study by Gopal et al. (2007) suggested that neem seed kernel extract reduces bacterial and fungal populations significantly, in which azadiractin being the main active ingredient of the extract.

However, the consistent increase in bacteria, actinomycetes and fungal population densities observed in the vermicompost with 10% neem leaf indicated that neem leaf, at 10% composition, is palatable to earthworms and favorable for microorganisms' growth. Neem leaf, which is well known as a powerful nematicide, has been shown to increase the growth and reproduction of *Eudrilus eugineae* (Gajalakshmi & Abbasi 2004). The action of earthworm could be the factor that contributes to the higher bacteria, actinomycetes and fungi population density demonstrated in T2 and T3 vermicompost in the present study. This may be due to neem leaf is nutrient rich (Lokanadhan et al. 2012), thereby increasing the microbial activity in earthworm gut and its vermicasts despite its antimicrobial activity. In addition to that, earthworm guts maintain a favorable condition for microorganisms, resulting in greater microbial activity, plant growth factors and pest repellants in the vermicasts produced. Bacteria that help in food digestion are found in the foregut, actinomycetes that are antagonistic against pathogens are found in the midgut, and fungi that help in the formation of vermicast are found in the hindgut (Kiyasudeen et al. 2014).

TABLE 2. Bacteria, actinomycete, fungi and total microbial population density (Log_{10} $cfug^{-1}$) in vermicompost and control of different treatments. Values are mean ± standard error of the mean (n = 4)

	Bacteria		Actinomycetes		Fungi		Total	
	VC	C	VC	C	VC	C	VC	C
T1	0.00 ± 0.00^{Bd}	7.58 ± 0.09^{Ab}	8.21 ± 0.05^{Aab}	7.53 ± 0.04^{Bab}	5.96 ± 0.03^{Ac}	5.94 ± 0.03^{Ac}	14.17 ± 0.04^{Bd}	21.05 ± 0.10^{Ab}
T2	8.84 ± 0.02^{Aa}	7.67 ± 0.14^{Bb}	8.21 ± 0.05^{Aab}	7.59 ± 0.04^{Bab}	6.53 ± 0.05^{Aa}	6.37 ± 0.02^{Ba}	23.59 ± 0.03^{Aa}	21.62 ± 0.14^{Bab}
T3	8.62 ± 0.06^{Aa}	7.79 ± 0.12^{Bb}	8.25 ± 0.02^{Aa}	7.61 ± 0.01^{Bab}	6.42 ± 0.05^{Aa}	6.20 ± 0.06^{Bb}	23.29 ± 0.11^{Aa}	21.60 ± 0.12^{Bab}
T4	7.55 ± 0.12^{Ac}	7.64 ± 0.09^{Ab}	7.97 ± 0.03^{Ab}	7.52 ± 0.03^{Bb}	5.85 ± 0.07^{Bc}	6.10 ± 0.06^{Ab}	21.37 ± 0.10^{Ac}	21.26 ± 0.07^{Ab}
T5	8.40 ± 0.02^{Ab}	8.11 ± 0.04^{Ba}	8.06 ± 0.13^{Aab}	7.75 ± 0.03^{Aa}	6.16 ± 0.04^{Ab}	6.24 ± 0.02^{Aab}	22.62 ± 0.10^{Ab}	22.10 ± 0.02^{Aa}

T1: 40%N:40%EFB:20%CD, T2: 20%N:60%EFB:20%CD, T3: 10%N:70%EFB:20%CD, T4: 80%EFB:20%CD, T5: 100%EFB, VC: vermicompost, C: control. Means with different uppercase letter (horizontal comparison between VC and C) and different lowercase letter (vertical comparison) showed significant difference ($p < 0.05$)

The chitinase activity was higher in vermicompost (VC) than the control (C), except in VC5 (Figure 1). In vermicompost, VC3 (10% neem) showed the highest chitinase activity, followed by VC4 (0% neem, 80% EFB: 20% CD), VC2 (20% neem) and VC1 (40% neem). The higher chitinase activity in the vermicompost was most probably contributed by the chitinolytic activity of actinomycetes present in the earthworm gut and vermicompost. This is supported by the positive relationship ($r = 0.571$, $p < 0.01$) between the chitinase activity and the actinomycetes' population density demonstrated in this study. According to Yasir et al. (2009), chitinase gene diversity is increased by the action of earthworm *via* the enrichment of chitinolytic bacteria in vermicompost. In general, earthworm uses fungi as a food source. Since the cell wall of fungi is made of chitin, the feeding activity of earthworms and their excretion of casts are enriched with chitin-decomposing microorganisms and enzymes. This subsequently contributes to the higher chitinase activities in vermicompost (Devi et al. 2009).

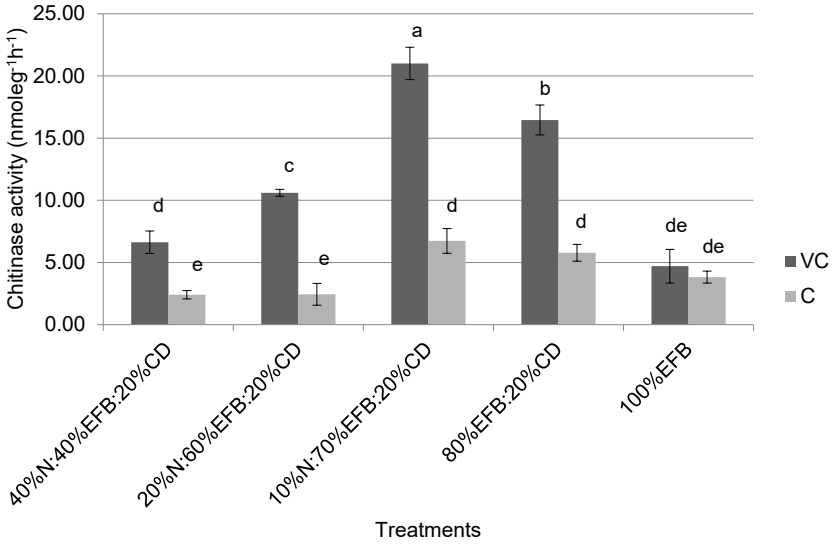

FIGURE 1. Chitinase activity in vermicompost (VC) and control (C) of different treatments. Values are mean ± standard error of the mean. Different letter showed significant difference ($p < 0.05$), $n = 4$

In contrast, protease activity was lower in VC than C (Figure 2). The protease activity was highest in C3 (10% neem), followed by C4 (0% neem, 80% EFB: 20% CD) and C2 (20% neem), and the lowest activity was in C1 (40% neem) and C5 (0% neem, 100% EFB), respectively. The low protease activity in vermicompost might be caused by the depletion of protein content during vermicomposting. According to Aira et al. (2006), earthworms exploit the organic carbon and nitrogen pools in vermicompost during vermicomposting. The depletion of available organic substrates subsequently decreases the enzyme synthesis by microorganisms (Garcia et al. 1994). Our result is in agreement with Lazcano et al. (2008), in which the earthworm-free compost has higher protease activity than the treatments with earthworms. The low protease activity in vermicompost could be attributed to the dependency of enzyme activity on substrate availability. A study by Devi et al. (2009) demonstrated that casein-hydrolysing protease activity declines sharply as protein content decreases in the ageing substrate.

Additionally, protease activity has a positive relationship with microbial population density ($r = 0.321$,

$p < 0.05$). Though prematurely, the result suggested that microorganisms might play a part in affecting the patterns of protease activity. The result of this study corroborates the finding by Benitez et al. (2002), in which significant correlations between protease activity and microbial population density were observed. Together with enzyme activity decline, the progressive deficient in substrate also reduces the microbial population density, which affects the synthesis of new enzymes. This explains the dependency of the microbial population and protease activity on substrate availability.

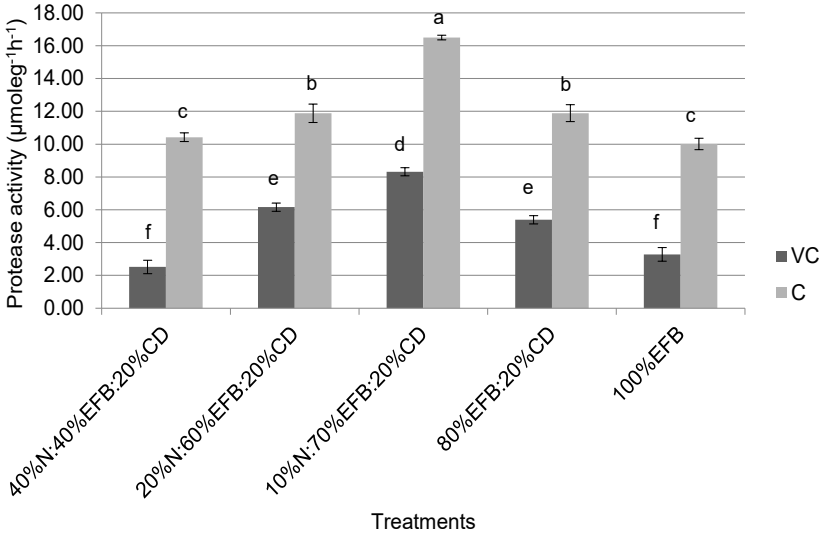

FIGURE 2. Protease activity in vermicompost (VC) and control (C) of different treatments. Values are mean ± standard error of the mean. Different letter showed significant difference ($p < 0.05$), n = 4

From the results, β-1,3-glucanase activity did not show any clear pattern as the case for chitinase and protease (Figure 3). Our results demonstrated that VC1 and VC5 possessed higher β-1,3-glucanase activity than that of their controls, whereas VC2, VC3 and VC4 were not significantly different from their controls. This indicated that the active ingredient in neem leaf might not have any significant effect on β-1,3-glucanase activity, as no clear pattern was recorded for this enzyme. To understand the activity of β-1,3-glucanase enzyme, its relationship with other enzymes was analyzed by conducting Pearson correlation analysis. The results showed that the β-1,3-glucanase activity has a negative relationship with protease activity ($r = - 0.468$, $p < 0.01$). The negative correlation suggested that the former was probably suppressed by the latter. According to Pitson et al. (1996), the presence of protease inactivates one of the three extracellular β-1,3-glucanase activities produced by a filamentous fungus *Acremonium persicinum* at neutral or alkaline medium pH. The pH of vermicompost usually falls in the range of neutral to alkaline (Padmavathiamma et al. 2008), and the pH range for protease activity was observed at 7.0 to 9.2 with an optimum pH7.8 (Akel et al. 2009). This phenomenon could contribute to an increase in protease activity, which in turn inactivates the β-1,3-glucanase produced by the microorganisms.

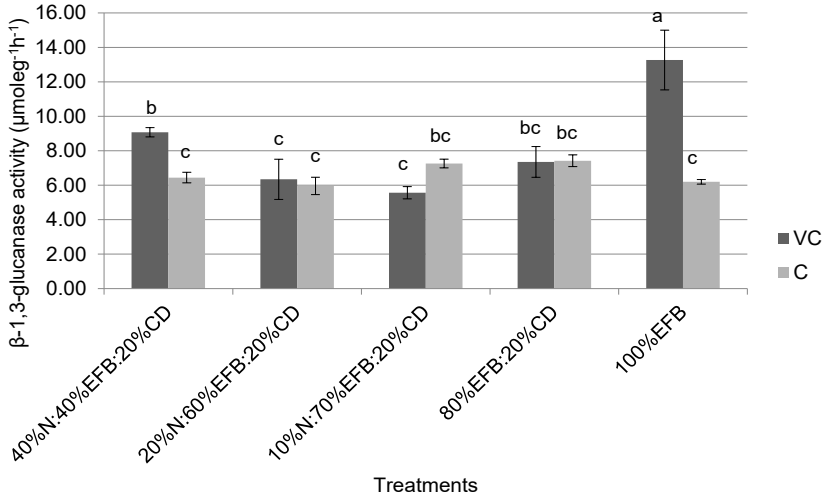

FIGURE 3. β-1,3-glucanase activity in vermicompost (VC) and control (C) of different treatments. Values are mean ± standard error of the mean. Different letter showed significant difference ($p < 0.05$), n = 4

The present study showed that treatment with 10% neem resulted in the highest chitinase and protease activity in both vermicompost and control. However, a decrease in enzyme activity was observed at higher neem composition. The pattern of enzyme activity coincides with the microbial population density. This indicated that neem composition plays a major role in determining the microbial population density, and subsequently the enzymatic activity of the vermicompost. In this context, the active ingredients introduced by the addition of neem affects the biocontrol properties of the vermicompost indirectly *via* the action of earthworms. The findings suggested that there is a threshold level (about 20% neem) at which the microorganisms can withstand the bioactivity of compounds introduced by the addition of neem leaves, with 10% neem being the most suitable composition for the enhancement of microbial and enzyme activity in vermicompost. According to Gopal et al. (2007), the application of azadirachtin granules into soil at a recommended dose has an enhancing effect on the microbial population density and enzyme activity, whereas higher doses would give a negative impact. The reason could be that the enhanced microbial population subsequently contributed to the increase in the enzymes' activity in vermicompost.

Conclusion

Our study suggested that the addition of an appropriate composition (10%) of neem leaf as one of the raw materials in vermicompost would enhance the microbial population and increase the chitinase and protease activities, which are important fungus-controlling enzymes. Thus, further study on the biopesticide value of the neem leaf-based vermicompost is worth being carried out by conducting bioassays on certain insect pests of economic importance or by identifying the bioactive compounds present in the vermicompost.

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