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Isolation and characterization of thermophilic fungi from deep layers of compost during the pre-decomposition phase

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Abstract

Compost is a product of the fermentation of organic materials derived from plant leaves, vegetables, fruits, organic waste, and animal and livestock manure. Composting is a decomposition process carried out by decomposer microorganisms. Thermophilic fungi play a crucial role in the decomposition of organic materials at high temperatures, making it essential to study their diversity and characteristics in organic waste management. This study aims to isolate and characterize thermophilic fungi present in the pre-decomposition phase at the Compost Plant of PT. Great Giant Pineapple. The study employs an exploratory descriptive method, including sample collection, homogenization, serial dilution, isolation, purification, macroscopic, microscopic, and physiological characterization, as well as enzymatic index determination. The results of this study yielded six thermophilic fungi isolates capable of producing cellulase enzymes in isolates BIO-PK 1, BIO-PK 2, and BIO-PK 5; protease enzymes in BIO-PK 1 and BIO-PK 2; and ligninase enzymes in BIO-PK 4. Enzyme testing results showed 3 fungi positive for cellulase, 2 fungi positive for protease, and 1 fungus positive for ligninase. The highest enzymatic index for each enzyme was: cellulase 3.73 (BIO-PK 2), protease 0.77 (BIO-PK 5), and ligninase scoring 1 (BIO-PK 4). The isolation and characterization results obtained will then be analyzed and described descriptively, quantitatively, and qualitatively.

Keywords: Thermophilic Fungi; Compost; Composting; Physiological Activity

1. Introduction

Composting is the microbial decomposition of organic matter into a stable product known as compost [22;3]. This process transforms non-hazardous organic materials into soil-like substances through enzymatic activity, which requires a balanced C/N ratio, adequate moisture, optimal pH, and sufficient oxygen levels [6;32]. Enzymes secreted by microorganisms break down compounds such as carbohydrates, proteins, lipids, and lignin, highlighting the vital role of microbes in composting [17]. Composting occurs in two main phases: pre-decomposition and decomposition. The pre-decomposition phase initiates the transformation of organic matter, while the decomposition phase involves active microbial breakdown by fungi, bacteria, actinomycetes, and others [25].

The process includes four temperature-based stages: mesophilic (10–42 °C), thermophilic (45–70 °C), cooling (65–50 °C), and maturation (50–23 °C) [36]. Thermophilic microbes degrade complex compounds such as proteins and cellulose. As these compounds are depleted, the temperature drops, marking the transition to the maturation phase, dominated by fungi capable of surviving harsh conditions [21;1]. Despite the key role of thermophilic fungi in composting, studies on their isolation and characterization remain limited. Therefore, this research focuses on thermophilic fungi isolated from 1-meter depth compost in the pre-decomposition phase at PT. Great Giant Pineapple.

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2. Tools and Materials

This study used the following equipment: test tubes, Petri dishes, beakers, measuring cups, 250-ml Erlenmeyer flasks, micropipettes, Drigalski pipettes, round-tipped needles, stirring rods, a pH meter, an object glass, a cover glass, dropper pipette, a vortex mixer, an incubator, an autoclave, a hot plate, an analytical balance, a biological safety cabinet, a microscope, a spoon, an icebox, sterile plastic, matches, scissors, gloves, and sterile bottles.

The materials used were pre-decomposition phase compost samples obtained from the Compost Plant PT at a depth of 1 meter. GGP; distilled water; chloramphenicol; skim milk agar; PDA; peptone; yeast extract; guaiacol; Czapek agar dox; carboxymethyl cellulose (CMC); Congo red 0.1%; 1 M NaCl; olive oil; methyl red; lactophenol cotton blue; a spirit lamp; 70% alcohol; 96% alcohol; filter paper; tissue; aluminum foil; and heat-resistant plastic.

2.1. Serial Dilution

Serial dilution was performed aseptically in a Biological Safety Cabinet (BSC). A 25 g sample of compost was weighed and placed in a sterile Erlenmeyer flask containing 225 mL of 0.9% NaCl, then homogenized to obtain a 10-1 dilution. One milliliter of the 10-1 dilution was taken using a micropipette and added to a reaction tube containing 9 mL of 0.9% NaCl, then homogenized using a vortex to obtain a 10-2 dilution. The procedure was repeated in the same manner until a 10-10 dilution was obtained [28].

2.2. Isolation of Thermophilic Fungi

Then, isolation was carried out to obtain thermophilic fungi on PDA solid media that had been added with 1% streptomycin. The addition of streptomycin to the media was intended to protect the fungi from bacterial contamination [11]. The solidified PDA medium was then inoculated with 200 µl of compost sample suspension using the spread plate method with a Drigalski loop. The Petri dishes containing the isolates were then incubated at 30 °C for 7 days [12].

2.3. Purification of Thermophilic

Fungi Seven-day-old fungi isolates were purified by reinoculating each colony onto fresh PDA medium. One colony was inoculated onto fresh PDA medium using the spot method and then incubated again in an incubator at 30°C for 3 days [10]. The purpose of purification is to obtain pure cultures from each isolate. A pure culture is a culture consisting of cells that are identical in shape and size [7].

2.4. Morphological Characterization Macroscopic of Thermophilic Fungi

Macroscopic characterization was performed to determine the color of the colony, colony size, and hyphae observed in each culture in the purified petri dish [31]. Macroscopic observations were performed using a dissecting microscope.

2.5. Morphological Microscopic Characterization of Thermophilic Fungi

Fungi colonies also need to be observed microscopically by making slide cultures. The purpose of making slide cultures is to observe the microscopic morphology of fungi such as hyphae, sporangia, and conidia, and to identify them based on the observed fungi cell structure [32]. When preparing a culture slide, it is important to maintain the sterility of the tools and materials. The culture slide is prepared by growing fungi on a PDA medium cut into approximately 1 x 1 cm squares, placed on an object glass, and covered with a cover glass. When placing the object glass, a micro tip is used as a support and placed inside a petri dish.

2.6. Physiological Characterization (Enzymatic Activity Test)

2.6.1. Cellulase Test

Isolates were inoculated on Czapek-Dox agar medium + 1% CMC and chloramphenicol, pH 6.8, and incubated at 30°C for 5 days. Cellulase activity was indicated by a clear zone after Congo red staining and rinsing with 1 M NaCl [27].

2.6.2. Protease Test

The isolates were inoculated onto skim milk agar medium (pH 7), and incubated at 50°C for 5 days, and the clear zones formed were observed [16].

2.6.3. Ligninase Test

Isolates were inoculated onto Boyd and Kohlmeyer medium (containing guaiacol), pH 7, incubated at 30°C for 7 days in the dark. A positive reaction was indicated by the formation of a brown color around the colony [13].

2.7. Determination of Enzymatic Index

The physiological activity of protease, amylase, and cellulase enzymes is determined by calculating the colony area and the clear zone area formed using the gravimetric method [29]. The gravimetric method can be calculated as follows:

- Using colony patterns and clear zones (replicas) drawn on clear plastic mica
- Colony replicas and clear zones were weighed using an analytical balance
- Cut a 1 cm x 1 cm piece of paper and weigh it
- Calculate the area of the colony and clear zone using the following formula.

$$\text{Colony Area} = \frac{\textbf{Weight of colony replica}}{\textbf{Paper weight 1 cm x 1 cm}} + 1cm^2$$

$$\text{Clear Zone Area} = \frac{\textbf{Weight of clear zone replica}}{\textbf{Paper weight 1 cm x 1 cm}} + 1cm^2$$

- The calculation results are then entered into the enzymatic index formula:

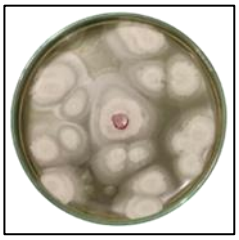
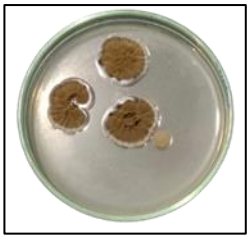
$$\text{Enzymatic Index} = \frac{\textbf{Clear zone area} - \textbf{Colony size}}{\textbf{Colony size}}$$

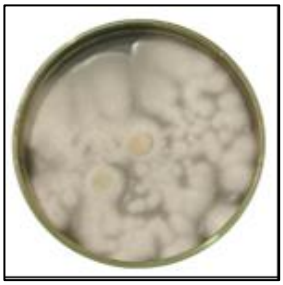
3. Results and discussion

3.1. Isolation and Macroscopic Characterization of Thermophilic Fungi

The results of macroscopic observations of thermophilic fungi in Table 1 yielded six fungi isolates with the codes BIO-PK 1, BIO-PK 2, BIO-PK 3, BIO-PK 4, BIO-PK 5, and BIO-PK 6. These six fungi have different macroscopic characteristics. The macroscopic characteristics observed include the color and shape of the fungi. Among the six isolates, two isolates, BIO-PK 1 and BIO-PK 3, have the same macroscopic characteristics.

Table 1 Macroscopic thermophilic fungi obtained from a depth of 1 meter in the pre-decomposition phase

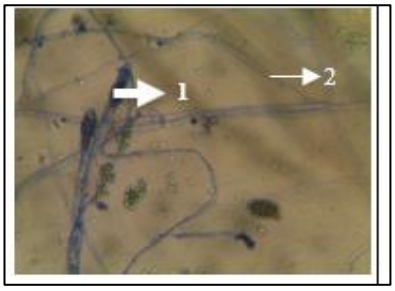
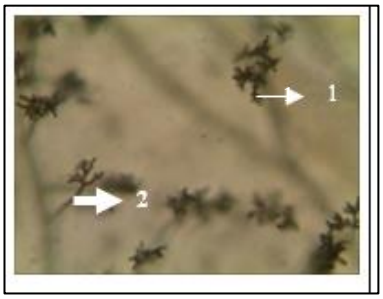
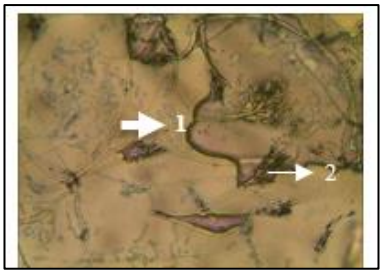
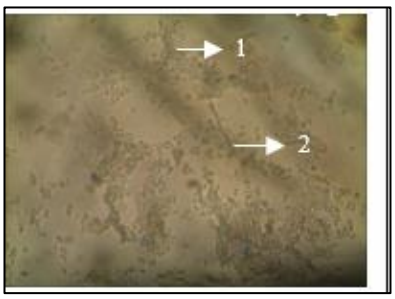
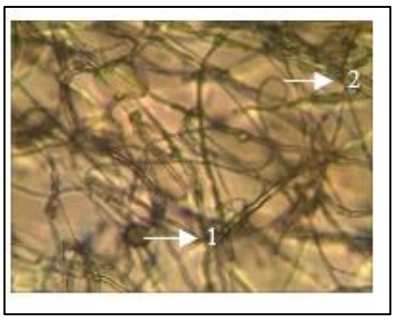
Code	Image	Description
BIO-PK 1		Isolate BIO-PK 1 is white in color, with grayish-green edges around the colony. Isolate BIO-PK 1 has a velvety, wool-like appearance and a cotton-like texture [4].
BIO-PK 2		Isolate BIO-PK 2 is brown in color, with gray edges and a faint green color. Isolate BIO-PK 2 has a compartmentalized texture in each colony, and isolate BIO-PK 2 tends to grow in agar [15].

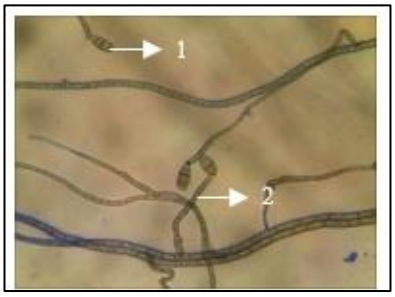
BIO-PK 3		Isolate BIO-PK 3 is white in color with faded gray edges around the colony. Isolate BIO-PK 3 has a velvet-like appearance, similar to wool, and a texture resembling cotton [4].
BIO-PK 4		Isolate BIO-PK 4 has a yellowish-green color and gray edges around its colony. Isolate BIO-PK 4 has a velvety appearance and a dust-like texture.
BIO-PK 5		Isolate BIO-PK 5 has a white color in the center and edges of the colony. Isolate BIO-PK 5 has a shape similar to fungi in tempeh, and a texture resembling cotton.
BIO-PK 6		Isolate BIO-PK 6 has a grayish-black color on the top and edges, and the center of the colony is grayish-white. Isolate BIO-PK 6 has a circular shape and a smooth, cotton-like surface texture [30].

3.2. Isolation and Microscopic Characterization of Thermophilic Fungi

Microscopic observations of thermophilic fungi in Table 2 revealed six types of fungi with different genera and microscopic characteristics. The six fungi were obtained using the slide culture method. The microscopic characteristics observed included hyphae, sporangia, and conidia in fungi using a microscope. Of the six isolates, two isolates (BIO-PK 1 and BIO-PK 3) were found to belong to the same genus, Penicillium sp. The other four isolates (BIO-PK 2, BIO-PK 4, BIO-PK 5, and BIO-PK 6) were identified as belonging to the genera *Odiodendron* sp., *Cylindrocladium* sp., *Verticillium* sp., and *Curvularia* sp.

Table 2 Microscopic thermophilic fungi obtained from a depth of 1 meter pre-decomposition phase

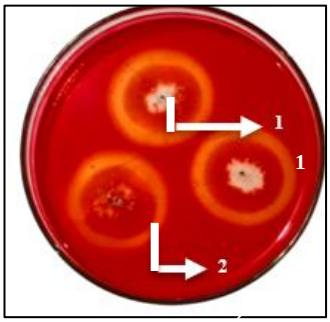
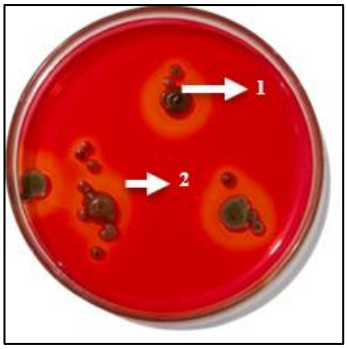
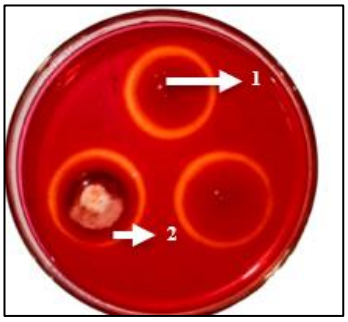
Code	Image	Information	Description	Genus
BIO-PK 1		1: conidia 2: hypha	Isolate BIO-PK 1 has septate hyphae (septate) and spore bodies called conidia. The conidia of isolate BIO-PK 1 are spherical and easily spread [4].	*Penicillium* sp. [34].
BIO-PK 2		1: conidia 2: hypha	Isolate BIO-PK 2 has coenocytic hyphae (without septa), colorless spores (conidia), single cells, and is located in a single layer on the surface of the vesicles. Isolate BIO-PK 2 is characterized by upright colorless conidia with enlarged vesicles [15].	*Odiodendron* sp. [34]
BIO-PK 3		1: conidia 2: hypha	BIO-PK 3 isolate has coenocytic hyphae (without septa) and spore bodies called conidia. The conidia of the BIO-PK 3 isolate are spherical and easily spread [4].	*Penicillium* sp. [34].
BIO-PK 4		1: spora 2: hypha	Isolate PK 4 has septate hyphae (septate) and easily dispersible spores. The conidia of isolate PK 4 are elongated and easily dispersible.	*Cylindrocladium* sp. [34].
BIO-PK 5		1: conidia 2: hypha	Isolate PK 5 has septate hyphae (septate) and spore bodies called conidia. The conidia of isolate PK 5 are spherical in shape.	*Mortierella* sp. [15].

BIO-PK 6		1: conidia 2: hypha	Isolate PK 6 has septate hyphae and pale to black conidia. The conidia of isolate PK 6 are slightly curved and elongated with three septa [34].	*Curvularia* sp. [34].

3.3. Testing Thermophilic Fungi Cellulase Enzymes

From the six thermophilic fungi isolates the results of the cellulase enzyme test are presented in Table 3. In the cellulase enzyme test, three isolates were found to be positive for cellulase enzyme (BIO-PK1, BIO-PK 2, and BIO-PK 3), indicated by the formation of a clear zone around the colony. The remaining three isolates (BIO-PK 4, BIO-PK 5, and BIO-PK 6) were negative, indicated by the absence of a clear zone around the colonies.

Table 3 Testing of thermophilic fungi cellulase enzymes obtained from a depth of 1 meter in the pre-decomposition phase

Code	Image	Information	Description
BIO-PK 1		1: isolate 2: clear zone	Isolate BIO-PK 1 showed positive results, marked by the formation of a clear zone around the colony.
BIO-PK 2		1: isolate 2: clear zone	Isolate BIO-PK 2 showed positive results, marked by the formation of a clear zone around the colony.
BIO-PK 3		1: isolate 2: clear zone	Isolate BIO-PK 3 showed positive results, marked by the formation of a clear zone around the colony.

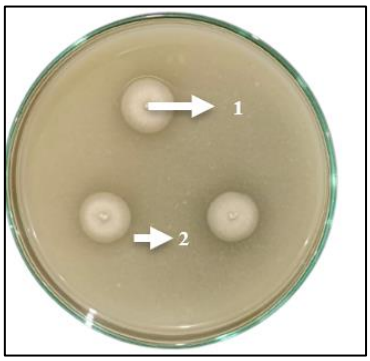
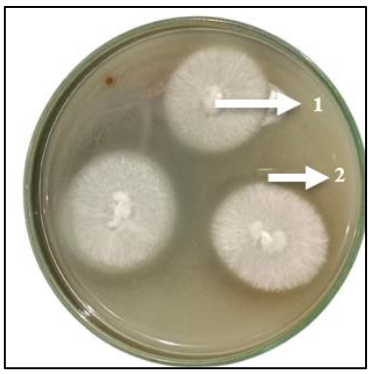
Based on the cellulase activity test results (Table 3), only isolates BIO-PK 1, BIO-PK 2, and BIO-PK 3 formed clear zones, indicating cellulase production. In contrast, BIO-PK 4, BIO-PK 5, and BIO-PK 6 showed no clear zones, suggesting an absence of cellulase activity. The enzyme activity in this study was higher, likely due to the abundance of cellulose [20]

the presence of cellulolytic microbes, and favorable environmental conditions during composting [23]. Clear zones reflect cellulase activity via β-1,4 glycosidic bond hydrolysis [2]. Congo red staining helps detect this process, as bond cleavage prevents dye interaction, forming clear zones [18]. Complete hydrolysis yields glucose, while incomplete hydrolysis forms cellobiose. Enzyme production is influenced by incubation time and pH. Reducing sugars appear on day one from CMC medium conversion but decline as biomass increases and consumes glucose. Optimal pH and temperature enhance enzymatic efficiency [20].

3.4. Thermophilic Fungi Protease Enzyme Test

Enzyme protease test results for the six thermophilic fungi isolates are shown in Table 4. The enzyme protease test identified two isolates that were positive for protease enzymes (BIO-PK 1 and BIO-PK 5), indicated by the formation of a clear zone around the colony. The remaining four isolates (BIO-PK 2, BIO-PK 3, BIO-PK 4, and BIO-PK 6) were negative, as indicated by the absence of a clear zone around the colonies.

Table 4 Testing of thermophilic fungi protease enzymes obtained from a depth of 1 meter in the pre-decomposition phase

Code	Image	Information	Description
BIO-PK 1		1: Isolate 2: Clear zone	Isolate BIO-PK 1 showed positive results, marked by the formation of a clear zone around the colony.
BIO-PK 5		1: Isolate 2: Clear zone	The BIO-PK 5 isolate showed positive results, marked by the formation of a clear zone around the colony.

Based on Table 4, only isolates BIO-PK 1 and BIO-PK 5 formed clear zones, indicating protease enzyme activity. In contrast, BIO-PK 2, BIO-PK 3, BIO-PK 4, and BIO-PK 6 showed no clear zones, suggesting the absence of protease production. The activity observed in this study was lower, possibly due to limited protein availability in compost during the pre-decomposition phase [14], which restricts microbial induction of protease production [24].

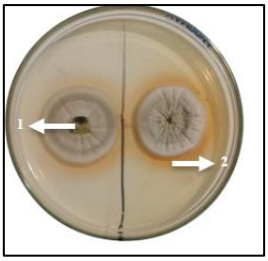
Protease enzymes catalyze the hydrolysis of peptide bonds in proteins [14]. Positive isolates are indicated by clear zones on skim milk medium, which contains proteins degraded by proteolytic microbes into soluble nitrogen compounds [8]. The size of the clear zone correlates with the level of enzyme activity. The presence of clear zones confirms proteolytic activity, where proteases hydrolyze proteins into peptides and amino acids [14]. Proteins in the skim milk medium act as inducers, and the resulting clear zones reflect the enzymatic hydrolysis of these substrates [36].

3.5. Testing Thermophilic Fungi Ligninase Enzymes

From the six thermophilic fungal isolates, the results of the ligninase enzyme test are presented in Table 5. In the ligninase enzyme test, only one isolate tested positive for ligninase enzyme (BIO-PK 4), indicated by the appearance of

a brownish color around the colony. The remaining five isolates (BIO-PK 1, BIO-PK 2, BIO-PK 3, BIO-PK 5, and BIO-PK 6) were negative, as indicated by the absence of a brownish color around the colony.

Table 5 Testing of thermophilic fungal ligninase enzymes obtained from a depth of 1 meter in the pre-decomposition phase

Code	Image	Information	Description
BIO-PK 4		1: Isolate 2: Hydrolysis zone	BIO-PK 4 isolate showed positive results, indicated by a color change around the colony.

Based on ligninase enzyme activity testing (Table 5), only isolate BIO-PK 4 showed a brown halo around the colony, indicating ligninase production. Isolates BIO-PK 1, 2, 3, 5, and 6 showed no color change, suggesting an inability to produce ligninase and degrade lignin. Ligninase activity in this study was lower [19], likely due to the limited number of ligninase-producing isolates. Lignolytic fungi are generally less abundant than carbohydrate-degrading bacteria, and compost microorganisms tend to produce more cellulase than ligninase [26].

Fungi degrade lignocellulosic materials by producing enzymes such as Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP), oxidize lignin structures. Ligninase production increases with fungal growth, as fungi utilize lignin-rich substrates as nutrient sources during their lag and active growth phases. Lignolytic fungi can reduce lignin content more efficiently due to their ability to secrete LiP, MnP, and laccase enzymes, with LiP and MnP, are considered the main agents of lignin degradation [19].

3.6. Thermophilic Fungi Enzymatic Index Value

From the six thermophilic fungal isolates, the results of the cellulase, protease, and ligninase enzymatic indices are presented in Table 6. Based on the enzymatic index values, the highest and lowest values were obtained. The cellulase enzymatic index value of the isolate with code (BIO-PK 2) had the highest value at 3.73, while the isolate with code (BIO-PK 3) had the lowest value at 0.49. The protease enzymatic index value of the isolate with code (BIO-PK 5) had the highest value with a result of 0.77, while the isolate with code (BIO-PK 1) had the lowest value with a result of 0.62. For ligninase, scoring was performed, and only one isolate with code (BIO-PK 4) was obtained with a score of 1.

Table 6 Enzymatic Index Values of Thermophilic Fungi Obtained from a Depth of 1 Meter in the Pre-decomposition Phase

Code		Enzymatic Index	
	Cellulase	Protease	Ligninase
BIO-PK 1	RLk = 4,4 RLz= 9,06 IE = 1,05	RLk = 1,76 RLz = 2,86 IE = 0,62	Skor = 0
BIO-PK 2	RLk = 0,73 RLz = 3,46 IE = 3,73	RLk = 0 RLz = 0 IE = 0	Skor = 0
BIO-PK 3	RLk = 4,6 RLz = 6,86 IE = 0,49	RLk = 0 RLz = 0 IE = 0	Skor = 0
BIO-PK 4	RLk = 0 RLz = 0	RLk = 0 RLz = 0	Skor = 1

	IE = 0	IE = 0	
BIO-PK 5	RLk = 0 RLz = 0 IE = 0	RLk = 6,9 RLz = 12,26 IE = 0,77	Skor = 0
BIO-PK 6	RLk = 0 RLz = 0 IE = 0	RLk = 0 RLz = 0 IE = 0	Skor = 0

Information	**Ligninase scoring**
LRK = Average area of 3-point colonies	4 = dark brown
LRZ = Average area of 3-point zones	3 = brown
IE = Enzymatic Index	2 = light brown
	1 = brownish (thinner)
	0 = no brown spots

Based on Table 6, after calculations were performed using the Enzymatic Index (EI) formula, a variety of EI results were obtained. This is due to environmental factors such as pH, water content, the number of components within, and nutrients that can influence optimal fungal growth [9]. Sufficient nutrients can influence the clear zone produced. Incubation time can also affect the formation of the clear zone. In the ligninase test, scoring was used to determine positive results.

In the cellulase enzyme test, the lowest IE was 0.49 and the highest IE was 3.73, while in the cellulase enzyme test, the lowest IE was 0.49 and the highest IE was 3.73. For the ligninase enzyme test, the highest score obtained was 1 (light brown (thinner)). From the enzyme testing results, it was found that the cellulase enzyme was the most effective in producing isolates that could form a clear zone. This indicates that during the pre-decomposition phase of composting, cellulolytic activity is more dominant than proteolytic and ligninolytic activity.

4. Conclusion

Six thermophilic fungal isolates were obtained, designated BIO-PK 1, BIO-PK 2, BIO-PK 3, BIO-PK 4, BIO-PK 5, and BIO-PK 6. The thermophilic fungi exhibited diverse morphological characteristics, including shape and color, belonging to the genera *Penicillium* sp. (BIO-PK 1 and BIO-PK 3), *Odiodendron* sp. (BIO-PK 2), *Cylindrocladium* sp. (BIO-PK 4), *Verticillium* sp. (BIO-PK 5), and *Curvularia* sp. (BIO-PK 6). All six fungal isolates demonstrated the ability to degrade cellulose (BIO-PK 1, BIO-PK 2, and BIO-PK 3) with the highest Enzymatic Index of 3.73 (BIO-PK 2), proteolytic activity (BIO-PK 1 and BIO-PK 3) with the highest Enzymatic Index of 0.77 (BIO-PK 5), and lignolytic activity (BIO-PK 4) with the highest score of 1 (BIO-PK 4).

Compliance with ethical standards

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Disclosure of conflict of interest

All authors have no conflicts of interest.

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