IdentificationAndIsolationOfThermophilic Fungi In Pre-DecompositionOrganicWaste

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Abstract: Indonesia is one of the world's leading agrarian countries, boasting a vast and fertile agricultural sector. This significantly impacts the structure of the national economy. As the temperature rises, mesophilic microorganisms become less competitive and are replaced by thermophilic ones. The thermophilic phase is an important stage in composting. In this phase, there is an increase in decomposition or the initial formation of humus. This study aims to isolate and characterize thermophilic fungi in the compost base during the pre-decomposition phase at PT. Great Giant Pineapple. This descriptive exploratory study determined the presence of thermophilic fungi through several stages: sampling the compost, fungal isolation, purification, macroscopic and microscopic characterization, enzymatic characterization, and determination of the enzymatic index value. Six isolates (Bio PD 1, PD 2, PD 3, PD 4, PD 5, and PD 6) with various colors, including white, gray, and pink, and a predominantly smooth and dense texture were isolated. The six isolates have the following microscopic characteristics: septate hyphae, small round conidia, and conidiofores. The isolates that showed positive activity for the cellulase enzyme were Bio PD 3, PD 4, PD 5, and PD 6, with Bio PD 5 showing the highest index value (0.90). Bio PD 1, PD 2, PD 3, and PD 5 showed protease enzyme activity, with the highest value for the index on Bio PD 2 and 5 (0.14). Meanwhile, ligninase enzyme activity indicated that the six isolates did not have the ability to break down lignin compounds in the test conducted.

Keywords: Composting, Thermophilic fungi, Isolation and Characterization.

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I. INTRODUCTION

Indonesia is an agrarian country with a large and fertile agricultural sector, contributing to national economic growth. One of the largest agricultural companies in Indonesia is PT. The third largest pineapple industry globally is PT. *Great Giant Pineapple* (GGP), located in Lampung. This company produces 2,500 tons of pineapples daily for export to over 63 countries. Due to the large-scale production process, there is a need for environmentally friendly waste management. One of the programs operated is integrated solid waste management (*zero solid waste*), utilizing manure (animal waste), bromelain fiber, and other materials to be processed into compost(Susanto & Lubis, 2017).

Before entering the composting process, there is an initial stage in the decomposition of organic materials known as the pre-decomposition stage. During this stage, organic materials are prepared for decomposition by microorganisms. The pre-decomposition stage is an important step that includes sorting materials, cutting them into small pieces, and regulating moisture levels. This aims to make the composting process more effective, thereby accelerating decomposition and ensuring that the composting process runs optimally (Marlina*etal.*, 2017).

Initial pre-decomposition is carried out by mesophilic microorganisms that quickly break down easily soluble and easily degradable compounds. The heat generated causes the compost temperature to rise rapidly. As the temperature rises, mesophilic microorganisms become less competitive and are replaced by thermophilic microorganisms(Handrah *et al.*, 2021).

During the thermophilic phase, high temperatures accelerate the breakdown of complex carbohydrates such as cellulose and hemicellulose, kill pathogenic microbes, and destroy harmful organic substances. As energy sources in this phase begin to deplete, the compost temperature gradually decreases until it reaches mesophilic temperatures. Over time, the activity of mesophilic microorganisms decreases due to the decreasing availability of food, so this phase is called the cooling phase, which ends with the maturation phase (Maksudi *et al.*, 2022).

After the cooling phase in composting, the maturation phase begins where fungi continue to play a crucial role in completing pre-decomposition and forming humus, improving soil structure, water and nutrient retention, and long-term soil fertility. Thermophilic fungi activity generates ammonia and nitrogen gas, raising compost pH to become more alkaline(Hamidah & Gawy, 2023).

This study aims to isolate and characterize thermophilic fungi in the pre-decomposition phase compost base of PT. *Great Giant Pineapple'scompost plant*. Additional research on thermophilic fungal inoculum is deemed essential, with a study focusing on isolating and characterizing thermophilic fungi during predecomposition at a compost plant. The study aims to enhance understanding of thermophilic fungi and optimize microorganism efficiency in composting processes.

II. EXPERIMENTAL PROCEDURE

This study was conducted from December 11, 2024, to March 11, 2025, at the *Compost Plant* of PT. GGP. The equipment used included test tubes, Petri dishes, micropipettes, pH meters, incubators, autoclaves, hot plates, biosafety cabinets, and microscopes. The materials used were compost samples, 0.9% NaCl solution, distilled water, PDA medium, 1% CMC medium, skim milk agar, 1% olive oil, 1% chloramphenicol, Czapek medium, Boyd and Kohlmeyer medium, and Guaiacol medium. Sampling was conducted at 1 point with 3 repetitions, and 100 grams were collected at a depth of 2 meters. Data on fungal isolation, characterization, and identification were analyzed descriptively and quantitatively through macroscopic and microscopic observations, as well as enzymatic testing via clear zone observation and enzymatic index analysis.

1.1 Serial Dilution

A 25 g sample of compost was placed in an Erlenmeyer flask containing 225 mL of 0.9% NaCl and homogenized for a 10^{-1} dilution. Next, 1 mL of this dilution was taken and mixed with 9 mL of 0.9% NaCl in a test tube, homogenized with a vortex for a 10^{-2} dilution. This procedure is repeated until a dilution of 10^{-9} is achieved.

1.2 Isolation of Thermophilic Fungi

A total of 100 μ l of suspension was inoculated into Potato Dextrose Agar (PDA) medium with 1% chloramphenicol using the spread method and a drigalski tool, then incubated at 30°C in the dark for 7 days(Ibraheem *et al*, 2021).

1.3 **Purification of Thermophilic Fungi**

Each fungal colony with different morphology was inoculated onto new PDA medium using the spot method, then incubated at 30°C for 7 days. The purification results were stored as stocks for further characterization, including macroscopic and microscopic morphological analysis and enzymatic testing according to established procedures

1.4 Macroscopic Characterization of Fungi

The macroscopic characteristics of fungi observed include color, shape, and texture on PDA media. Samples were inoculated into Petri dishes containing PDA and incubated for 7 days at 30°C. After the colonies grew, visual observations were made with the naked eye and the results were documented (Heirina *et al.*, 2020).

1.5 Microscopic Characterization of Fungi

Microscopic observation of fungi was performed using the slide culture method, which involves growing fungi on PDA media $(1 \times 1 \text{ cm})$ that has been inoculated and covered with a glass cover, then incubated on wet cotton in a Petri dish at a temperature of 30°C. After incubation, the cover slip covering the agar medium is transferred to an object slide that has been stained with *Lactophenol Cotton Blue* (LPCB). The agar medium on the object slide is discarded, stained with LPCB, and covered with a cover slip. The preparation is observed under a microscope to examine fungal structures such as hyphae, conidia, and spores. LPCB stainsfungalcellwallsblue, facilitatingmicroscopicidentification (Tjampakasari*etal.*, 2024).

1.6 Enzymatic Characterization

a. Cellulase Test

Fungal isolates were inoculated onto Czapek medium and incubated for 5 days at 30°C Sari *et al.*, (2017). After growth, the medium was poured with a 0.1% Congo Red solution for 20 minutes, then washed with a 1 M NaCl solution for 20 minutes. Clear zones around the colonies indicate cellulolytic activity.

b. Protease Assay

Fungal isolates were inoculated onto skim milk agar medium and incubated at 30°C for 5 days. Clear zones around the colonies indicated protease activity (Mahardika *et al.*, 2021).

c. Ligninase Assay

Fungal isolates were grown on guaiacol medium containing glucose, peptone, yeast extract, agar, guaiacol at pН 7.0, and incubated for 7 and 4 mМ days at 30°C. Darkbrowncoloraroundthecoloniesindicatesligninaseactivitythroughguaiacoloxidation (Irawan etal., 2022).

1.7 Determination of Enzymatic Index

The level of enzymatic activity (cellulase and protease) was determined by measuring the area of the fungal colony and the area of the clear zone formed using the gravimetric method. The steps taken were as follows, according to Sumardi *et al.* (2021).

a. Calculation of colony area

1. Draw the colony pattern on transparent plastic mica as a colony replica.

2. Weigh the colony replica using an analytical balance.

3. Cut paper using HVS paper with dimensions of 1 cm x 1 cm, then weigh it.

Calculate the colony area using the following formula:

Colony replica weight $\times 1 \ cm^2$

Colony area = Paper weight 1 cm x 1 cm

Clear zone replica weight \times 1 cm² Clear zone area = Paper weight 1 cm x 1 cm

b. Enzymatic index calculation

Calculated using the following formula (Rosa et al., 2020).

Enzymatic Index = Clear zone area - Colony area

III. RESULTS AND DISCUSSIONS

3.1. Isolation and Macroscopic Characterization of Thermophilic Fungi

Fungal isolates were incubated for 7 days on Potato Dextrose Agar medium. They were then observed conventionally through morphological observations, including color and shape. Six fungal isolates were obtained, coded as Bio PD 1, Bio PD 2, Bio PD 3, Bio PD 4, Bio PD 5, and Bio PD 6, each with distinct characteristics. The results of the macroscopic observations are presented in **Table 1** below.

 Table 1. Macroscopic Characterization Results of Thermophilic Fungal Isolates Obtained from the Basic Predecomposition Phase

Code	Picture	Description
Bio PD 1		 Color: The center is blackish grey. Around it is a pink or brick red color, and yellow on the outside. On the 14th day the color of the zone becomes more contrasting. Texture: The fungal colony is fluffy, especially at the edge of the colony. The center is denser and somewhat powdery. On the 14th day the texture of the fungus appears dry.

D ! :	
Bio PD 2	Color: Pure white and there are green dots in the middle that spread to the edges. The edges of the colony look like a thin mist. On day 14 there was no color change in the fungal colony. Texture: The colony has a fine, dense, cotton-like texture. On the 14th day there was no change.
Bio PD 3	Color: Fungal colonies are light gray to whitish in color, with a slightly darker center. On day 14, the surface of the colony will be evenly gray to the edge of the colony. Texture: The texture of the colony is smooth and dense, with a slightly hairy surface. On day 14, there is no change.
Bio PD 4	Color: Fungal colonies show a dominant color of whitish gray to dark gray, The center of the colony looks darker (dark gray) than the surrounding area. Day 14 will be more dominated by dark gray Texture: The texture of the colony is fluffy. The surface of the colony forms a wavy circular pattern. Day 14 the texture of the colony looks dry-dense.
Bio PD 5	Color: The colony has a dominant pure white color evenly from the center to the edge of the colony. On the 14th day the color of the colony remains white with no change. Texture: The texture of the colony looks very smooth, dense, and hairy like cotton, the surface of the colony looks flat. While on the 14th day the texture of the colony will be thicker and cover the entire cup.
Bio PD 6	Color: The colonies are bright white evenly from the center to the edges. On the 14th day the color of the colony will remain white. Texture: The texture of the colony looks like fluffy, fibrous cotton. On the 14th day, the colony texture was slightly denser.

3.2. Microscopic Characterization of Thermophilic Fungi

Based on the results of microscopic morphological identification of hyphae, conidiophores, and the shape and arrangement of conidia, it was found that the six isolates originated from five different fungal genera. The five genera successfully identified include *Penicillium* sp. (with two distinct isolates), *Paecilomyces* sp. (one isolate), *Geotrichum* sp. (one isolate), *Deuteromycotina* sp. (one isolate), and *Gliocladium* sp. (one isolate). The morphological descriptions and initial classifications of each fungal isolate obtained were based on references from Malloch (1981) and Watanabe (2002), which discuss fungal characteristics. The initial classifications and characteristics of each isolate are presented systematically in **Table 2** below

 Table 2. Microscopic Characterization Results of Thermophilic Fungal Isolates Obtained from the Basic Predecomposition Phase

Isolate	Description	Genus	Description
Bio PD 1			
	 Small round conidia 	Paecilomyces sp.	Conidiophores are colorless, simple or branched, spores in chains from the tips of the phialids, colorless or brightly pigmented, hyphae are septate, and conidia are arranged in chains.
Bio PD 2			
	 Smallroundconid ia Conidiophore 	Penicillium sp.	Septate hyphae, single or branched conidiophores, hyaline (colorless), conidia are round to oval.
Bio PD 3			
	 Conidia Conidiophore Septatehyphae 	<i>Geotrichum</i> sp.	Chains of colorless, slimy spores (conidia) through segmentation of vegetative filaments. septate, conidiophores, and cylindrical conidia.
Bio PD 4			
	 Septatehyphae Conidiophore 	Deuteromycotin a sp.	Conidia are round, oval, to cylindrical, hyaline or colored and septate hyphae.

Bio PD 5	 Smallandscatte redconidia Conidiophore Phialides 	Penicillium sp.	Conidiophores erect, unbranched hyphae septate, hyaline (colorless), and branched
Bio PD 6	 Conidia Conidiophore Septatehypha e 	<i>Gliocladium</i> sp.	Conidiophores are erect, phialids are tapering, spores (conidia) are colorless, pink, or green, the upper part has penicillic branches, commonly found in soil.

3.3 Fungal Enzymatic Activity and Index Values

The composting process takes place under aerobic conditions with a balance of carbon and nitrogen, as well as sufficient moisture and aeration. Fungi play an important role in the further decomposition of complex organic materials through the production of extracellular enzymes such as cellulase, protease, and ligninase, whose activity is influenced by temperature during composting. Cellulase is active during the thermophilic phase ($50-60^{\circ}$ C), when thermophilic fungi thrive and degrade fibrous materials. Protease is more active during the early to mid-phase ($30-50^{\circ}$ C), when protein-rich substrates are still available, but its activity decreases as temperature increases and proteins begin to deplete (Ray & Kumar, 2020). Meanwhile, ligninase reaches optimal activity in the late phase ($30-45^{\circ}$ C), when easily degradable materials have been depleted and lignin begins to be broken down by ligninolytic fungi (Rahmasyitha, 2019).

3.3.1 Cellulase Enzyme Activity

Of the six fungal isolates tested on CMC medium, four isolates (Bio PD 3, Bio PD 4, Bio PD 5, and Bio PD 6) showed significant cellulolytic activity, indicated by the formation of clear zones around the colonies after *Congo red* staining. The cellulase activity index of each isolate is shown in the following figure.



Figure 1. Thermophilic fungal isolates in cellulase enzyme activity test Description: (1) Fungal colonies; (2) Clear zone formed.

Four isolates (Bio PD 3, Bio PD 4, Bio PD 5, and Bio PD 6) exhibited clear zones around the colonies, indicating the production of cellulase enzymes, particularly endoglucanase (CMCase), which can degrade CMC. The other two isolates did not show clear zones, and were therefore considered unable or less capable of producing cellulase enzymes. The enzymatic index (EI) value, which is the ratio of the diameter of the clear zone to the diameter of the colony, was measured in the four positive isolates with a range of 0.48–0.90. Isolate Bio PD 5 had the highest EI value, while Bio PD 3 had the lowest. These differences in IE values reflect variations in cellulase production capacity among isolates, indicating that not all fungal isolates possess the same cellulolytic potential.

Table 3. Cellulase Enzymatic Index Values			
Code	RLK (cm ²)	RLZ (cm ²)	IE
Bio PD 1	-	-	-
Bio PD 2	-	-	-
Bio PD 3	2,21	3,28	0,48
Bio PD 4	2,01	3,28	0,63
Bio PD 5	0,76	1,45	0,90
Bio PD 6	2,26	1,41	0,60

Description: (RLK) Average total colony area of 3 points

(RLZ) Average total clear zone area of 3 points

(IE) Enzymatic Index

3.3.2 Protease Enzyme Activity

Of the six fungal isolates tested on Skim Milk Agar medium, four isolates (Bio PD 1, Bio PD 2, Bio PD 3, and Bio PD 5) showed proteolytic activity, indicated by clear zones around the colonies. These positive data were then used to calculate the enzymatic index value based on colony diameter and clear zone diameter.



Figure 2. Thermophilic fungal isolates in protease enzyme activity test. Description: (1) Fungal colony; (2) Clear zone formed.

The four positive isolates exhibited varying sizes of clear zones, reflecting differences in protease enzyme activity. The highest enzymatic index (EI) values were observed in Bio PD 2 and Bio PD 5 (0.14), followed by Bio PD 1 (0.13) and Bio PD 3 (0.02). High proteolytic activity is characterized by a wide and clear zone, but the size of the zone and colony does not always correlate visually with the enzymatic index value.

Table 4. Protease Enzymatic Index Values			
Code	RLK (cm ²)	RLZ (cm ²)	IE
Bio PD 1	1,9	1,68	0,13
Bio PD 2	1,33	1,16	0,14
Bio PD 3	2,0	1,6	0,02
Bio PD 4	-	-	-
Bio PD 5	3.26	2,85	0,14
Bio PD 6	-	-	-

Description: (RLK) Average total colony area of 3 points

(RLZ) Average total clear zone area of 3 points

(IE) Enzymatic Index

3.3.3 Ligninase Enzyme Activity

Testing of ligninase enzyme activity in six fungal isolates (Bio PD 1 to Bio PD 6) using media with guaiacol as an indicator did not show any reddish-brown hydrolysis zones. This indicates that none of the isolates were capable of decomposing lignin.



Figure 3. Thermophilic fungal isolates in ligninase enzyme activity test Description: (1) Fungal colonies.

The research results showed that the six fungal isolates from the pre-decomposition phase compost did not exhibit ligninase activity, as indicated by the absence of a brown zone on the guaiacol medium. Ligninase enzyme activity is significantly influenced by environmental factors such as temperature, pH, humidity, and nutrients. Ligninase enzymes, such as laccase, MnP, and LiP, function optimally at acidic pH and high temperatures (around 60°C), while lower compost temperatures (25–35°C) do not support their optimal activity.According to Yakin & Mulyono (2017), ligninase enzymes have a relatively high optimal temperature of 60°C. At lower temperatures, such as the compost environment temperature ranging from 25°C to 35°C, the activity of ligninase enzymes tends to decrease significantly. In previous enzyme tests, some isolates, even those with the same code, could produce cellulase and protease enzymes, as the production of ligninolytic enzymes is highly dependent on the fungal species and its environmental conditions.

Additionally, not all fungi possess the genes encoding for ligninase enzyme production. Some fungal species only have the ability to produce other enzymes such as cellulase or protease, which play a more significant role in degrading other components of organic material like cellulose or protein. According to research by Subowo (2010), only a small fraction of fungi naturally produce ligninase enzymes, while the majority produce enzymes like cellulase or protease without ligninolytic capability.

IV. CONCLUSION

This study isolated six thermophilic fungi from the pre-decomposition phase compost of PT. *Great Giant Pineapple* (Bio PD 1–6) with variations in colony color and texture. Microscopically, the isolates showed septate hyphae, conidiophores, and small round conidia. Four isolates (Bio PD 3, 4, 5, 6) exhibited cellulase activity, with Bio PD 5 having the highest enzymatic index (0.90). Four other isolates (Bio PD 1, 2, 3, 5) actively produced protease, with Bio PD 2 and Bio PD 5 having the highest indices (0.14). None of the isolates exhibited ligninase activity. It is recommended to conduct phylogenetic analysis and further testing on the best isolates (Bio PD 2 and 5) to optimize their utilization in biodegradation and composting.

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