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Characterization of thermophilic fungal communities during the thermophilic stage of composting

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Abstract

PT. Great Giant Pineapple is one of the companies operating in the food industry. During its production process, the company generates a significant amount of waste, including fruit peels, pineapple cores, livestock manure, and other parts not used in the final product. Efforts to address this issue involve processing the waste into compost through a decomposition process involving microorganisms, including thermophilic fungi. Thermophilic fungi possess thermostable properties and play a crucial role in accelerating chemical reaction rates by releasing extracellular enzymes, thereby speeding up the composting process. This study aims to isolate and characterize thermophilic fungi in deep compost during the composting process at PT. Great Giant Pineapple. The methods used include sampling, homogenization, and serial dilution, isolation, purification, macroscopic, microscopic, and physiological characterization, and determination of enzymatic indices. The results of this study yielded four isolates indicated as thermophilic fungi. Macroscopic isolates Bio DK 1, Bio DK 2, and Bio DK 3 showed green colonies with white to grayish-white edges, smooth and thick in texture. Meanwhile, the Bio DK 4 isolate appears different with white-colored colonies and a thin, smooth, fibrous texture. Microscopic all isolates have hyphae, with Bio DK 1, 2, and 3 isolates having conidia, while the Bio DK 4 isolate has sporangia. In the enzyme activity test, Bio DK 1 showed the highest ability to produce cellulose enzymes with an enzymatic index of 7.52, while Bio DK 4 had the highest enzymatic index for protease enzymes at 3.05. In the ligninase enzyme activity test, all isolates were unable to degrade lignin.

Keywords: Thermophilic Fungi; Isolation; Characterization; Compost; Composting

1. Introduction

The agroindustry sector plays a crucial role in Indonesia's national economy and serves as the primary source of livelihood for the population [39]. PT. Great Giant Pineapple, part of Great Giant Foods, is an agroindustry company managing various business units, including pineapple processing, banana processing, bromelain enzyme production, and livestock farming. These intensive production activities generate large amounts of organic waste, such as fruit peels, pineapple cores, and livestock manure, which need to be managed sustainably through methods such as composting [2].

Composting is the process of decomposing organic materials with the help of soil microorganisms such as fungi and bacteria. These microorganisms produce extracellular enzymes that accelerate the degradation of organic materials into high-quality compost [22, 16]. Environmental conditions such as moisture content, temperature, pH, and nutrients significantly influence microbial development during composting [38].

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Thermophilic fungi play a crucial role in high-temperature composting due to their ability to produce enzymes such as cellulase, protease, and ligninase that remain stable and active under extreme conditions [13, 7]. The adaptation of thermophilic fungi to high temperatures is supported by factors such as membrane permeability and cellular chemical stability [3].

Given the importance of thermophilic fungi in composting, this study aims to isolate and characterize thermophilic fungi from compost at a depth of 1 meter at the PT. Great Giant Pineapple composting facility. This depth was chosen because it influences temperature, humidity, and the types of fungi that grow [40]. It is hoped that the isolated fungi will be able to survive and function optimally at high temperatures, thereby improving the efficiency of organic waste composting.

2. Material and methods

2.1. Sample Preparation

This study used an exploratory descriptive method to identify and characterize thermophilic fungi in compost at a depth of 1 meter during the composting process at PT. Great Giant Pineapple. Sampling was conducted using purposive sampling at one location with three replicates. Samples were collected aseptically using gloves and spoons sterilized with 70% alcohol, with each replicate placed in a separate sterile plastic bag. The samples were then stored in heat-resistant containers and immediately transported to the laboratory for analysis.

2.2. Homogenization and Serial Dilution

Weigh 25 g of compost sample, then put the sample into an Erlenmeyer flask containing 225 mL of NaCl and homogenize it. Next, suspend 1 mL of the sample in a test tube containing 9 mL of NaCl and vortex it [33].

2.3. Isolation of Thermophilic Fungi

A total of 100 µL of sample was taken and then inoculated into a Petri dish containing PDA medium containing 1% chloramphenicol. The inoculation process was carried out using the spread plate method and then incubated at 30ºC for 7 days [13].

2.4. Purification of Thermophilic Fungi

From the various fungal colonies growing on Petri dishes, each colony was observed to determine morphological differences. Next, one colony was aseptically inoculated to new PDA media using the spot method. The Petri dishes containing the isolates were then incubated at 30°C for 7 days [12].

2.5. Macroscopic Characterization of Thermophilic Fungi

Macroscopic observation can begin approximately 7-14 days after purification, depending on the growth rate of the fungi. The first step is to visually observe each fungal colony with the naked eye to analyze various morphological aspects. Observations include colony color and colony texture [10].

2.6. Microscopic Characterization of Thermophilic Fungi

This was performed using the slide culture technique and lactophenol cotton blue staining to observe the structure of hyphae, sporangia, and conidia using an optical microscope. The steps are as follows: prepare a sterile Petri dish containing filter paper and a support, then place a object glass on top of it. Cut the PDA medium into 1 x 1 cm squares. Next, place the PDA pieces on the object glass. Take a sample of the colony using an ose needle. The ose needle is then inserted/rubbed on all sides of the agar, after which it is covered with a cover glass. Using a sterile pipete, sterile distilled water is added to the filter paper under the slide glass until it is wet and pooled. The sample is then incubated at room temperature and observed daily until growth is visible (2x24 hours). Next, the object glass and cover glass are stained with lactophenol cotton blue and observed under an optical microscope [35]

2.7. Physiological Characterization (Enzymatic Activity Test)

2.7.1. Cellulose 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 [28].

2.7.2. Protease Test

The isolates were inoculated onto skim milk agar medium (pH 7), incubated at 50°C for 5 days, and the clear zones formed were observed [17].

2.7.3. Ligninase Test

Isolates were inoculated onto Boyd and Kohlmeyer medium (containing guaiacol), pH 7, incubated at 30°C for 7. A positive reaction was indicated by the formation of a brown color around the colony [15].

2.7.4. Determination of Enzymatic Index

The enzymatic index was calculated by comparing the colony area and clear zone using the gravimetric method : replicas of the colony and clear zone were drawn on clear mica plastic, weighed, and then calculated using the gravimetric formula [32].

$$\text{Colony Area} = \frac{\text{colony replica weight}}{\text{paper weight 1 cm x 1 cm}} \text{ x 1 cm}^2$$

$$\text{Clear Zone Area} = \frac{\text{clear zone replica weight}}{\text{paper weight 1 cm x 1 cm}} \text{ x 1 cm}^2$$

The enzyme index is calculated using the following [24] :

$$\text{Enzymatic Index} = \frac{\text{average clear zone area - average colony size}}{\text{average colony size}}$$

2.8. Data Analysis

Data analysis, including macroscopic and microscopic characterization, was performed by comparing the visualization results with existing references, while physiological characterization analysis in the form of enzyme activity, including cellulase and protease, was performed by calculating the enzymatic index. Meanwhile, ligninase enzyme activity was performed by scoring.

3. Results and discussion

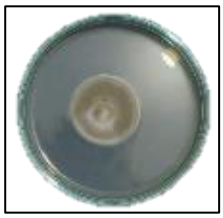
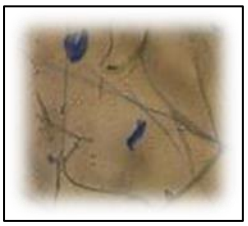
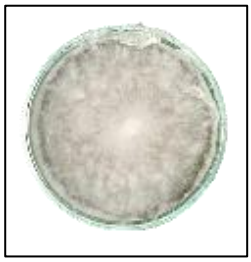
3.1. Macroscopic and Miscroscopic Characterization of Thermophilic Fungi

In this study, four isolates were obtained that were indicated as thermophilic fungi. The macroscopic characteristics of all isolates showed morphological differences, so they were coded as Bio DK 1, Bio DK 2, Bio DK 3, and Bio DK 4 to facilitate observation and data recording during the study.

Microscopic characteristics were presented using the slide culture method. Observations included hyphae, sporangia, and conidia. Based on the results obtained, all isolates were successfully identified and were suspected to belong to a specific genus, referring to Watanabe (2002) and descriptions of the characteristics of these fungi in Malloch (1981).

All isolates showed hyphae as the main structure of the fungal body, which are tubular in shape, resembling long threads, and may or may not be septate depending on the species [10]. All four isolates have septate hyphae (septum), which separate intercellular spaces and facilitate cytoplasmic transport, as well as function in nutrient absorption and reproduction through the formation of asexual and sexual spores [4, 25]. In addition to hyphae, some isolates were also found to have conidia and sporangia. Conidia are asexual spores formed at the tips or sides of specialized hyphae (conidiophores), while sporangia are sacs at the tips of sporangiophores that produce sporangiospores as asexual spores.

Table 1 Macroscopic and Microscopic Characteristic of Thermophilic Fungi Obtained from Compost at a Depth of 1 m during the Process Phase

Isolate	Macroscopic	Microscopic	Description
Bio DK 1			Macroscopic: The center of the fungal colony is dark green, gradually fading to white at the edges. Over 7-14 days, the colony darkens and spreads.It has a smooth and thick texture. Microscopic: Brown to colorless conidia are produced by fragmentation of the conidiophore branches.
Bio DK 2			Macroscopic: The entire fungal colony appears green, with the edges of the colony appearing whitish gray. Over the course of 7-14 days, the green area of the colony darkens and expands. It has a smooth and thick texture. Microscopic: The conidia are dark green/black in color and arranged in long, branched chains. The conidiophores are dark in color.
Bio DK 3			Macroscopic: The colony is green, with a whitish gray lower edge. Over 7-14 days, the colony darkens and spreads. It has a smooth and thick texture. Microscopic: Clear, curved conidia grow toward the tip.
Bio DK 4			Macrocopic : The entire colony is white in color. Over the course of 7-14 days, the colony expands and fills the Petri dish. It has a texture like fine, thin fibers or threads. Microscopic : The sporangium is round and transparent.

Isolate Bio DK 1 is suspected to be *Oidiodendron* sp., characterized by septate hyphae and cylindrical to oval conidia formed through hyphal fragmentation [5]. This genus thrives in soil rich in organic matter and plays a role in decomposition and soil fertility enhancement [18, 42]. Isolate Bio DK 2 is likely *Cladosporium* sp., with septate hyphae and dark-colored elliptical to cylindrical conidia formed in branched chains [6]. *Cladosporium* sp. acts as a decomposer of organic material and produces extracellular enzymes, and has potential as a biofertilizer [27, 43].

Isolate Bio DK 3 is suspected to be *Codinaea* sp., characterized by septate hyphae and hyaline, long, curved needle-shaped conidia [42]. *Codinaea* sp. acts as an important saprophytic fungus in the decomposition of organic matter [8]. Isolate Bio DK 4 is suspected to be *Gongronella* sp., with white colonies, sporangia, and hyphae as the main structures. *Gongronella* sp. plays a role in the decomposition of organic matter and composting, and is capable of producing biodegradative enzymes such as protease to break down proteins in organic waste [20, 36].

3.2. Physiological Characterization (Enzymatic Activity Test)

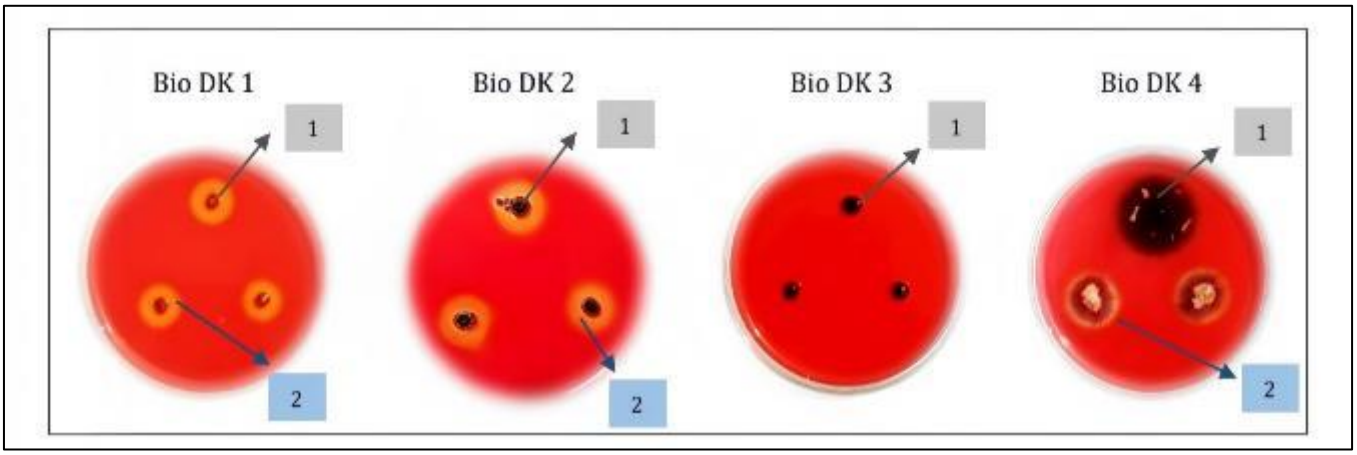
3.2.1. Cellulase Test

Four isolates obtained were tested for their cellulolytic activity to determine their ability to hydrolyze cellulose substrates. Based on Table 2, the isolate with the code Bio DK 1 showed the highest enzyme index of 7.52, indicating very strong enzyme activity. Conversely, the Bio DK 3 isolate had the lowest enzyme index because it did not exhibit cellulolytic activity, as evidenced by the absence of a clear zone around the medium where the isolate grew. This indicates that the isolate does not produce cellulase enzymes capable of degrading the substrate in the test medium.

Media that can be degraded by fungi will show a clear zone around the colony, indicating the presence of cellulolytic activity (Figure 1).

Table 2 Enzymatic Index of Cellulase Activity

Isolate Code	Average total colony are of 3 points (cm^2)	Average total clear zone area of 3 points (cm^2)	Enzymatic index
Bio DK 1	0.23	1.96	7.52
Bio DK 2	0.66	2.7	3.09
Bio DK 3	-	-	-
Bio DK 4	1.73	6.1	2.52



Description: (1) Fungal colonies; (2) Clear zone formed.

Figure 1 Thermophilic Fungal Isolates in Cellulase Assay.

In the cellulase enzyme activity test, the thermophilic fungal isolates Bio DK 1, Bio DK 2, and Bio DK 4 were able to form clear zones in the medium, indicating the production of active cellulase enzymes that hydrolyze cellulose substrates, resulting in degradation and the formation of clear areas around the colonies. This indicates that all three isolates have potential cellulolytic capabilities. Isolate Bio DK 1 is suspected to belong to the genus *Oidiodendron* sp., an ericoid mycorrhizal fungus that produces extracellular enzymes including cellulase [1]. Isolate Bio DK 2 is suspected to belong to the genus *Cladosporium* sp., which is also known as a producer of cellulase enzymes in soil fungi [11]. Isolate Bio DK 3 is suspected to be *Codinaea* sp., but it does not show cellulase activity because it does not form a clear zone [32]. Isolate Bio DK 4 is suspected to be *Gongronella* sp., which is capable of producing hydrolytic enzymes, including cellulase, for cellulose degradation and nutrient cycling [45].

Based on Table. the enzymatic index values were calculated from the comparison of colony area and clear zone using the gravimetric method. Most Bio DK isolates had enzymatic indices between 2 and 7, indicating sufficiently high cellulase activity in the 1-meter-deep compost during the process phase. Isolate Bio DK 3 showed the lowest index because it did not form a clear zone, while isolate Bio DK 1 had the highest index of 7.52, far exceeding isolate RB1 in, which ranged from 1.03 to 1.33 [12].

In Figure 1. isolate Bio DK 1 suspected to be *Oidiodendron* sp., demonstrated high ability to break down cellulose through cellulase enzymes. The mechanism of this enzyme involves endo-β-1,4-glucanase, which randomly breaks glycosidic bonds, exo-β-1,4-glucanase, which cuts the ends of oligosaccharide chains, and β-glucosidase, which converts cellobiose into glucose [12]. Environmental factors such as medium pH (6.8) and incubation temperature (30°C) play a significant role in cellulase enzyme activity [30]. Testing using Congo red dye binds to cellulose polysaccharides, forming a clear zone in areas hydrolyzed into glucose, which does not bind to this dye. Rinsing with a 1 M NaCl solution clarifies the clear zone, facilitating observation of enzymatic activity [9].

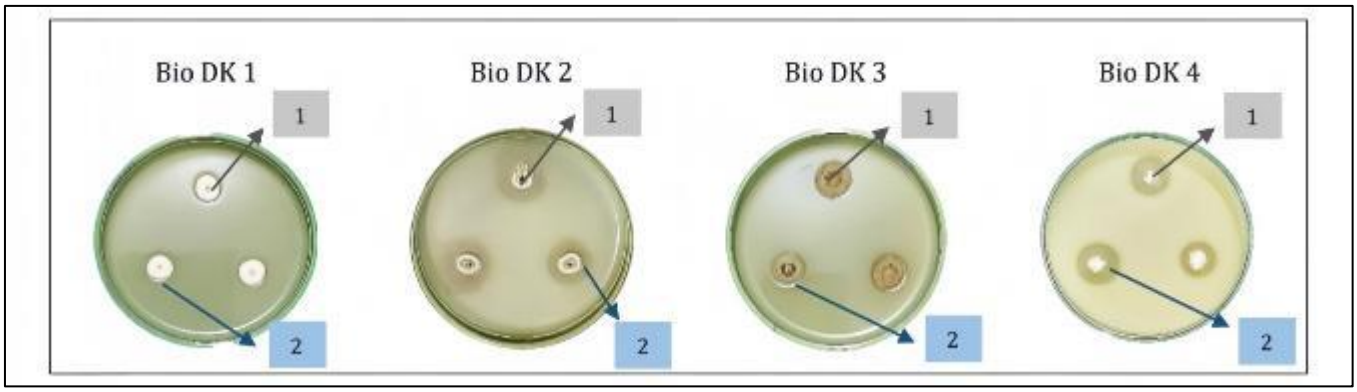
3.3. Protease Test

All isolates were tested for protease activity to determine their ability to degrade protein substrates. Based on the data in Table 3. all isolates showed enzymatic index values indicating the ability to produce protease enzymes. The highest index was achieved by isolate Bio DK 4 at 3.05, while the lowest index was achieved by isolate Bio DK 1 at 0.32.

Proteolytic activity is characterized by the formation of a clear zone around the colony on the medium degraded by the fungi. Figure 2. shows isolate Bio DK 4, which has the highest protease activity.

Table 3 Enzymatic Index of Protease Activity

Isolate Code	Average total colony are of 3 points (cm^2)	Average total clear zone area of 3 points (cm^2)	Enzymatic index
Bio DK 1	1.1	1.46	0.32
Bio DK 2	1.26	3.9	2.09
Bio DK 3	1.2	1.73	0.44
Bio DK 4	0.6	2.43	3.05



Description: (1) Fungal colonies; (2) Clear zone formed.

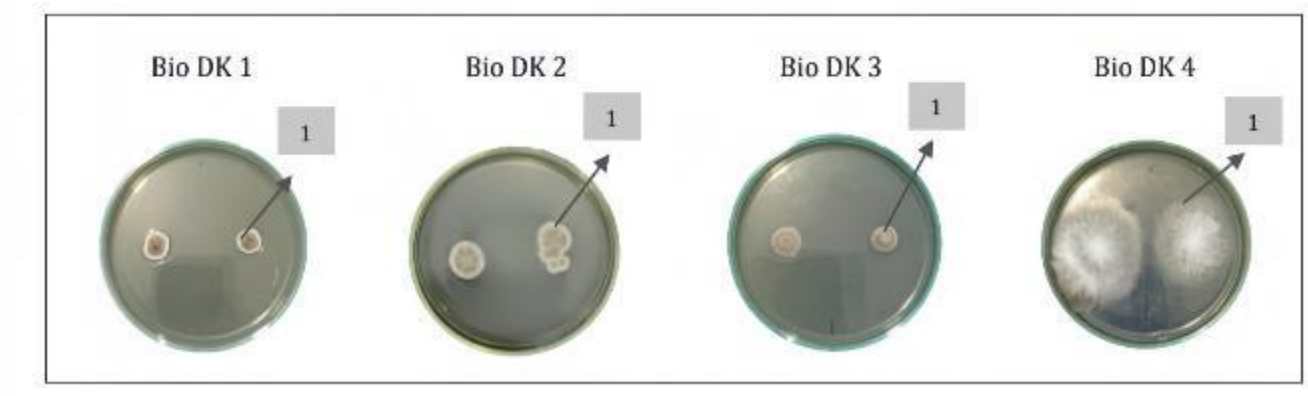
Figure 2 Thermophilic Fungal Isolates in Protease Assay

In the protease enzyme activity test, all isolates were able to form clear zones, indicating the production of protease enzymes that hydrolyze proteins in the medium, resulting in degradation and the formation of clear areas around the colonies. Isolate Bio DK 1, suspected to be *Oidiodendron* sp., was able to secrete protease [21]. Isolate Bio DK 2 belongs to the genus *Cladosporium* sp., which also produces active protease in protein degradation [23]. Isolate Bio DK 3, suspected to be *Codinaea* sp., showed active protease activity with a clear zone [34]. Isolate Bio DK 4, suspected to be *Gongronella* sp., had the highest protease activity, supporting the important role of this fungus in the biodegradation of organic material [45].

Enzymatic index analysis (Table 4) showed variations in protease production among isolates, with Bio DK 4 having the highest value of 3.05 and Bio DK 1 the lowest at 0.32. These values are higher than the index range *for Aspergillus section Nigri* DUCC, which ranges from 1.06 to 1.51 [26]. In Figure 2, the Bio DK 4 isolate shows a high protease enzyme index value, reflecting its ability to degrade proteins. *Gongronella* sp. protease works by binding to proteins as substrates and breaking peptide bonds through hydrolysis, producing peptides and amino acids that are easily absorbed for fungal growth. This enzyme is effective under specific pH and temperature conditions that support its optimal activity [36]. Differences in enzymatic index values among isolates are influenced by variations in isolate type, testing methods, and incubation conditions. In this study, pH 7 and 30°C were used, consistent with the findings of Sedijani *et al*. (2023) that the highest protease activity occurs at neutral pH. This indicates that fungal proteases function optimally at neutral to slightly alkaline pH, consistent with the general characteristics of microbial protease enzymes.

3.4. Ligninase Test

All isolates were tested to determine their ability to degrade lignin substrates. Based on these tests, it was found that none of the isolates showed a brown color change in the medium surrounding the colonies. This indicates that the four isolates tested lack the ability to degrade lignin substrates, which is the primary indicator of ligninase enzyme activity.



Description: (1) Fungal colonies.

Figure 3 Thermophilic Fungal Isolates in ligninase Assay

All isolates from the 1 m deep compost during the process phase did not show active ability in producing ligninase enzymes, as indicated by the absence of hydrolysis zones. This indicates low or no production of ligninase enzymes by these isolates. Since no hydrolysis zones were formed, the enzymatic index as a quantitative parameter for lignin degradation could not be obtained. Variations in ligninase production capacity are influenced by the type of fungi, which have different enzymatic characteristics, resulting in varying effectiveness of lignin degradation. State that only a few fungal species are effective in producing ligninase enzymes [44]. Additionally, aerobic and anaerobic fungi play different roles in the degradation of organic matter [46].

Environmental factors such as medium conditions, incubation time, pH, temperature, and nutrients significantly influence enzyme activity and fungal growth. The expression of ligninase-producing genes depends on these conditions. Found that optimal temperature and pH (pH 6 and 35°C) are important for lignin peroxidase activity in Gliomastix sp., emphasizing the need to adjust environmental conditions for maximum enzyme activity [14].

Thus, the absence of ligninase activity in fungal isolates is likely due to a combination of factors such as suboptimal pH and temperature, fungal species, and insufficient nutrients supporting ligninase enzyme gene expression. Optimizing culture conditions is crucial for enhancing ligninase enzyme production in the lignin biodegradation process.

4. Conclusion

Four thermophilic fungal isolates were obtained from compost samples at a depth of 1 meter in the composting process phase at PT. Great Giant Pineapple. Each isolate exhibited distinct macroscopic and microscopic characteristics, and were assigned identification codes: Bio DK 1, Bio DK 2, Bio DK 3, and Bio DK 4. All isolates obtained possessed hyphae. Isolate Bio DK 1 had a dark green color with white edges that were smooth and thick in texture, indicating the highest cellulase enzyme production capacity with an enzymatic index of 7.52. Meanwhile, isolate Bio DK 4 was white in color with a smooth and thin fibrous texture, exhibiting the highest protease activity with an enzymatic index of 3.05. In the ligninase enzyme activity test, no isolates were found capable of degrading lignin

Compliance with ethical standards

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Disclosure of conflict of interest

All author have no conflict of interest.

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