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Thermophilic bacteria from surface layer of compost: Isolation and characterization during the processing stage

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Abstract

Composting is a decomposition process of organic waste that occurs aerobically with the assistance of decomposer agents under controlled conditions. Thermophilic bacteria are one group of decomposer microorganisms that play a role in composting during the thermophilic phase. This study aims to isolate and characterize thermophilic bacteria from the surface layer of compost during the processing stage at PT Great Giant Pineapple's Compost Plant, with the expectation of accelerating the composting process. This research was conducted using an exploratory descriptive method, with stages including sampling of surface-layer compost, isolation of thermophilic bacteria, purification of bacterial isolates, macroscopic and microscopic morphological characterization, and enzymatic activity assays. The results showed that seven bacterial isolates were obtained. The colony morphology of the seven isolates was dominated by irregular and spreading forms with varied margins, such as irregular, wooly, undulate, and entire. Most colonies showed raised elevations and shared a uniform cream coloration. Microscopically, all isolates were rod-shaped, Gram-positive, and capable of forming spores. Isolates ALV1, ALV2, ALV3, ALV4, ALV5, ALV6, and ALV7 demonstrated the ability to produce amylase and protease enzymes, while only ALV1, ALV2, ALV3, ALV5, and ALV6 were capable of producing cellulase enzymes. The highest enzymatic index (EI) values for amylase and cellulase were observed in ALV6, at 8.38 and 14.66 respectively, whereas the highest protease EI value was recorded in ALV4, at 4.39.

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

1. Introduction

Composting is a process of decomposition, or the breakdown of organic waste, that can occur aerobically through the activity of organisms and microorganisms under controlled conditions [1]. According to [2], several factors can influence the composting process, including the texture and particle size of the raw material, the volume of waste or raw material, moisture content, oxygen availability, pH level, C/N ratio, and temperature. Temperature is one of the most important factors that can influence the survival and activity of microorganisms in the composting process.

During the composting process, there are four phases with different temperature ranges. These phases are the mesophilic phase, thermophilic phase, cooling phase, and maturation phase [3]. The thermophilic phase is when the composting process occurs most efficiently, with temperatures ranging between 45–70°C [4]. During this phase, thermophilic microorganisms generate significant amounts of heat and gas from the breakdown of organic materials such as glucose and other carbohydrates, utilizing thermostable extracellular enzymes [5]. Examples of thermophilic bacteria involved in composting include *Brevibacillus borstelensis*, *Bacillus cereus*, *Bacillus paralicheniformis* [6], *Bacillus subtilis* [7], and *Thermoanaerobacterium* sp. [8].

PT Great Giant Pineapple (PT GGP) is a private agribusiness company in Indonesia engaged in large-scale pineapple cultivation and processing. The composting process at PT GGP's Compost Plant is divided into two distinct stages: the

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pre-decomposition stage and the processing stage. The pre-decomposition stage serves as the initial phase, during which organic waste materials from PT GGP such as bromelain residue, cassava peels, and animal manure remain largely intact and have not yet undergone significant decomposition. Following this, the composting process enters to the processing stage, where the organic waste begins to decompose more intensively. This stage is characterized by a significant increase in temperature (ranging from 60–65°C) and a pH range of 4.6–4.8, indicating that the composting process has entered the thermophilic phase.

The main substrates present during the processing stage at PT GGP's Compost Plant include starch, cellulose, and protein. According to [9], the addition of microbial inoculum can improve the quality of the final compost and reduce the composting time. The presence of thermophilic bacterial inoculum is expected to accelerate the composting process because the enzymes produced are thermostable and capable of breaking down organic materials under extreme conditions [10]. Some of the thermostable enzymes produced by these bacteria such as amylase, cellulase, and protease are key drivers of the composting process [4].

Based on the description above, it is necessary to isolate and characterize thermophilic bacteria and their physiological characteristics, including the enzymatic activity obtained from the surface layer of compost during the processing stage, that may serve as potential inoculants to accelerate the composting process.

2. Material and methods

2.1. Sample collecting

Compost samples were collected from the surface layer of compost during the processing stage using purposive sampling at PT Great Giant Pineapple's Compost Plant, located in Central Lampung Regency, Lampung Province, Indonesia. Samples were taken from one point with three replications at a depth of 5–10 cm from the surface. The collected samples were placed in sterile plastic bags, stored in a cooler box to maintain temperature, and transported to the laboratory for further analysis.

2.2. Isolation and purification of thermophilic bacteria

A total of 25 g of compost sample was suspended in a sterile bottle containing 225 mL 0.9% NaCl. The mixture was homogenized to create a 10^{-1} dilution. A 10^{-2} dilution was then prepared by transferring 1 mL of the 10^{-1} dilution into a tube containing 9 mL of 0.9% NaCl, followed by homogenization using a vortex. This procedure was repeated until a 10^{-10} dilution was obtained.

Thermophilic bacteria were isolated using the spread plate method on nutrient agar (NA) medium modified with nutrient broth (NB) and 6% agar, adjusted to pH 4.7. A total of 0.1 mL from dilutions 10^{-6} to 10^{-10} was pipetted and inoculated onto Petri dishes, then spread using a Drigalski spatula. Each dilution was plated in duplicate and incubated at 60 °C for 24 h [11]. After incubation, single bacterial colonies were selected using a sterile loop and transferred to modified NA plates using the streak plate method to obtain pure cultures, followed by incubation at 60°C for 24 h. Pure isolates were subsequently stored on slanted agar (NB + 6% agar pH 4.7) for macroscopic, microscopic, and enzymatic assays.

2.3. Macroscopic identification of thermophilic bacteria

Macroscopic identification of bacterial colonies included observation of colony morphology such as form, color, margin, and elevation. Observations were conducted after 24 h of incubation at 60 °C.

2.4. Microscopic identification of thermophilic bacteria

2.4.1. Gram staining

Gram staining was performed by smearing a 24-hour-old bacterial culture onto a clean glass slide previously dripped with 1–2 drops of distilled water. The smear was air-dried and heat-fixed over a spirit lamp. The slide was stained with crystal violet for 1 min and rinsed with distilled water, followed by staining with Lugol's iodine solution for 1 min and rinsing again. The slide was decolorized with 96% ethanol for 30 s until it appeared pale, then counterstained with safranin for 1 min and rinsed with distilled water. The preparation was observed under a light microscope starting from the lowest magnification. Purple-colored cells indicated Gram-positive bacteria, while red-colored cells indicated Gram-negative bacteria [12].

2.4.2. Spore staining

Spore staining was conducted by smearing a 72-hour-old bacterial culture onto a glass slide previously dripped with 1–2 drops of distilled water. The smear was spread evenly and heat-fixed over a spirit lamp. The slide was stained with malachite green for 10 min, then rinsed with distilled water. It was subsequently counterstained with safranin for 1 min and rinsed again. Observations were made under a light microscope beginning with the lowest magnification. Vegetative cells appeared red, while spores appeared green [13].

2.5. Enzymatic Activity Assay of Thermophilic Bacteria

2.5.1. Amylase activity assay

Amylase activity was determined by inoculating a 24-hour-old bacterial isolate onto modified NA medium (NB + 6% agar) containing 1% starch using the spot method. Incubated at 60°C for 24 hours. Plates were incubated at 60 °C for 24 h. After incubation, the plate was flooded with an iodine solution (2% I_2 and 0.2% KI). A clear zone around the colony indicated positive amylase activity [11]. The enzymatic index (EI) was subsequently calculated to quantify enzymatic activity.

2.5.2. Cellulase activity assay

Cellulase activity was evaluated by inoculating a 24-hour-old bacterial isolate onto modified NA medium (NB + 6% agar) containing 1% carboxymethyl cellulose (CMC) using the spot method. Incubated at 60°C for 24 hours. Plates were incubated at 60 °C for 24 h. After incubation, the plate was flooded with 0.1% Congo red solution for 30 min, followed by rinsing with 1 M NaCl for 15 min. The formation of a clear zone around the colony indicated positive cellulase activity [11]. The enzymatic index (EI) was subsequently calculated to quantify enzymatic activity.

2.5.3. Protease activity assay

Protease activity was conducted by inoculating 24-hour-old bacterial isolates onto skim milk agar medium using the spot method. Plates were incubated at 60 °C for 24 h. A clear zone surrounding the colony indicated positive protease activity [11]. The enzymatic index (EI) was subsequently calculated to quantify enzymatic activity.

2.5.4. Catalase activity assay

Catalase activity was assessed by placing one drop of 3% H_2O_2 on a glass slide and mixing it with a loopful of a 24-hour-old bacterial isolate. The reaction was observed for 20 s. The appearance of gas bubbles indicated a positive catalase reaction, while the absence of bubbles indicated a negative result [14].

2.6. Enzymatic index (EI) calculation

The enzymatic activities of amylase, cellulase, and protease were quantified by measuring the areas of bacterial colonies and the corresponding clear zones using the gravimetric method described by [15]. The first step is to draw the colony pattern (colony replica) on transparent mica plastic, then weigh the colony replica using an analytical balance. Next, cut a piece of HVS paper with dimensions of 1 cm x 1 cm, then weighed. The same procedure is used to determine the area of the clear zone. Once the weights of each are obtained, the colony area and clear zone area can be calculated using the following formulas.

$$\text{Colony Area (CA)} = \frac{\text{Weight of colony replica}}{\text{Weight of 1 cm x 1 cm paper}} x 1\ \text{cm}^2$$

$$\text{Clear Zone Area (ZA)} = \frac{\text{Weight of clear zone replica}}{\text{Weight of 1 cm x 1 cm paper}} x 1\ \text{cm}^2$$

$$\text{Average Total Colony Area (ATC)} = \frac{\text{CA1} + \text{CA2} + \text{CA3}}{3}$$

$$\text{Average Total Clear Zone Area (ATZ)} = \frac{\text{ZA1} + \text{ZA2} + \text{ZA3}}{3}$$

The enzymatic index (EI) was calculated as the ratio of the clear zone area to the colony area using the following formula [16].

$$\text{Enzymatic Index (EI)} = \frac{\text{ATZ} - \text{ATC}}{\text{ATC}}$$

3. Result and discussion

3.1. Isolation and macroscopic identification of thermophilic bacteria

Isolation of thermophilic bacteria from surface layer of compost samples taken during the processing stage at PT Great Giant Pineapple's Compost Plant, resulted seven bacterial isolates with distinct colony morphologies. The seven thermophilic bacterial isolates are ALV1, ALV2, ALV3, ALV4, ALV5, ALV6, and ALV7.

Table 1 Macroscopic observation results of seven thermophilic bacterial isolates

Isolate Codes	Colony Morphology Characteristic			
	Form	**Color**	**Margin**	**Elevation**
ALV1	Irregular and spreading	Cream	Irregular	Flat
ALV2	Rhizoid	Cream	Wooly	Flat
ALV3	Irregular and spreading	Cream	Irregular	Raised
ALV4	Irregular and spreading	Cream	Undulate	Flat
ALV5	Round with scalloped margin	Cream	Wooly	Raised
ALV6	Round with scalloped margin	Cream	Entire	Raised
ALV7	Rhizoid	Cream	Entire	Raised

Based on macroscopic observations (Table 1), colony morphology among the isolates was dominated by irregular and spreading forms with varied margins, such as irregular, wooly, undulate, and entire. Most colonies showed raised elevations and shared a uniform cream coloration. According to [17], differences in bacterial morphology can be influenced by several factors such as species and environmental conditions (pH, temperature, and substrate availability).

3.2. Microscopic identification of thermophilic bacteria

3.2.1. Gram staining

Gram staining of the seven thermophilic bacterial isolates, observed at 1000× magnification, showed that all isolates were Gram-positive and rod-shaped. The Gram-positive nature of the isolates was indicated by the presence of blue to purple-colored cells under the microscope (Figure 1). The purple color in Gram-positive bacteria after Gram staining is due to their thick cell walls rich in peptidoglycan, which retains the crystal violet stain during the decolorization step [18].

During the Gram staining process, bacterial cells are initially stained with crystal violet, followed by the application of iodine solution, which forms a crystal violet–iodine complex. When subjected to a decolorizing agent such as alcohol or acetone, Gram-positive bacteria retain the dye complex due to their thick peptidoglycan cell wall, resulting in a purple coloration. In contrast, Gram-negative bacteria possess a thinner peptidoglycan layer and an outer lipid membrane that is disrupted by the decolorizer, allowing the dye complex to be washed out. Consequently, these cells are counterstained with safranin, which imparts a red or pink coloration [19].

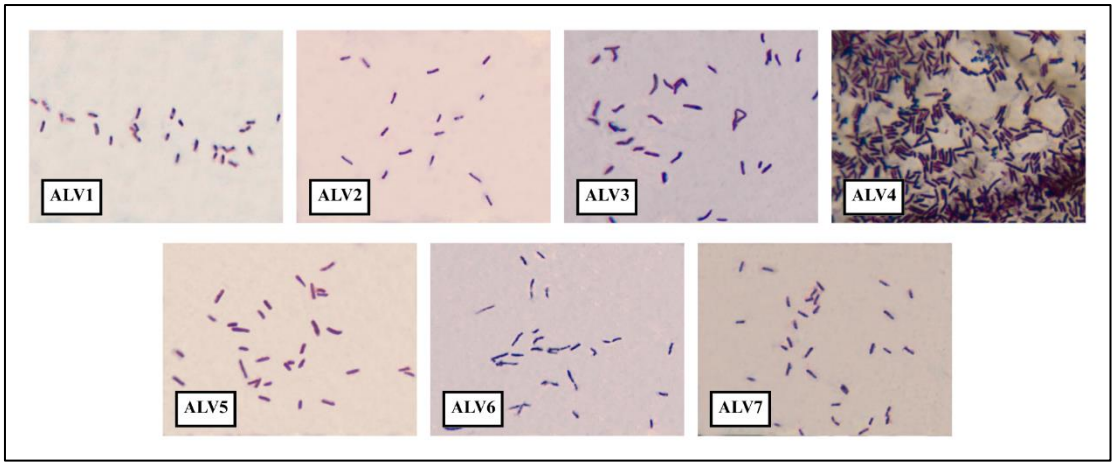

Figure 1 Gram staining observation of seven thermophilic bacterial isolates at 1000x magnification

3.2.2. Spore staining

All isolates showed similar results in spore staining. Microscopic examination at 1000× magnification revealed green-colored spores and red-colored vegetative cells (Figure 2). Spore staining is a standard technique used to identify endospores, which are highly resistant structures formed by certain types of bacteria, particularly those belonging to the genera *Bacillus* and *Clostridium*. In this study, the Schaeffer-Fulton method was employed, which uses malachite green as the main dye and safranin as a contrast dye. Malachite green will penetrate the spores, while the vegetative cell part will be stained by safranin. As a result, the spores will appear green, while the vegetative cells will be red [20].

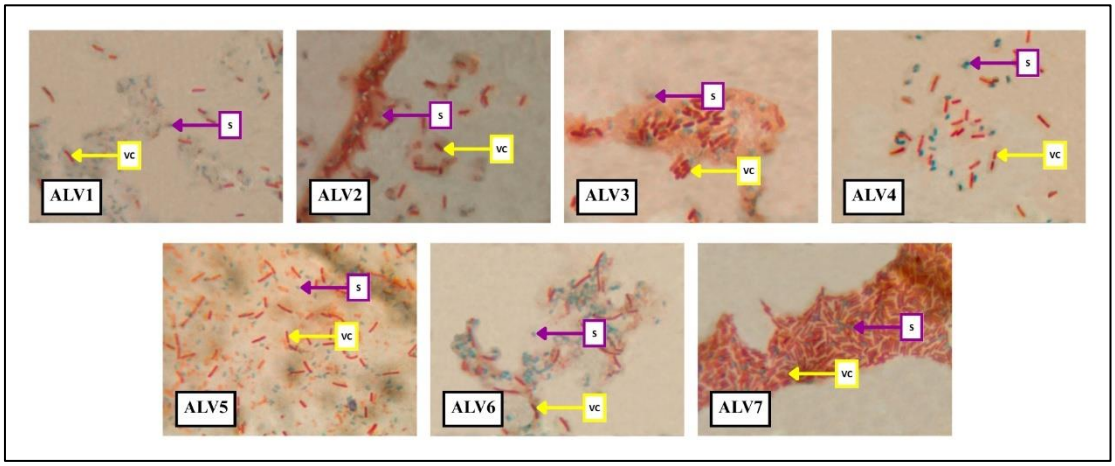

Figure 2 Spore staining observation of seven thermophilic bacterial isolates at 1000x magnification. (S) spore; (VC) vegetative cell

According to [21], Gram-positive bacteria form spores as a response to unfavorable environmental conditions, such as nutrient limitation, chemical exposure, high temperatures, radiation, or desiccation. In this study, the seven isolates formed spores as a response to high temperatures or extreme heat of the composting process. Spores serve as a dormant form that is highly resistant to environmental stress, allowing bacteria to survive for long periods until conditions become favorable for growth. This ability is closely related to the thick, peptidoglycan-rich structure of the Gram-positive cell wall, which provides additional protection and stability during the sporulation process [22].

3.3. Enzymatic activity assay of thermophilic bacteria

3.3.1. Amylase activity assay

In this study, all seven thermophilic bacterial isolates (ALV1–ALV7) demonstrated the ability to degrade starch substrates in media, as indicated by the formation of clear zones around their colonies (Figure 3). The formation of clear zones around the colonies indicates that the seven thermophilic bacterial isolates are capable of producing amylase enzymes [11].

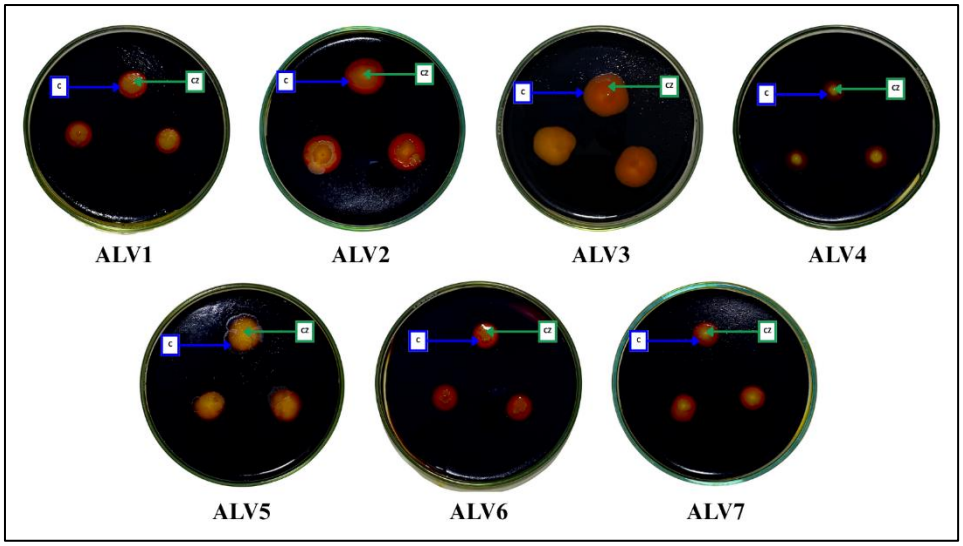

Figure 3 Result of amylase enzyme activity assay for seven isolates. (C) colony; (CZ) clear zone

The amylase enzyme secreted by bacteria will break down starch molecules in the media into simple sugars such as maltose and glucose. The addition of iodine solution to the plates after incubation will react with the starch molecules in the media so that a blue-black color will appear. However, if the starch in the media has been hydrolyzed by the amylase enzyme secreted by bacteria, then iodine will not react to form a blue-black complex. As a result, a clear zone forms around the bacterial colony indicating that the bacteria have produced amylase and hydrolyzed starch [23].

3.3.2. Cellulase activity assay

Positive cellulase enzymatic activity assay is indicated by the formation of a clear zone around the colony after the medium containing carboxymethyl cellulose (CMC) is stained with Congo red [24]. The clear zone formed around the colony indicates the presence of cellulose degradation activity in the media by the cellulase enzyme produced by the bacteria [25]. In this study, only five out of the seven thermophilic bacterial isolates were capable of producing cellulase enzymes (Figure 4). The five isolates were ALV1, ALV2, ALV3, ALV5, and ALV6.

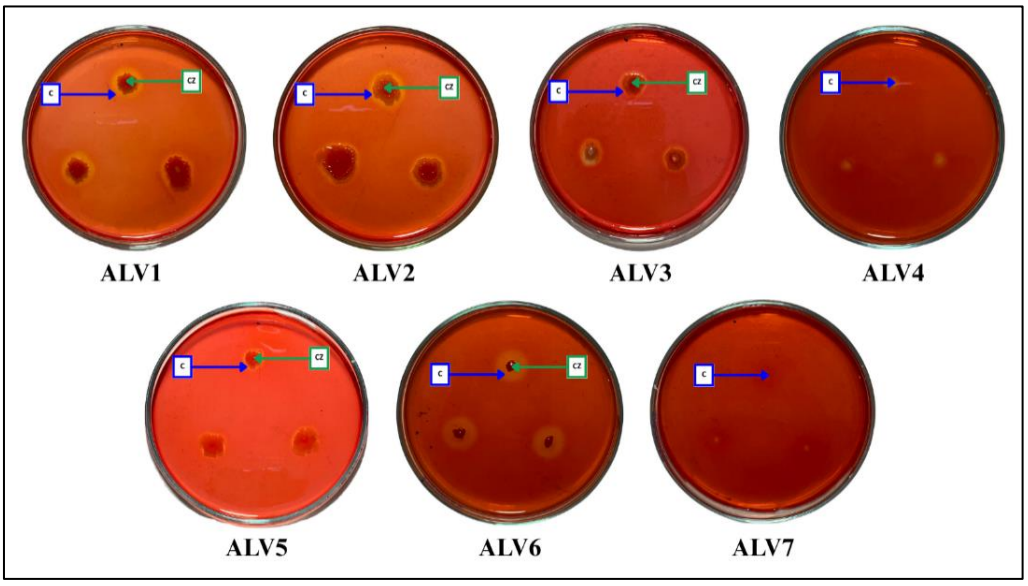

Figure 4 Result of cellulase enzyme activity assay for seven isolates. (C) colony; (CZ) clear zone

This study found that only isolates ALV 4 and ALV7 did not produce clear zones around the colonies in the cellulase activity assay. According to [26], the absence of a clear zone in the enzymatic activity assay is related to enzyme production by bacteria, which can be influenced by several factors such as pH and temperature. In addition to pH and

temperature, according to [27], bacterial enzyme production can also be influenced by species and growth rate in the medium.

3.3.3. Protease activity assay

In this study, it was found that all seven thermophilic bacterial isolates (ALV1–ALV7) assayed were capable of producing protease enzymes. This was indicated by the formation of clear zones around the colonies after the incubation period (Figure 5).

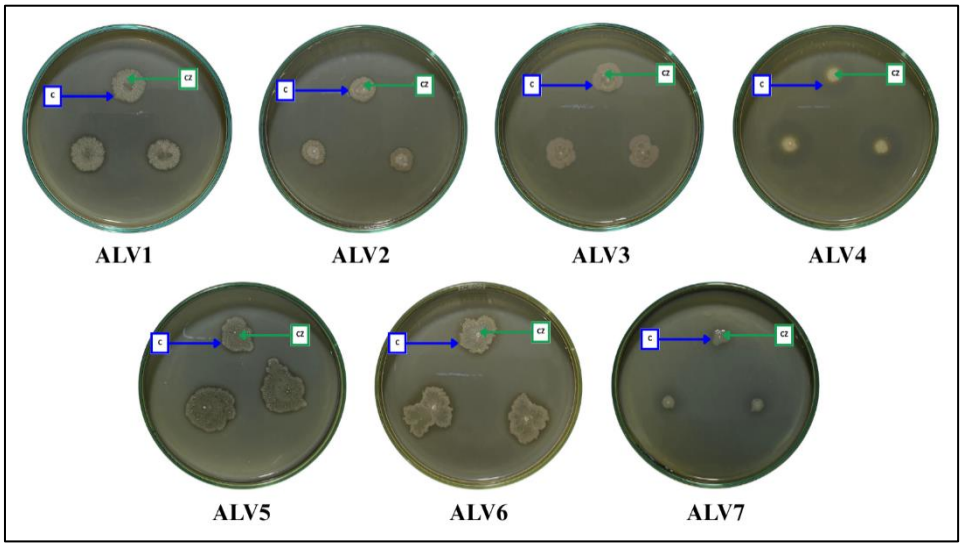

Figure 5 Result of protease enzyme activity assay for seven isolates. (C) colony; (CZ) clear zone

Positive results in the protease enzymatic activity assay are indicated by the formation of a clear zone around the colony [11]. The formation of a clear zone around the colony in the protease activity assay indicates the hydrolysis of peptide bonds in casein or milk protein in the medium, which previously had an opaque (cloudy) appearance, becoming clear. As a result, the portion of the medium that has not undergone hydrolysis will appear cloudier [28].

3.3.4. Catalase activity assay

The catalase assay in Figure 6 shows positive results for all seven thermophilic bacterial isolates (ALV1–ALV7), indicated by the formation of air bubbles after the addition of 3% H_2O_2 (hydrogen peroxide) solution to the bacterial cultures. This finding is consistent with the research by [29], who also reported that four thermophilic bacterial isolates obtained from compost pile samples collected from residential areas in Vereeniging, South Africa, exhibited bubble formation indicating a positive reaction in the catalase assay.

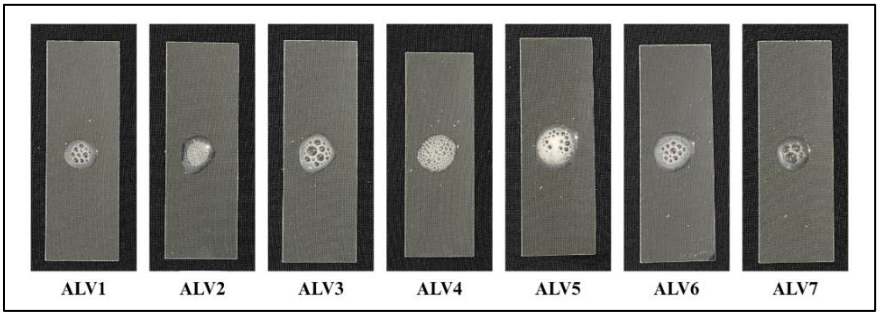

Figure 6 Result of catalase enzyme activity assay for seven isolates

The appearance of air bubbles when 3% H_2O_2 is dripped onto the tested thermophilic bacterial isolate indicates the formation of oxygen gas (O_2) due to the breakdown of H_2O_2 by the catalase enzyme produced by the bacteria [30]. Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide into water and oxygen, while hydrogen peroxide is toxic to cells because it can deactivate enzymes in cells. Catalase-positive bacteria can be interpreted as aerobic bacteria because hydrogen peroxide is formed during aerobic metabolism, so aerobic bacteria must decompose

toxic hydrogen peroxide [31]. According to [32], during the composting process, the catalase enzyme produced by bacteria plays a role in breaking down hydrogen peroxide and reducing its toxic effects on other microorganisms. Furthermore, the presence of catalase activity may also reflect the degree of oxidation occurring during composting [33].

3.4. Enzymatic index (EI)

The results of the average colony area and average clear zone area calculations in the amylase activity assay (Table 2) show differences among the thermophilic bacterial isolate. The highest average colony area and average clear zone area were produced by ALV3 (2.93 cm^2 and 3.2 cm^2), while the lowest were observed in ALV6 (0.2 cm^2 and 1.5 cm^2). Interestingly, isolate ALV6 exhibited the highest enzymatic index (EI) value of 8.38, whereas the lowest EI value, 0.09, was observed in isolate ALV3. This condition indicates that the extracellular enzymes produced by the isolate with the smallest average colony area are more efficient in degrading the surrounding substrate compared to isolates with similar average values of both colony area and clear zone area.

The study by [34] reported that two selected isolates produced amylase enzymes with amylolytic index values ranging from 3.04 to 3.52 at 55°C. This suggests that ALV6 has an enzymatic index significantly higher than the reported average. Therefore, ALV6 deserve attention as a promising candidate in the exploration of amylase-producing microorganisms, particularly for applications in composting processes.

Table 2 Average colony area, average clear zone area, and enzymatic index values from amylase, cellulase, and protease activity assay of seven thermophilic bacterial isolates

Type of Enzymatic Activity Assay	Isolate Codes	ATC (cm^2)	ATZ (cm^2)	EI
Amylase	ALV1	0,8	1,53	0,99
	ALV2	1,4	2,86	1,06
	ALV3	2,93	3,2	0,09
	ALV4	0,33	1,56	3,75
	ALV5	1,63	1,96	0,2
	ALV6	0,2	1,5	8,38
	ALV7	1	1,53	0,53
Cellulase	ALV1	1,33	2,13	0,63
	ALV2	1,33	1,76	0,33
	ALV3	0,66	1,2	0,87
	ALV4	0,26	0	-
	ALV5	1,16	1,4	0,2
	ALV6	0,1	1,56	14,66
	ALV7	0,1	0	-
Protease	ALV1	2,1	2,76	2,1
	ALV2	0,86	1,5	0,73
	ALV3	1,53	1,9	0,24
	ALV4	0,73	3,33	4,39
	ALV5	2,9	3,83	0,32
	ALV6	2,83	4,5	0,58
	ALV7	0,4	1,9	3,54

(ATC) average total colony area; (ATZ) average total clear zone area; (EI) enzymatic index; (-) does not produce enzyme.

The results of the average colony area and average clear zone area calculations in the cellulase activity assay (Table 2) show variations among the thermophilic bacterial isolate. The highest average colony area and average clear zone area were produced by ALV1 (1.33 cm^2 and 2.13 cm^2), while the lowest were observed in ALV7 (0.1 cm^2 and 0 cm^2). The highest EI value was obtained from ALV6, at 14.66, while the lowest was from ALV5, at 0.2. Isolates ALV4 and ALV7 did not have EI values. This was because in the cellulase activity assay, both isolates did not produce clear zones, making the calculation of their enzymatic index values impossible.

Compared to a previous study by [35] which reported that the cellulolytic index ranged from 2.61 to 3.42 for bacterial isolates grown at 55°C, ALV6 demonstrated significantly higher enzymatic activity than average. This highlights ALV6 as a promising candidate for further exploration as a cellulase enzyme producer, particularly in applications involving the processing of lignocellulosic waste during composting.

Similarly, the results of the protease activity assay (Table 2) revealed notable differences among the thermophilic bacterial isolates. The highest average colony area was produced by ALV5 (2.9 cm^2), and the highest clear zone area was produced by ALV6 (4.5 cm^2), while the lowest colony area was recorded for ALV7 (0.4 cm^2), and the lowest clear zone area for ALV2 (1.5 cm^2). The highest EI value in this assay was obtained from ALV4 (4.39), and the lowest from ALV3 (0.24).

A study by [36] reported that thermophilic bacterial isolates incubated at 55°C produced proteolytic index ranging from 1.09–3.50. When compared to these values, the enzymatic index of ALV4 was higher than those reported in previous studies. Therefore, ALV4 has great potential as a source of thermophilic proteases.

The higher number of positive results in the amylase and protease activity assays compared to the cellulase activity assay suggests that starch and protein are the predominant substrates present in the surface layer compost of the processing stage during composting at PT GGP's Compost Plant. However, cellulose may also be present in considerable amounts. These substrates primarily originate from PT GGP's production waste, such as bromelain, cassava peels, and livestock manure. This is further supported by the presence of thermophilic bacterial isolates exhibiting high and diverse EI values. According to [37], variations in EI values among isolates may be influenced by differences in bacterial species.

4. Conclusion

Based on this study, seven thermophilic bacterial isolates were obtained from surface layer of compost samples during the processing stage at PT Great Giant Pineapple's Compost Plant, namely ALV1, ALV2, ALV3, ALV4, ALV5, ALV6, and ALV7. The colony morphology of the seven isolates was dominated by irregular and spreading forms with varied margins, such as irregular, wooly, undulate, and entire. Most colonies showed raised elevations and shared a uniform cream coloration. Microscopically, all isolates were rod-shaped, Gram-positive, and capable of forming spores. Isolates ALV1, ALV2, ALV3, ALV4, ALV5, ALV6, and ALV7 demonstrated the ability to produce amylase and protease enzymes, while only ALV1, ALV2, ALV3, ALV5, and ALV6 were capable of producing cellulase enzymes. The highest enzymatic index (EI) values for amylase and cellulase were observed in ALV6, at 8.38 and 14.66, respectively, whereas the highest protease EI value was recorded in ALV4, at 4.39.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest to be disclosed.

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