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Exploration Of Thermophilic Bacteria in The Basal Zone of Compost Through Isolation and Enzymatic Physiological Characterization

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Abstract: *Composting Is one example of aerobic and anaerobic waste treatment. The composting process generally takes place in several stages that canbe recognized from changes in temperature by microorganisms that degrade organic matter. Degradation of organic matter in compost at PT Great Giant Pineapple can be done with the help of thermophilic bacteria. Thermophilic bacteria have the ability to survive and adapt to extreme temperatures to produce an enzyme in the form of extracellular enzymes. The results showed that there were 4 isolates that had different morphological characteristics. The four isolates have a bacillus cell shape, are Gram positive, and have endospores. The four isolates of thermophilic bacteria are able to produce catalase enzyme, amylase enzyme, cellulase enzyme, and protease enzyme except SLI and SL3 isolates. The highest amylaseenzymaticindexvaluebyisolateSL1was6.06;thehighestcellulaseenzymaticindexby isolate SL2was2,03;andproteaseenzymaticindex byisolateSL4was11,11*

Keywords:Composting,thermophilicbacteria,isolation,characterization,enzymaticindex

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I. INTRODUCTION

Composting is an efficient process whereby organic waste can be recycled. The composting processcan be carried out aerobically oranaerobically. Both processes produce organic fertilizer in the form ofcompost. The composting process has parameters that need to be considered, namely temperature, moisture content, pH, and time. Scientifically, the composting process is characterized by four main phases: the mesophilic phase, the thermophilic phase, the cooling phase or mesophilic II phase, and the maturation phase (Papale *et al*., 2021). During the mesophilic phase, the compost temperature rises to around 40° C due to the presence of acid-forming bacteria. Next, the compost temperature will rise to enter the thermophilic phase between 40–70° C, which is dominated by thermophilic bacteria and involves the degradation of organic material. During the cooling phase, the compost temperature will decrease to 40–45° C, and in the maturation phase, the compost temperature will drop to ambient temperature 20–30° C.

Thermophilic bacteria are a group of bacteria that can live at high temperatures. Thermophilic bacteria are known to be able to survive in high temperatures because they contain thermostable extracellular enzymes. Thermophilic bacteria play a role in the ecosystem as agents of biodegradation of various organic materials because they possess these enzymes. The types of extracellular enzymes in thermophilic bacteria include protease, amylase, cellulase, and catalase. Thermophilic bacteria are commonly found invarious habitats suchas hot springs, volcanoes, compost, and various geothermal areas (Rukmi *et al*., 2018).

Observationofbacteriacan be carried out ifthebacteriaareseparated fromtheirenvironment and other microorganisms. Bacterial isolation is an attempt to grow bacteria outside their natural environment. The separation of bacteria outside their environment is intended to obtain a pure bacterial culture. This separation is carriedout bygrowingthemonsolidmedia,sothat permanent cell colonies will form.This studyaims toisolate thermophilic bacteria in the decomposition phase of compost and to determine their macroscopic and microscopic morphological characteristics, enzymatic characteristics, and Enzymatic Index (EI) at the Compost Plantof PT. Great Giant Pineapple. Thermophilic bacteria are isolated bygrowingthemina mediumtailored to the needs of theisolatedbacteria. PT. Great Giant Pineapple requires a bacterial inoculumto help accelerate the

wastecompostingprocesssothatthedecompositionoforganicmaterialscantakeplacemorequicklyand efficiently.

II. EXPERIMENTALPROCEDURE

Thisresearch wasconductinDecember2024–March2025attheResearch andDevelopment Laboratory, PT. Great Giant Pineapple, Central Lampung.

Sampling

Compost samples were taken from the bottom layer of compost at the PT. Great Giant Pineapple Compost Plant bydiggingdown to a depth of 2 meters fromthe surface. Compost samples were taken fromthe samepoint three times, with25 grams takeneachtime,andthen placedinsterileheat-resistant plasticbags. The compost samples were then stored in a storage box for isolation in the laboratory.

MacroscopicandMicroscopicIsolationandCharacterizationofThermophilicBacteria

Thesample was weighed at25gandthendissolvedin225 mLof0.9%NaClphysiologicalsolutionfor a 10-1 dilution. One milliliter was then taken using a micropipette and added to another test tube containing 0.9% NaCl physiological solution, then homogenized with a vortex for a 10-2 dilution. The procedure was repeatedinthesame manneruntil a10-9dilutionwas obtained.Isolationwas performedbytaking0.1 mLofthe dilution and pipetting it onto a Petri dish containing NB medium + 6% agar, then spreading it evenly. Two replicates were performed for each dilution. Incubation was carried out at 500 C for 24 hours. After isolation, bacterial purification was performed to obtain single colonies. Macroscopic observations were made after 24 hours of incubation on agar media, including the shape, elevation, edges, and color of the growing colonies. Microscopic identification of the bacteria was performed using a 3% KOH test, Gram staining, and endospore staining.

3%KOHtest;

The 3% KOH test is performed by placing 1 drop of 3% KOH solution on a glass slide, then taking 1 bacterial isolate and homogenizing it. Apositive test result is indicated by the formation of mucus on the glass slide. The purpose of the KOH test is to determine whether the bacteria obtained are Gram-negative or Gram- positive (Dash & Payyapilli, 2016)

Gramstaining

Abacterial cultureis taken using1 loop onto a glass slidein an aseptic manner, then spread evenly and fixed. The preparation is then stained with crystal violet and left for 1 minute, then rinsed with runningdistilled water and air-dried. The preparation is stained with Lugol's iodine and left for 1 minute, then rinsed withrunning distilled water andair-dried.The specimen is then stainedwith 70% alcohol and left for 30 seconds until the layer appears paler. Finally, the specimen is stained with safranin, left for 1 minute, then rinsed with flowing distilled water and air-dried.

EndosporeStaining

Abacterial culture was taken using a 1-inch loop onto a glass slide in an aseptic manner, then spread and fixed.Thespecimenisthenstained with malachite greenfor10 minutes,rinsed withrunningdistilledwater, and air-dried. Thespecimenis thenstained withsafraninfor10seconds,rinsed withrunningdistilledwater,and air- dried. The specimen can then be observed under a microscope, starting from the lowest magnification to the highest (Agustina dkk, 2013).

AmylaseEnzymeActivityTest

Amylase enzyme activity tests were conducted to identify the bacterial groups that produce amylase enzymes. The purified bacterial culture was streaked with 1 loop and then inoculated usingthe spot method on starch agar medium and incubated at 50°C for 24 hours. After incubation, the isolate was treated with a 1% iodine solution. A positive amylase enzyme test result is indicated by the formation of a clear zone around the growing colony (Saidan *et al*., 2024).

ProteaseEnzymeActivityTest

Protease enzyme activity tests were conducted to identify the groups of bacteria that produce protease enzymes. Purified bacterial cultures were streaked with 1 loop andthen inoculated usingthe dot method on NA media containing3% skim milk and incubated at 60^0 C for 24hours.Positive protease enzyme test results were indicated by the formation of a clear zone around the growing colonies (Saidan *et al*., 2024).

CellulaseEnzymeActivityTest

Cellulase enzyme activity tests were conducted to identify the bacterial groups that produce cellulase enzymes.Purifiedbacterial cultures werestreaked with1loopandtheninoculatedusingthespot methodonNB + agar5% mediumcontaining1%CMC and incubated at 60^0 C for24 hours. Afterincubation, theisolates were washed with congo red solution for 15 minutes, then discarded and rinsed with 1 M NaCl for 15 minutes. A positive cellulase enzyme test result is indicated by the formation of a clear zone (Saidan *et al*., 2024).

CatalaseEnzymeActivityTest

The catalase enzyme activity test was conducted to identify groups of bacteria that produce the catalase enzyme. This was done byplacinga drop of 3% H2O2 solution on a glass slide, then addinga bacterial colony culture and homogenizingit. The catalase enzyme test result was considered positive if air bubbles formed and negative if no bubbles formed (Khatoon *et al*., 2024).

DeterminationofEnzymaticIndex(EI)

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.According toSumardi *et al* (2021), the gravimetric method can be calculated as follows:

1. usingcolonypatternsandclearzones(replicas)drawnonclearplasticmica
2. colonyreplicasandclearzoneswereweighedusingananalyticalbalance
3. cuta1cmx1cmpieceofpaperandweighit
4. calculatetheareaofthecolonyandclearzoneusingthefollowingformula.

$$\text{ColonyArea}=\frac{\boldsymbol{Weightofcolonyreplica}}{\boldsymbol{Paperweight1cmx1cm}}\text{x1 cm}^2$$

$$\text{ClearZoneArea}=\frac{\boldsymbol{Weightofclearzonereplica}}{\boldsymbol{Paperweight1cmx1m}}\text{x1cm}^2$$

5. Thecalculationresultsarethenenteredintotheenzymaticindexformula:

$$\text{EnzymaticIndex}=\frac{\boldsymbol{Clearzonearea}-\text{Colonysize}}{\boldsymbol{Colonysize}}$$

III. RESULTSANDDISCUSSIONS

MacroscopicandMicroscopicCharacterizationofThermophilicBacteria

Macroscopic observations were conducted on four selected isolatesoriginatingfromcompost samples from the bottomlayer of the decomposition phase. The observations consisted of colony shape, colony color, colony edge, and colony elevation, as shown in Table 1.

Table1.MacroscopicCharacteristicsofThermophilicBacteria

IsolateCode	MorphologicalCharacteristics			
	Form	Color	ColonyEdge	Elevation
SL1	*Wrinkled*	Cream	*Irregular*	*Flat*
SL2	*Round*	Cream	*Undulate*	*Flat*
SL3	*Round*	Cream	*Cilliate*	*Flat*
SL4	*Round*	Cream	*Entire*	*Flat*

Based on Table 1, the morphology of bacterial isolates obtained from the bottom layer of compost during the decomposition phase had the same elevation and colony color, namely flat and cream-colored. Isolate SL1 has a wrinkled colony shape with irregular colony edges, isolate SL2 has a round colony shape with undulate colony edges, isolate SL3 has a round colony shape with ciliate colony edges, and isolate SL4 has a round colony shape with entire colony edges.

3%KOHTest

The results of the 3% KOHtest showed that all isolatesgave negative results, indicated bythe absence of mucus formation. These negative results indicate that the four thermophilic bacterial isolates have Gram- positive properties, as described in Table 2.

Table2.3%KOH TestforThermophilicBacteria

Morphological Characteristics	IsolateCode SL1	SL2	SL3	SL4
3%KOHTest	-	-	-	-

Description:

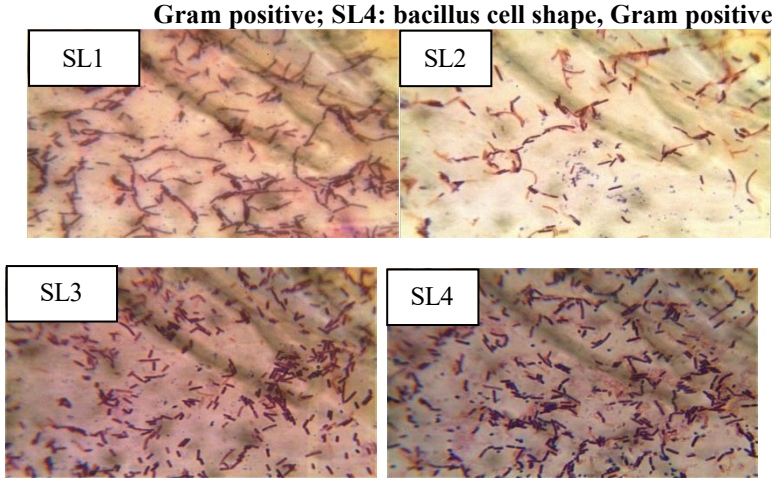
- 3%KOHtest(-):Gram-positive

Testing 3% KOHon bacteria indicates that gram-positive(+)bacteria havethick cell walls and thin fat, while gram-negative(-)bacteriahavethickfat andthincell walls.KOHreacts withfat (lipidbilayer) andcauses gram-negative cells to rupture. The ruptured cells will release genetic material DNA, which is abundant in bacterial cells. The results of the 3% KOHtest indicate thatthermophilic bacterial isolatesin the decomposition phase of the bottom layer have cell walls that can withstand alkaline solutions (3% KOH), so no slime is produced. Conversely, if the bacterial isolate being tested isGram-negative, the alkaline solution (KOH 3%)will break down the bacterial cell wall and release substances that formslime (Agung *et al*., 2024).

GramStaining

Gram staining was performed on thermophilic bacterial isolates to determine cell shape and bacterial propertiesat1000xmagnification. (Figure1)showsthatallfourisolateshavethesame Gramproperties,namely positive, marked by cells stained purple, and a uniformcell shape, namely bacilli.

Figure1. Results ofobservations ofcell shapeand Gramstaining offourthermophilicbacterial isolates: SL1:bacillus cell shape,Grampositive;SL2:bacillus cell shape, Grampositive;SL3:bacillus cell shape, Gram positive; SL4: bacillus cell shape, Gram positive

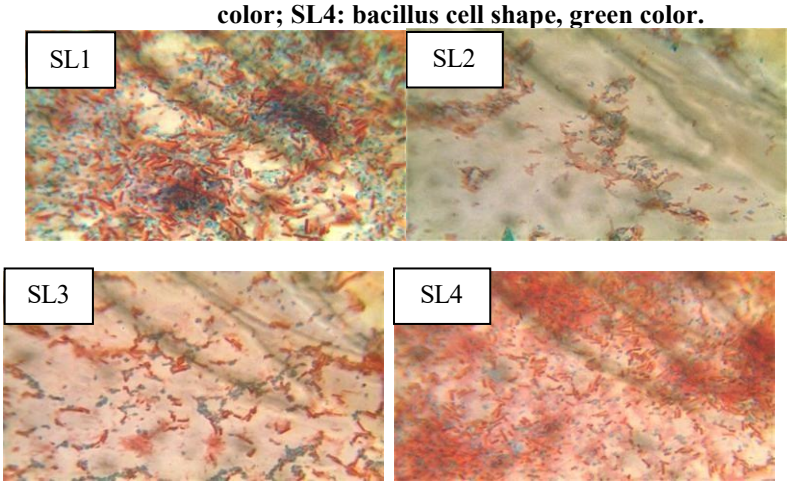


Gram staining distinguishes between Gram-positive and Gram-negative bacteria by staining the cells purple (Gram-positive) or red (Gram-negative). The Gram staining technique provides insights into the morphological shape and structure of bacteria (Paray *et al*., 2023). The characterization results indicate that thermophilic bacteria exhibit Gram-positive properties, supported by Tan *et al*., (2023) research, which found that microorganisms most commonly found in thermophilic compost phases typically belong to the genus Bacillus, which are Gram-positive rod-shaped bacteria.

EndosporeStaining

Endosporestainingwas performed to determine whetherthebacterial isolates had theabilityto produce spores. Observation of (Figure 2) at 1000x magnification of the four thermophilic bacterial isolates found showed that all four isolates were capable of producing green spores and red vegetative cells.

Figure2. Results ofobservations ofsporeshapeand coloroffourthermophilicbacterial isolates,namely SL1:bacillus cell shape,green color;SL2:bacillus cellshape,green color;SL3:bacillus cell shape,green color; SL4: bacillus cell shape, green color.

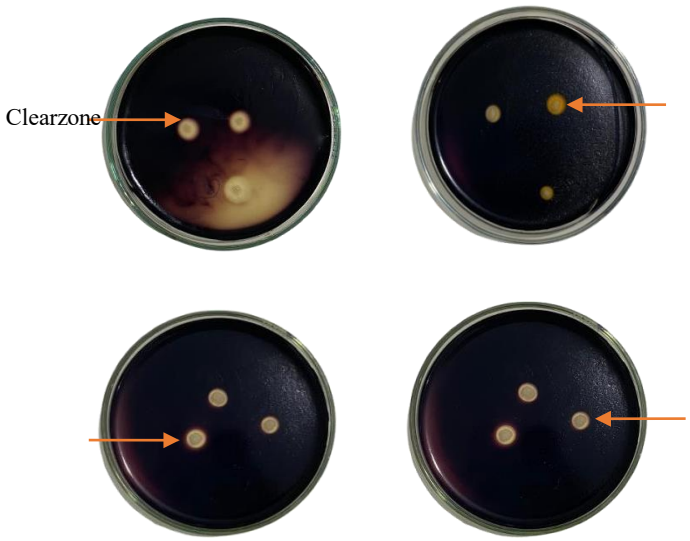


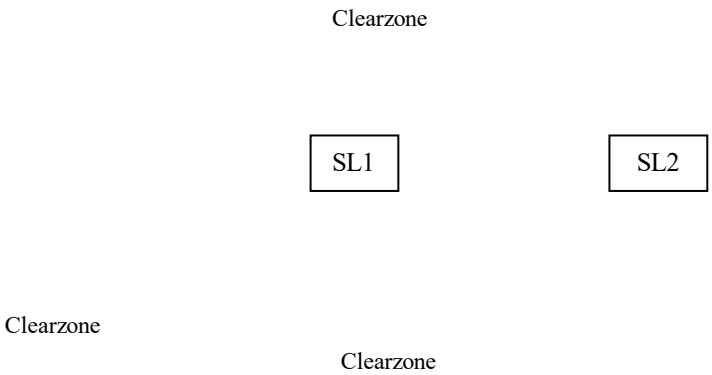
Endospore staining aims to determine whether bacterial isolates have the ability to produce spores or not. All four thermophilic bacterial isolates from the decomposition phase of the compost base have the ability to form endospores, indicating that the presence of endospores in the four isolates is a form of adaptation to the physicaland chemicaltreatmentsof anextreme environment,such ashigh ambienttemperatures(thermophilic). This is in accordancewith Prescott *et al*, 2002; Pratiwi, (2022), who state that bacterial endospores arestructures that are resistant to extreme conditions and that some spore-forming bacteria are found in soil. Generally, endospore formation is carried out by Gram-positive bacteria. Gram-positive bacteria such asBacillus are capable of formingendospores because theyhave thick cell walls and the genetic abilityto activate the sporulation process. Endospores produced by Gram-positive bacteria allow the bacteria to remain dormant (inactive) for a long time until environmental conditions improve, at which point they germinate into active bacterial cells (Pereira, 2014).

AmylaseEnzymeActivityTest

The results of the amylase enzyme test in(Figure 3) show that all four isolates were able to form clearzones around the bacterial colonies with varying clear zone areas. Positive results in the amylase enzyme test indicate that the bacterial isolates obtained fromthe bottomlayer of the decomposition phase compost have the ability to degrade amylum, thereby forming clear zones.

Figure3.Results ofamylaseenzymetestingon thermophilicbacterial isolates,namelySL1:clearzone formed; SL2: clear zone formed; SL3: clear zone formed; SL4: clear zone formed




Clearzone
SL1
SL2
Clearzone
Clearzone

SL3

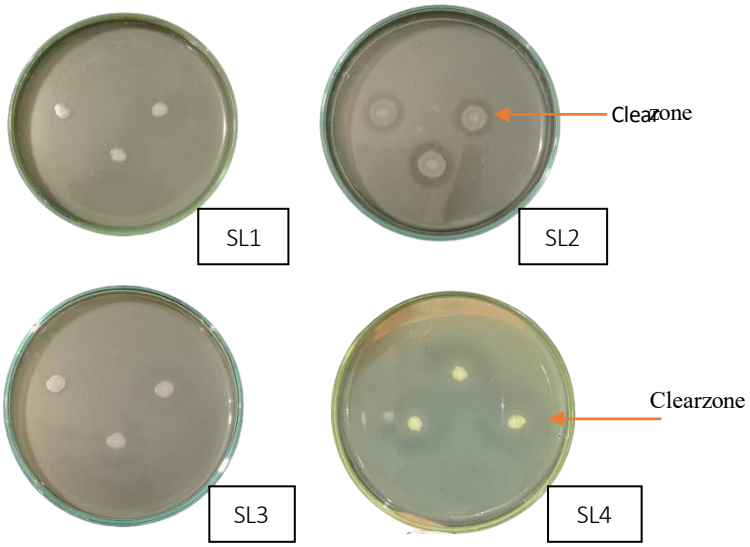
SL4

The clear zoneis formed becausetheamylaseenzymehas theabilityto hydrolyzestarch in solid mediainto simple compounds. To clarify the presence of the clear zone, the starch media that has been colonized by bacteria is sprayed with Lugol's iodine. The area outside theclear zone will turn purple-blue after beingsprayed with the solution, because Lugol's iodine will react with the unhydrolyzed starch. The clear zone is not colored because the starch in that zone has been hydrolyzed into simpler compounds such as disaccharides or monosaccharides. Amylase is a group of extracellular enzymes that induce substrates. The size of the clear zone produced depends on the amount of glucose monomers produced fromthe starch hydrolysis process. Therefore, the greater the number of glucose monomers produced, the larger the clear zone that will form around the bacterial colony.

ProteaseEnzymeActivityTest

The results of theprotease enzyme test in (Figure 4) showthatisolateSL1 was unable toforma clear zone, isolateSL2 was ableto forma clear zone, isolateSL3 was unableto forma clearzone, and isolateSL4 was able to form a clear zone. The clear zones formed in isolates SL2 and SL4 indicate that thermophilic bacteria in the decomposition phase of compost have the ability to produce protease.

Figure4.Results ofproteaseenzyme tests on thermophilicbacterial isolates,namelySL1:noclearzone formed; SL2: clear zone formed; SL3: clear zone formed; SL4: no clear zone formed

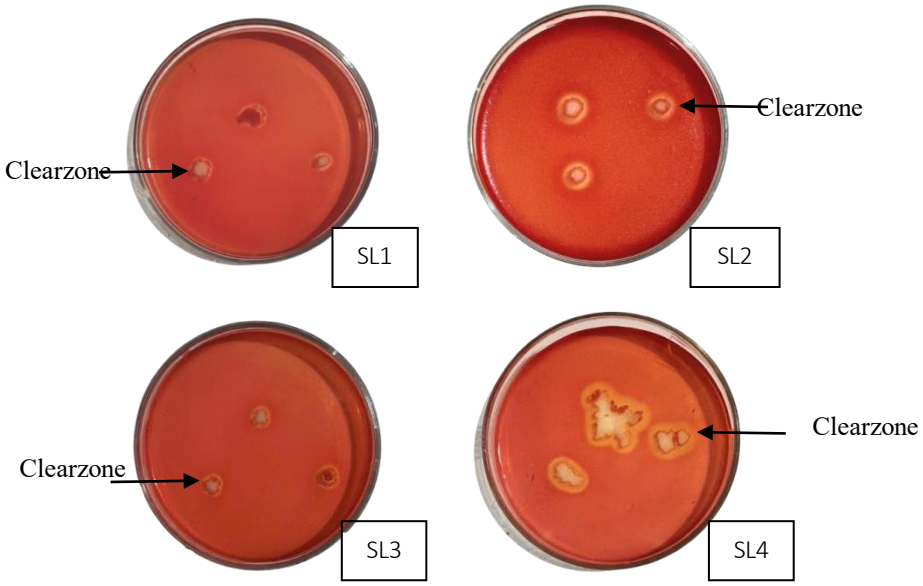


The clear zonethat forms is causedbybacterial colonies that havetheabilitytosecreteneutral proteaseinto their environment, causing the proteins in skim milk to be hydrolyzed and resulting in a clear zone around the colony. Soeka & Sulistiani (2017) added that the formation of a clear zone produced by test bacteria indicates that the bacteria are capable of producing extracellular protease enzymes. Of the four isolates tested, only two isolates were able to formclear zones. The isolates that didnotproduce clear zones were due to several factors, namely differences in bacterial types, the growth rate of each isolate on the medium, and the type of enzyme produced. Litrinopiza *et al*. (2021) also noted that the factors causing the absence of a clear zone include incompatibility between the casein substrate in skim milk medium and the protease produced, or the isolate simply does not produce protease enzymes.

SelulaseEnzymeActivityTest

The results of the cellulase enzyme test in (Figure 5) show that the four isolates, SL1, SL2, SL3, and SL4, have the ability to form clear zones around the colonies. This indicates that thermophilic bacteria at the bottom of the decomposition phase have the ability to degrade cellulose.

Figure5. Results ofcellulaseenzymetestingon thermophilicbacterial isolates,namely SL1:clearzone formed; SL2: clear zone formed; SL3: clear zone formed; SL4: clear zone formed

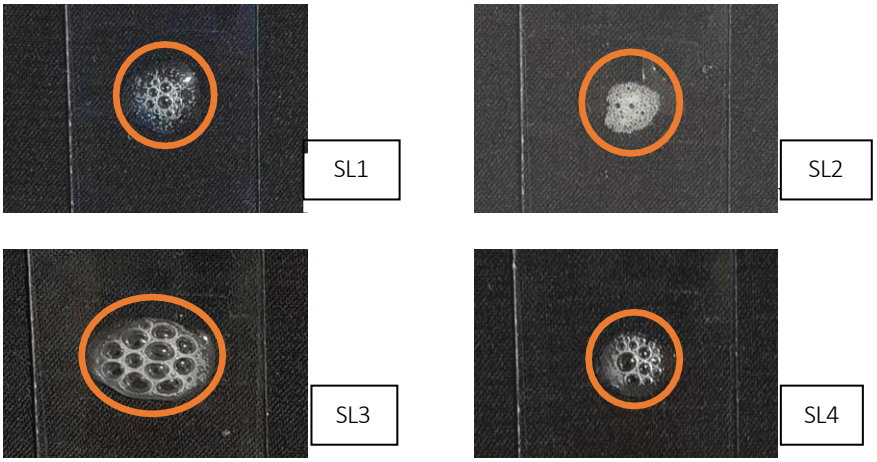


The formation of a clear zone indicates that the bacterial isolate has the ability to degrade cellulose. CMC (carboxymethyl cellulose) is used as a substrate to detect the presence of cellulase enzymes. In this reaction, CMC will be hydrolyzed by cellulase into glucose. The hydrolysis test result is considered positive if a clear zone forms around the bacterial colony when congo red is poured. Congo red is commonly used for staining microorganisms that can produce cellulase enzymes. After applyingcongo red, rinsing with NaCl is performed to remove congo redthat is not bound to polysaccharides.Rinsing with NaCl serves to remove unusedcongo red dye and to clarify the clear zone that forms, as congo red is the sodium salt of benzidinediazo-bis-1- naphthylamine-4-sulfonic acid (C32H22N6Na2O6S2) and is therefore soluble and rinsable by other sodium salts. Theclear zoneformed on the mediumis dueto thebacterial isolatehydrolyzingtheβ-1,4 glycosidicbonds into glucose, which forms the clear zone (Majidah *et al*., 2023).

CatalaseEnzymeActivityTest

The results of the catalase enzyme test in (Figure 6) show that all four isolates reacted positively, as indicated by the formation of gas bubbles after the bacterial isolates were dripped with hydrogen peroxide solution H2O2. The test results indicate that thermophilic bacteria in the decomposition phase of compost have the abilityto produce catalase enzymes that function tobreak down hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2).

Figure6.Resultsofcatalaseenzymetestsonthermophilicbacterialisolates,namelySL1:bubblesformed (a); SL2: bubbles formed (b); SL3: bubbles formed (c); SL4: bubbles formed (d)



The enzyme catalase plays an important role in reducing oxidative stress by catalyzing the breakdown of hydrogen peroxide (H2O2) into water and oxygen (Rasheed, 2024). The enzyme catalase is a hemoprotein consisting of four heme groups. It is the heme that enables catalase to react with peroxide compounds. Under certain conditions, bacteria will produce hydrogen peroxide. Hydrogen peroxide is a toxin that can damage the metabolicsystemofbacteria, causingthemto die iftheyareunable to break down hydrogen peroxideinto other harmlesscompounds.Hydrogenperoxide canbe brokendowninthepresence ofthe enzyme catalase (Pulungan *et al*., 2018).

DeterminationofEnzymaticIndex(EI)

The clear zone formed was then measured for its area and the colonies formed to obtain the enzymatic index. Table3 shows theresults oftheenzymaticindex calculations fromseveral enzyme activitytests that have been conducted.

Table3.ResultsofEnzymaticIndexCalculationsforThermophilicBacterialIsolates

Isolatecode	EnzymaticIndex(EI)		
	AmylaseEnzyme Activity Test	ProteaseEnzyme Activity Test	CellulaseEnzyme Activity Test
SL1	6,06	-	0,86
SL2	1,1	1,16	2,03
SL3	3,15	-	0,60
SL4	6	11,11	1,13

The highest amylase enzymatic index was produced by isolate SL4 at 6.06, while the lowest amylase enzymatic indexwas 1.1. The highest index obtained was not significantly different from the study conductedbyNovitasari *et al* (2014),whofoundthreeisolates withthesame amylaseenzymeindexof6. Theresults ofthe amylase enzyme index for isolate SL4 indicate that this isolate has an advantage in amylase production, particularly for application in the composting process. This shows that environmental conditions such as temperature and pHare veryinfluential. Duringthe isolation process, amylase-positive isolates were grown in a neutral pH mediumof 6.8, which is in line with the research conducted by Ullah *et al* (2021) that maximum amylaseenzyme activity will occurin thepHrangeof5 to 8. Lowerand higherpHvalues will decreaseenzyme activity.

Intheprotease enzymaticactivitytest,thehighestenzymaticindexwasproducedbyisolate SL4at11.11. Sumardi *et al* (2021) stated that the isolate would produce a clear zone at neutral pH with an average colony areaof0.66 cm^2. ThepHofthe mediumgreatlysupported theformation ofaclearzonein thetested bacterial isolates. The absence of a clear zone in isolates SL1 and SL3 indicates no protease production. However, it cannot beconfirmedthat there areno genes encodingprotease enzymes. It is possiblethat thegenes encoding these enzymes are not expressed due to environmental factors such as pH, osmotic pressure, and temperature that are not suitable for the gene characteristics of the tested isolates, resulting in the isolates not producing protease enzymes and thus no clear zone being formed.

ThehighestenzymaticindexinthecellulaseenzymeactivitytestwasobtainedbyisolateSL2, whichwas 2.03. The bacterial isolate that produced the highest enzymatic index indicates that the bacteria produce cellulaseenzymes. Thisisinaccordance with what wasstatedbyMulyasari *etal*(2015)thatthehighestactivity index value indicates that the bacteria are capable of degrading cellulose well. Accordingto Wahyuni (2017), high enzyme activity values are caused bythe abilityof cellulase enzymes to hydrolyze cellulose into glucose. Majidah *et al* (2023) also added that the clear zone formed on the mediumindicates that the bacterial isolate is capable of hydrolyzing lignocellulose in the mediumcontent, resulting in a large clear zone.

IV. CONCLUSION

Based on the research conducted, four thermophilic bacterial isolates were obtained from the base compost in the decomposition phase, with isolate codes SL1, SL2, SL3, and SL4. The macroscopic characteristics of the fourisolatesobtained have different morphologies with cream-colored colonies.SL1 has a wrinkled colony shape, irregular colony edges, and a flat elevation; SL2 has a round colony shape, undulate colonyedges, and a flat elevation; SL3 has around colonyshape, ciliate colonyedges, and a flat elevation; SL4 has a round colony shape, entire colony edges, and a flat elevation. The microscopic characteristics of the four isolates include bacillus-shaped cells, Gram-positive nature, and the presence of endospores. All four isolates were capable of producingcatalase enzymes, while isolates SL1,SL2, SL3, and SL4 were capable of producing amylaseenzymes,withthehighestEnzymaticIndex(EI)of6.06byisolateSL1.IsolatesSL1andSL4are

capable of producing protease enzymes, with the highestIE of 11.11 by isolate SL4. IsolatesSL1, SL2, SL3,and SL4 are capable of producing cellulase enzymes, with the highest IE of 1.13 by isolate SL4.

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