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Analysis of Amylase Activity in Bacteria Isolated from Hot Springs of Pentadio Resort

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Abstract

The hot spring of Pentadio Resort is one of the geothermal areas likely to be the place of bacteria or microorganisms that can produce amylolytic enzymes. This research aims to analyze bacteria in the Pentadio Resort hot springs that possess the potential to produce amylase enzymes, analyze the characteristics of bacterial isolates that produce amylase enzymes in Pentadio Resort hot springs and determine the types of bacteria that produce amylase enzymes in Pentadio Resort hot springs. The research employs a qualitative and descriptive method. The research involved several methods, including sample collection, media preparation, isolation and selection of thermophilic bacterial isolates, purification of thermophilic bacterial isolates, selection of isolates producing amylase enzymes, identification of selected isolates (motility test, biochemical test, gram staining), and molecular identification. The research yielded seven isolates, and isolate code A6 demonstrated the potential to produce amylase enzymes with a clear zone size of 23,26 mm, bacillus, gram positive, and positive motility. Subsequently, molecular identifidation was carried out in which, based on the results of phylogenetic tree reconstractiopisalate A6 was related to the bacteria Bacillus cereus strain MD 152, which belongs to the B. cereus bacteria

Keywords: *Hot spring, Amylase enzyme, Bacillus cereus, Pentadio Resort*

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INTRODUCTION

Pentadio resort hot spring is one of the geothermal areas that allows the discovery of bacteria, namely microorganisms that can produce amylolytic enzymes. Enzymes themselves are catalysts that can increase the speed of specific chemical reactions, without enzymes a chemical reaction will take place very slowly. Currently, the role of enzymes as catalysts in the industrial field is increasingly important. The use of enzymes in various fields is increasingly widespread, such as the food industry, textile industry, paper industry, agriculture, pharmacy, medicine, and the environment (Irdawaty et al., 2015).

The application of amylolytic enzymes as biocatalysts has been increasing since the last few years. Amylolytic enzymes are generally known as enzymes that catalyze a hydrolysis reaction of starch into dextrin and simple sugars consisting of glucose units (Madonna, 2014). Many industrialized countries have extracted enzymes from various microorganisms. This is because microorganisms produce enzymes that can be utilized by humans in large quantities and of various types. In addition, microorganisms are also easy to cultivate and the growth rate is relatively fast, and the scale of cell production is easier to increase. One type of enzyme that is produced by microorganisms is amylase. Amylolytic bacteria isolated from amylum-rich sources generally have the potential to produce better amylase (Susilawati et al., 2015).

According to Novitasari & Herdyastuti (2014) that Amylase is an enzyme that is widely used in industry. Amylase is an enzyme that can hydrolyze starch to produce various products such as maltose, dextrin and especially glucose molecules as the smallest unit. Amylase enzymes can be derived from various sources, such as microorganisms, plants, and animals. Microorganisms provide particularly beneficial enzymes because they can grow faster than plants and animals. Amylase derived from microorganisms is widely used in industry, especially in processes that require high temperatures. Based on the research of Susilawati et al (2015) Amylase can be produced by several types of amylolytic bacteria, namely *Bacillus aquamaris MKSC*, *Bacillus amyloliquifaciens ABBD*, *Bacillus subtilis, Bacillus licheniformis ATCC 9945a, Streptomyces sp., Geobacillus thermodenitrificans, Klebsiela pneumoniae, Clostridium sp., Lactobacillus sp., Micrococcus sp., and Bacteriodes sp*. This is in line with the research of Tuntun et al (2014) Bacteria found in Pacet hot springs (8 bacterial genus: *Thermus sp, Acetogenium sp, Bacillus sp, Thermotrix sp, Thermodesulfo bacterium sp, Thermomicobrium sp, Pseudomonas sp, and Sulfobacillus sp*.) are examples of microorganisms successfully isolated in Indonesia. Given that amylase-producing bacteria are very potential in the industrial field, it is necessary to conduct research in an effort to find potential natural resources for human welfare, by isolating amylase-producing bacteria from Pentadio Resort hot springs. This needs to be done because the excavation of strains of thermostable enzyme-producing microorganisms from hot springs in Gorontalo has not progressed far. Based on this, the author is interested in isolating and identifying amylase enzyme-producing bacteria from Pentadio Resort hot springs.

METHOD

Time and Place of Research

This research was conducted in April-July 2023. Water sampling was conducted at Pentadio Resort hot spring in West Pentadio Village, Telaga Biru Subdistrict, Gorontalo Regency. Microbiological analysis was conducted at the Microbiology and Biotechnology Laboratory, Biology Department, Gorontalo State University. Molecular analysis was conducted at PT Genetika Science Indonesia.

Type of Research

This study uses a descriptive qualitative method that describes the morphological characteristics of amylase enzyme-producing bacterial isolates from Pentadio Resort hot springs.

Sampling

Sampling was carried out at Pentadio Resort hot water, Gorontalo Regency, by taking samples using a sterile bottle as much as 600 ml, then put into a thermos of hot water in order to maintain the temperature of the sample. In addition, the temperature and pH of Pentadio Resort water were measured.

Media Creation

Preparation of Nutrient Agar Media (NA)

NA medium is made by weighing 10.8 g of NA, then put it in a glass beaker and adding sterile distilled water to a volume of 540 ml. the mixture is heated using a hotplate, after homogeneous medium is poured into a sterile erlenmeyer then tightly closed with cotton and aluminum foil. The medium is sterilized in an autoclave at 121°C for 60 minutes (Mawati et al., 2021).

Selektif Making Selective Media

Amylolytic selective agar medium was made by weighing yeast extract as much as 2 g, peptone 5 g, $MgSO_4$. 7H2 O 0.5 g, NaCl 0.5 g, $CaCl_2.2H_2O$ 0.15 g, starch 10 g, and agar 20 g, then the ingredients are put into a glass beaker and added sterile aquadest to a volume of 1000 ml. the mixture is heated to boiling, after which the medium is poured into a sterile Erlenmeyer and then tightly covered with cotton and aluminum foil. The medium is sterilized in an autoclave at 121 ° C and a pressure of 15 psi or about 2 atm for 15 minutes (Mawati et al., 2021).

Isolation and Selection of Amylase Enzyme Producing Bacterial Isolates

Water samples were put into Erlenmey-er, then shaken for 20-24 hours after which the samples were diluted with a concentration of 10^{-1}, 10^{-2}, 10^{-3} after which they were poured into NA solid media and leveled using a stirring rod, then the samples were incubated at 37ºC for 48 hours. Isolates that grow well are then purified by scraping and reincubated at 37ºC (Panda et al., 2013).

Purification and Isolation of Amylase Enzyme-Producing Bacteria

Producing Bacteria Bacterial purification was carried out by taking one ose from each bacterial colony that grew differently on the previous NA medium and inoculated with a nose into another Petri dish containing NA medium, then incubated at 37ºC (Mawati et al., 2021).

Selection of Amylase Enzyme Producing Isolates

Bacterial isolates were inoculated on 1% starch medium and incubated for 24 hours at 37ºC. The growing isolates were then dripped with iodine solution to select bacteria that produce amylase. Isolates that produce amylase are indicated by the clear zone around the bacterial colony. The clear zone formed around the bacteria is measured in diameter using a caliper (Mawati et al., 2021).

Identification of Amylase Enzyme Producing Isolates

Gram Stain Test

Gram staining is done to observe colony morphology. First a review was made on the preparation and then fixed, then dripped solution A (crystal violet) as much as 2-3 drops, let stand for 60 seconds. The preparation is then washed using running water and dried, then dripped with solution B (lugol) as much as 2-3 drops on the preparation and let stand for 60 seconds and washed again then dried. Preparations are dripped with 2-3 drops of safranin solution, allowed to stand for 60 seconds and then washed and dried. After the gram staining test, the preparations were observed under a microscope, for morphological identification. Morphological identification of bacteria, which includes the shape of the colony, the shape of the elevation edge, growth on tilted media.

Motility Test

Were taken as much as 1 ose and then inoculated on semi-solid media by stabbing, then incubated at 37°C for 48 hours. If there is a sign of propagation around the ose needle puncture in the media, the result is positive (motile). If there is no sign of propagation, the result is negative (non-motile).

Molecular Test

The identification process of bacteria carried out is using the sequencing method by comparing the nucleotide database using the Basic Local Aligment Search Tool (BLAST) program through the site www.ncbi.nlm.nih.gov/BLAST . The results shown from this molecular bacterial identification are the name of the bacterial species and the level of similarity of the nucleotide sequence of the isolate 16s rRNA gene with the nucleotide sequence of the corresponding bacterial species in the Gene Bank database

RESULT AND DISCUSSION

Morphological Characteristics of Amylase Enzyme-Producing Bacteria in Pentadio Resort Hot Springs

The isolation results of purified bacteria obtained 7 bacterial isolates that have different characteristics, based on elevation, margin, shape and color. The results of bacterial characterization are presented in Table 1.

Tabel 1. Results of Macroscopic Characteristics of Thermophilic Bacteria

No	Isolat	Elevation	Margin	Shape	Color
1	A1	Flat	Lobatte	Ireguler	White
2	A2	Flat	Smooth	Round	Pale Yellow
3	A3	Flat	Rhizoid	Rhizoid	White
4	A4	Convex	Smooth	Round	Yellow
5	A5	Flat	Filamentous	Filamentous	White
6	A6	Flat	Smooth	Round	White
7	A7	Flat	Ireguler	Ireguler	White

Selection of Bacterial Isolates Producing Amylase Enzymes

Based on the results of the selection of amylase enzyme-producing bacterial isolates, 1 isolate was found that has the potential to produce amylase enzyme, namely isolate A6 with a clear zone distribution area of 23.26 mm in Figure 1 The test results of isolates that have the potential to produce amylase enzyme can be seen in Table 2.

Tabel 2. Amylase enzyme activity test on hot spring bacterial isolates

Isolate code	Amylase test
A1	-
A2	-
A3	-
A4	-
A5	-
A6	+
A7	-

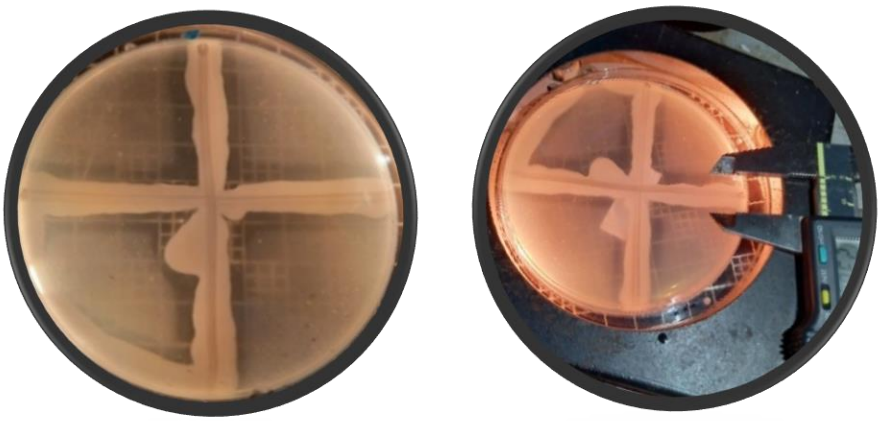

Figure 1. Wide distribution of the clear zone of isolate A6

Microscopic characteristics of isolates of bacteria that produce amylase enzymes

Based on the gram staining test of isolate A6, the results of gram-positive bacteria, bacillus-shaped (rod), purplish blue in color. The results of the gram staining test can be seen in Figure 2.

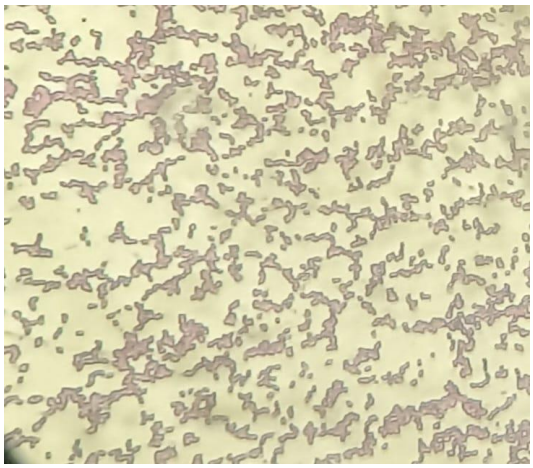

Figure 2. Gram staining test results on A6 bacterial isolates

Identification of bacterial isolates that produce amylase enzymes based on motility tests

Based on the results of the motility test of isolate A6, it is motile which is characterized by the presence of propagation around the tilted media. The results of the isolate motility test for A6 can be seen in Figure 3.

Figure 3. motility test results on isolate A6

Molecular Identification

Based on the reconstruction of phylogenetic trees with 16S rRNA gene sequences after 1000x boostrap to see the kinship relationship formed and get the results that isolate A6 is very closely related to *Bacillus cereus* strain MD 152 (Figure 4).

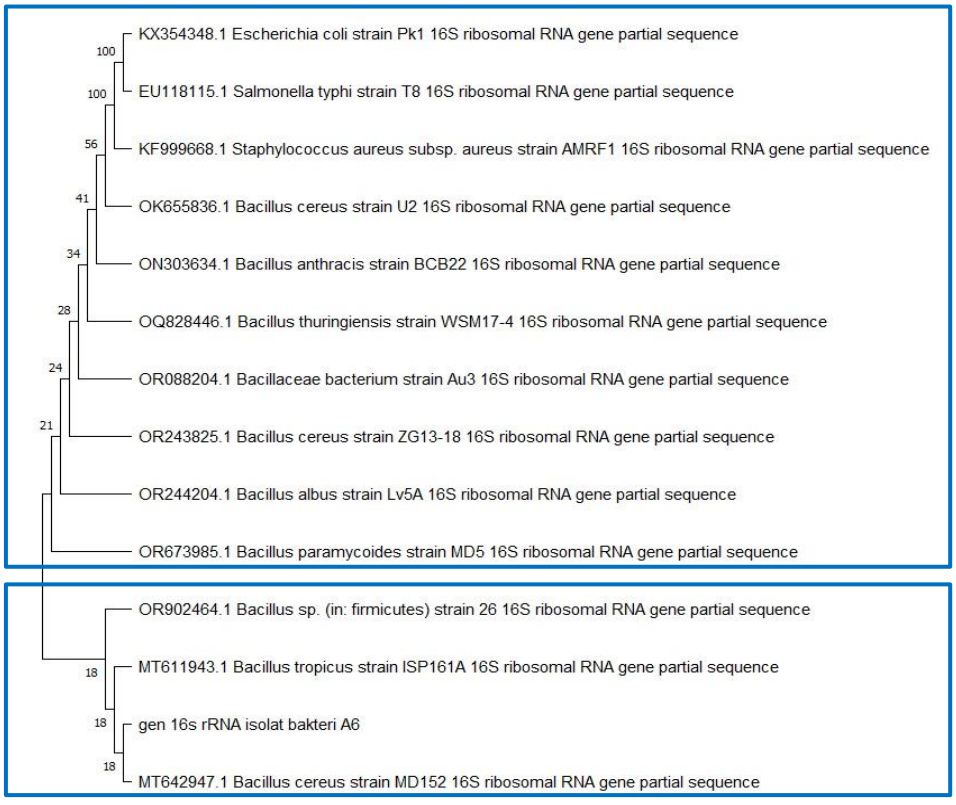

Figure 4. Phylogenetic Tree Reconstruction using Neighbor-joining (Bootsrap 1000x)

Discussion

Based on the isolation results obtained, there are 7 bacterial isolates with different macroscopic characteristics. Isolate A1 with flat elevation, lobate margin, with irregular shape and white in color, isolate A2 with flat elevation, smoth margin, with round shape and pale yellow in color, isolate A3 with convex elevation, smoth margin, with round shape and yellow in color, isolate A4 flat elevation, filamentous margin, with filamentous shape and white in color, isolate A5 elevation flat, margin smoth, with round shape and white in color, isolate A6 elevation flat, margin irregular, with irregular shape and white in color, and the last isolate A7 elevation flat, Subscribe to DeepL Pro to edit this document. Visit www.DeepL.com/pro for more information. margin rhizoid, with rhizoid shape and white in color. This is in line with the research of Zuraidah et al., (2020), that the isolates of thermophilic bacteria found are round, irregular, and filamentous, have a white and yellowish color and the colony surface is smooth and not slimy. Based on Kasi et al., (2020), the characteristics of bacterial isolates obtained are round and iregullary with convex and flat elevations and white in color.

Based on the results of the research that has been done, the bacterial isolate that is able to produce the enzyme amylase is isolate A6, this is indicated by the presence of a clear zone around the bacterial isolate that is tetesi liquid iodine, after being measured using a vernier term the clear zone distribution area is 23.26 mm. Wahyuni et al., (2020), stated that each isolate has a different ability to produce a clear zone.

Based on the research that has been done, bacterial isolate A6 is gram-positive, rod-shaped and purplish blue in color. According to Lande et al (2020), In the process of gram staining, bacteria are divided into 2 groups, namely gram-positive and gram-negative. This happens because there are differences in the structure of the bacterial cell wall. Gram-positive bacteria have cell walls that contain many peptidoglycans while the cell walls of gram-negative bacteria have thin peptidoglycans but contain many lipopolysaccharides. Panjaitan et al., (2020), stated that the difference in structure in gram-positive and gram-negative bacteria causes differences in color in bacterial isolates during the gram staining process.

In addition to macroscopic and microscopic testing that has been done. Furthermore, the biochemical test is carried out, namely the motility test. Motility test on bacteria aims to determine the presence or absence of cell movement in these bacteria. based on research that has been done for motility tests on amliase enzyme-producing bacteria, namely isolate A6. After the motility test was carried out on isolate A6, the results were positive motile, this was indicated by the presence of propagation around the ose needle puncture. According to Panjaitan et al., (2020), that the movement of motile bacteria can be characterized by the presence of a means of motion in the form of flagella while non-motile bacteria are bacteria that do not have flagella. this is in line with the research of Damayanti et al., (2018), that the motility test is one aspect to prove the presence or absence of bacterial movement that has been inoculated and based on research that has been done positive results are marked by the spread and propagation of wider colonies while negative results are marked by the absence of colony spread and only grow and accumulate in the center only.

To emphasize the results of bacterial characteristics that have the potential to produce amylase enzyme and microscopic characteristics, molecular tests were carried out. Bacterial isolate A6 was identified molecularly because it has the potential ability to produce amylase enzyme. Identification is done by analyzing based on the 16S rRNA gene sequence with primer 27F-1492R. The 16S rRNA gene sequence of bacterial isolate A6 obtained was then analyzed using BLAST on the site www.ncbi.nlm.nih.gov. From the blast results, 10 accessions of bacterial species were selected that had the highest percentage of similarity with isolae A6 and 3 different bacterial species were added as outgroups (accession KX354348.1 *Escherichia coli* strain Pk1, EU118115.1 *Salmonella typhi* strain T8, KF999668.1 *Staphylococcus aureus* strain AMRF1). All blast sequences were then aligned with Clustal W in the MEGA XI program. The aligned sequences were then reconstructed into a phylogenetic tree to determine the relationship between all bacterial species.

Based on the results of phylogenetic tree reconstruction performed with 1000x boostrap, 2 large clades were obtained, namely Clade A and Clade B (Figure 4). Clade A is divided into *Escherichia coli* strain Pk1, *Salmonella typhi* strain T8, *Staphylococcus aureus* strain AMRF1, *Bacillus cereus* strain U2, *Bacillus anthracis* strain BCB22, *Bacillus thuringiensis* strain WSM17-4, *Bacillaceae bacterium* strain Au3, *Bacillus cereus* strain ZG13-18, *Bacillus albus* strain Lv5A, *Bacillus paramycoides* strain MD5. Clade B bacterial isolate A6, *Bacillus cereus* strain MD152, *Bacillus tropicus* strain ISP161A, *Bacillus sp*. Strain 26. Based on the phylogenetic tree, it can be seen that isolate A6 has a very close relationship with *Bacillus cereus* strain MD 152 with a bootstrap of 18x.

Bacterial activity is influenced by the environment, one of which is the influence of temperature and pH. The existence of bacteria at high temperatures causes bacteria to produce stable enzymes, one of the enzymes that can be produced is the enzyme amylase. Pentadio Resort hot springs points 1 and 2 have a temperature of 70°C while point 3 has a temperature of 50°C. At point 1 the pH level of the water is 7.7, point 2 is 8 and at point 3 is 7.9. Based on the research of Mahestri et al., (2021), that the bacterial group *Bacillus cereus* is a bacterial isolate isolated from Kalianda South Lampung hot spring samples with temperatures reaching 55-65 °C and has the potential to produce amylase enzymes. This is in line with the research of Corneles et al., (2023), that one of the bacterial isolates isolated from Lahedong hot springs has a high similarity with the *Bacillus cereus group* capable of producing thermostable enzymes

According to Zuraidah et al., (2020), that bacteria capable of producing amylase enzymes are bacteria that show a clear zone, this is influenced by temperature factors and the ability of each bacterial isolate, but not all bacterial isolates can potentially produce amylolytic enzymes because each bacterial ability is different and not all thermophilic bacteria are amylolytic. This is in line with the opinion of Firliani et al., (2013), that biotic and abiotic conditions in the hot spring environment are very influential. According to Istia et al., (2020), one way to produce amylase enzymes using microorganisms, control of environmental factors is very important because microorganisms themselves are influenced by several things, namely temperature, incubation period and pH. The utilization of bacteria in producing amylase enzymes has an important role in the industrial field. From the results of the research conducted,

it can be seen that bacterial isolates taken from Pentadio Resort hot spring samples have the potential to produce amylase enzymes.

CONCLUSION

Based on the research conducted, it can be concluded as follows: (1) Bacteria in Pentadio Resort hot springs have the potential to produce amylase enzymes, because they have the ability to produce a clear zone when tested with iodine solution when grown on NA media that has been mixed with amylum. (2) Based on macroscopic characteristics, 7 bacterial isolates were obtained and those that have the potential to produce amylase enzyme are isolate A6, with morphological characteristics having flath elevation, smooth margins, round shape, and white color. Furthermore, the characteristics of amylase enzyme producing bacteria microscopically based on gram staining, namely Isolate A6 is rod-shaped including gram positive. Based on the results of biochemical tests, namely the motility test, the motility test shows that isolate A6 is positively motile. (3) Identification of bacteria in Pentadio Resort hot springs molecularly with phylogentic tree reconstruction showed that isolate A6 is a type of bacteria that is closely related to *Bacillus Cereus* strain MD 152 which is included in the *Bacillus Cereus group* as an isolate of amylase enzyme producing bacteria.

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