Isolation and Characterization of Amylase-Producing Amylolytic Bacteria from Rice Soil Samples

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Abstract. Amylase is an enzyme that plays a role in hydrolyzing starch to simple sugars. This protein is very important for various industrial processes. Microorganisms are considered as the best source of amylase production. This study was aimed at isolating and identifying amylolytic bacteria from rice soil samples that have the potential to produce amylase enzymes. Rice soil samples were collected from two locations, namely the Percut Sei Tuan area and Labu Beach. Samples were isolated, then macroscopically identified with microscopy, and microscopically characterized with catalase test, endospore staining, and gram staining. The identification results showed that 14 bacterial isolates from the two samples potential to produce amylase enzymes. The isolates which were characterized with catalase test showed positive results for all isolates. The characterization results with endospore staining showed 7 positive isolates and 7 negative isolates. Likewise with the results of gram staining characterization. Thus, from the two sample locations obtained amylolytic bacteria that potentially produce amylase enzymes.

1. Introduction

Enzymes are proteins synthesized by living cells and are catalysts of proteins that can accelerate chemical reactions that occur in living things in biological systems [1]. Amylase is an enzyme group that has the ability to break glycoside bonds in starch. Sources of α-amyrase can be obtained from plants, animals, and microorganisms. Amylase derived from microbes can produce large enzymes, so it is widely used as an industrial enzyme [2]. In addition, microorganisms are also easily cultured and their growth rates are relatively fast, and the scale of cell production is more easily increased. Amylase is a hydrolytic enzyme that has the ability to break glycoside bonds in starch. The hydrolysis results in the form of smaller molecules such as glucose, maltose, and dextrin [3]. Amylase can hydrolyze starch through three main stages namely gelatinization, liquidation, and saccharification [4]. All three processes have high levels of energy consumption [5].

Amylase use is reported to increase every year. Amylase is constitutionally a group of enzymes that are highly needed in the industrial sector with market control reaching nearly 25% of the world's enzyme market [6]. Amylase can be produced from amylolytic bacteria and amylolytic fungi originating from the soil or hot springs. Soil is a medium that is used as a place to live and growth of bacteria in a complex manner. Bacteria can live in the soil by utilizing all the nutrients in it [7]. Amylolytic bacteria in the soil are very abundant so that the production of amylase enzymes is also abundant and can meet the needs of amylase enzymes in several industries. Based on research conducted by [6], isolation of amylolytic bacteria producing amylase from paddy soil samples showed positive results, namely the highest number of bacterial isolates from all samples (field soil, bread,
flour, human saliva, horse manure etc.). In addition, amylolytic bacterial isolates from rice soil samples showed positive results after testing with the addition of iodine (the formation of a clear zone).

2. Materials and Methods

2.1. Media and chemicals
The media used in this study is agar nutrient media (Oxoid), NaCl 0.9% (Wida NS), Aquadest, Iodin (Merck), Crystal Violet (Merck), Lugol (Merck), Safranin (Sigma Aldrich), Malachite Green 5% (Sigma Aldrich) and H₂O₂ 3% (Merck).

2.2. Isolation amylolitic bacteria produce amylase
Amylolytic bacteria were isolated from the rice fields of Sei Rotan Village and the Labu Beach area, Lubuk Pakam. Each sample was taken as much as 25 grams and then mashed using mortar and pestle. Paddy soil samples were then taken as much as ± 1 gram and diluted in 9 mL 0.9% NaCl to 10⁻⁶ dilution. A total of 30μL of the sample was spread into the agar nutrient medium and subsequently incubated at 37°C for 24 hours. Colony isolates formed were counted and marked based on the morphological characteristics of each.

2.3. Qualitative Test of amylolytic bacteria produce amylase enzymes
Colony isolates formed were taken as many as 3 colonies and inoculated into a nutrient agar medium which was then incubated at 37°C for 24 hours. Isolation is repeated until a single colony is obtained for each colony. Then dripping iodine solution on the agar surface which contains isolates. If there is a clear zone in the media, the condition indicates the amylase enzyme is produced by the isolate so that the starch has been hydrolyzed in the area, while the blackish media indicates that the starch in the media has not been hydrolyzed.

2.4. Characterization of amylase producing bacteria macroscopically
Bacteria isolated from the two samples, then macroscopically characterized using a microscope. Every bacterial colony growing on nutrient media from positive isolates was observed for morphology. Morphological observations of each isolate included the color, shape and edge of the colony observed from the top, while the surface of the colony was observed from the side [8].

2.5 Characterization of amylase-producing bacteria microscopically with gram staining
Positive isolates were taken using an ose needle, then a smear preparation was made (on a glass object) using physiological NaCl and dried in the air then fixed over Bunsen. The preparation is dropped with a Gram A (violet crystal) solution and left for 3 minutes then rinsed with running water. Next drop with Gram B (Lugol) solution for 1 minute, then wash with running water. Then dropped with alcohol until the remaining dye disappears or for 30 seconds and rinsed again with running water. In the final stage, the preparation is dropped with gram C (safranin) solution and allowed to dry. Then it is dripped with immersion oil to be observed under a microscope at 40X magnification. Observations were made by looking at cell morphology and color. Gram-positive bacteria will turn purplish blue, while Gram-negative bacteria will turn red [8].

2.6 Endospore staining
The method used in staining endospores is the Schaeffer-Fulton method. Using Malachite green 5% and safranin to color endospores. Spores are colored by coloring agents by heating. The color of the spores is green or appears to be refractile under a microscope [9].
2.7 Catalase test
Catalase test is carried out by inserting isolates into a test tube that already contains a solution of Hydrogen Peroxide. Isolates are carefully taken using an ose needle. Positive results are shown by the emergence of gas bubbles in the tube [9].

3 Results and Discussion

3.1 Isolation and characterization of amylolytic bacteria produce amylase enzymes
Iodine test results obtained as many as 14 bacterial isolates that have the potential to produce amylase enzymes marked by the formation of clear zones around amylolytic bacterial isolates. The most bacterial isolates were produced in Percut soil samples, where 9 bacteria isolates were obtained which could potentially produce amylase enzymes. LubukPakam rice field samples produced as many as 5 bacterial isolates that could potentially produce the amylase enzyme show in Table 1.

| Location      | Number of isolates |
|---------------|--------------------|
| Percut        | 9 isolates         |
| Pantai Labu   | 5 isolates         |

Bacteria that have the potential to produce amylase enzymes are based on the formation of clear zones around bacterial isolates after the addition of iodine solution. Clear zones around bacterial colonies indicate that starch in the media has been degraded by extracellular amylase enzymes produced by bacteria into simple sugar compounds that do not show color reactions with iodine [10]. In this case, starch is used as a carbon source as an energy source for amylolytic bacteria and the resulting over haul of starch due to amylase activity is characterized by the formation of clear zones around amylolytic bacteria. Iodine solution does not provide color with carbohydrate polymers that are less than five monosaccharide groups, for example glucose [11]. Media around the colony that does not produce amylase will turn blue if drops of iodine solution. This shows that starch in the media is not degraded to simple sugars, which means that bacteria do not produce amylase.

According to [11] iodine solution will react with carbohydrates that contain more than 20 glucose groups (complex sugars or polysaccharides) to form a blue color. The ability of a bacterium to produce amylase is determined by the presence or absence of structural genes that regulate the synthesis of amylase proteins in bacterial cells. In addition, the expression of structural genes that encode amylase protein formation is also influenced by environmental factors. The presence of glucose in the medium at certain concentrations can inhibit the formation of protein amylase.

| Isolates Code | Shape  | Colour | Edge    | Elevation |
|---------------|--------|--------|---------|-----------|
| PC 4          | Coccus | Beige  | Slippery| Raised    |
| PC 5          | Coccus | White  | Slippery| Raised    |
| PC 6          | Coccus | Beige  | Slippery| Raised    |
| PC 7          | Irregular | White | Branched | Raised    |
| PC 8          | Irregular | White | Slippery| Raised    |
| PC 9          | Coccus | White  | Waved   | Flat      |
| PC 10         | Coccus | Beige  | Slippery| Raised    |
| PC 11         | Irregular | Beige | Waved   | Raised    |
| PC 12         | Irregular | Beige | Slippery| Flat      |
| PKM 1         | Circles | White | Waved   | Convex    |
| PKM 2         | Irregular | Beige | Waved   | Convex    |
| PKM 3         | Circles | White | Slippery| Raised    |
| PKM 7         | Coccus | White  | Waved   | Raised    |
| PKM 8         | Coccus | White  | Waved   | Convex    |
3.2. Characterization of amylase-producing bacteria macroscopically
The results of isolation of bacterial isolates produced from paddy soil, as many as 14 isolates were able to produce the amylase enzyme which was marked by the formation of clear zones around the isolates. The resulting isolates were then macroscopically characterized, including: colony shape, colony color and colony elevation (Table 2). Bacterial isolates macroscopically characterized have different shapes, colors, edges and elevations. Most bacteria that are characterized have irregular shapes, white and beige. The edges and elevations of each colony also have different shapes. Macroscopic observations were made using a microscope. Observation of the shape of the colony seen from above, the surface of the colony seen from the side and the edge of the colony seen from above.

3.3. Characterization of amylase-producing bacteria microscopically with gram staining
Gram positive bacteria will retain the purple color of violet crystals so that when observed under a microscope it will show purple while Gram negative bacteria cannot maintain the purple color of violet crystals but safranin dyes can be absorbed on the cell wall so that when viewed using a microscope will show red [12, 13] (Table 3).

| Table 3. Characterization of Amylolytic Bacteria Microscopically with Gram staining |
|----------------------------------------|-------------------------------|-------------------------------|
| Samples Source | Gram Positive (+) | Gram Negative (-) |
| Percut | PC7, PC8, PC10, PC11, PC12 | PC4, PC5, PC6, PC9 |
| Pantai Labu | PKM7, PKM8 | PKM1, PKM2, PKM3 |

Gram staining is done to group bacteria into 2 namely Gram positive bacteria and Gram negative bacteria. In Gram staining, the results obtained are determined from the cell wall composition of bacteria. The reaction to gram staining is based on differences in the chemical composition of the bacterial cell walls. Gram-positive bacteria have a thicker peptidoglycan layer while the gram-negative peptidoglycan layer is thinner and contains lipids on the outside. Peptidoglycan is the main polysaccharide consisting of 2 chemical subunits which are only found in bacterial cell walls. These subunits are N-acetylglosamine and N-acetylmeramic acid [14,15,16].

3.4. Catalase test
The catalase test results of amylolytic bacterial isolates showed positive results. This is marked by the formation of bubbles which are oxygen gas when inserted into a tube containing Hydrogen Peroxide (Table 4).

| Table 4. Catalase Test of Amylolitic Bacteria |
|-------------------------------------------|-----------------|-----------------|
| Samples Source | Positive | Negative |
| Percut | PC4, PC5, PC6, PC7, PC8, PC9, PC10, PC11, PC12 | - |
| Pantai Labu | PKM1, PKM2, PKM3, PKM7, PKM8 | - |

Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide and oxygen. Hydrogen peroxide is toxic to cells, so it activates enzymes in cells. Hydrogen peroxide is formed when aerobic metabolism, so that microorganisms that grow in the environment of aerob will produce a catalase enzyme that can decompose the toxic (Figure 1).
Figure 1. Catalytic Test for Amylolytic Bacteria (a) PC 10, a bacterial isolate from the Percut paddy soil; (b) PKM 8 bacterial isolates from Lubuk Pakam rice fields.

3.5. Endospore staining

The test results obtained that as many as 7 isolates that showed positive results and 7 isolates showed negative results. Positive results are shown by the formation of green in bacterial isolates after being seen using a microscope which shows that the bacteria have endospores. According to the second edition of the Bergeys Manual to find out the genus of gram-positive bacteria, endospores should be stained. The results of staining endospores in bacterial isolates after being seen using a microscope shows a green color which indicates that the bacteria have endospores. Endospores are structures that are resistant to extreme environmental conditions such as dry, heating and acidic conditions. Endospores are very dense and refractile because they have a very low water content. Bacteria that have endospores are very difficult to color so specific staining is needed. The specific coloring used is malachite green. Spore-producing bacteria are resistant to staining. Bacteria that produce spores will bind strongly to the dye compound namely malachite green and when it is carried out further staining using safranin, spore cells cannot bind to other dyes because it is bound to malachite green. Therefore the color of spore bacteria is green [14] (Table 5).

Table 5. Endospore staining of amylolytic bacteria

| Samples Source   | Positive         | Negative         |
|------------------|------------------|------------------|
| Percut           | PC7, PC8, PC10, PC 11, PC12 | PC4, PC5, PC6, PC9 |
| Pantai Labu      | PKM7, PKM 8      | PKM1, PKM2, PKM3 |

Bacteria that do not have spores tend to be resistant to painting because they only have vegetative cells. When colored with malachite, vegetative cells will be able to bind to the dye but can be worn off after washing because it does not bind strongly to the malachite green dye. After that, painting is done by using safranin and vegetative cells will bind to safranin dyes so that the color produced when observed by a microscope will show a pink color [17] (Figure 2).

Figure 2. Amylolytic Bacteria Endospora Staining Test (a) Positive results from Percut paddy soil samples and (b) Negative results from Lubuk Pakam paddy soil samples.
4 Conclusions
Amylolytic bacteria producing amylase isolates were obtained from two paddy soil locations and the potential to produce amylase enzymes was 14 isolates. Amylolytic bacterial isolates from the two paddy soil locations have different characteristics while the results of microscopic characterization based on catalase test showed that all isolates showed positive results. Based on the results of the characterization by gram staining obtained that as many as 7 isolates were gram-positive bacteria while 7 other isolates were gram-negative bacteria.

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