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Isolation, Screening, and Optimization of Amylase-Producing Endophytic Bacteria from *Rhizophora apiculata*

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ABSTRACT
High amylase enzyme production requires optimal supporting factors for enzyme production. The purpose of this study was to determine the optimum conditions for endophytic bacterial fermentation, including substrate concentration, pH, and fermentation temperature, for the production of amylase from endophytic bacteria of mangrove plants Rhizophora apiculata. Analysis of amylase enzyme activity was carried out using the 3,5-dinitrosalicylic acid (DNS) assay. The results obtained from this research were from a total of 18 endophytic bacteria R. apiculata that were successfully isolated, 6 bacterial isolates were obtained which showed the ability to produce extracellular amylase. The potential of bacteria for amylase production grown on 0.5% starch media was determined based on the ratio between the diameter of the halo zone and the diameter of the bacterial colony. Three isolates were selected, EB.5, EB.10, and EB.12 for further optimization of the fermentation conditions for amylase production. Based on the results obtained, it was shown that the fermentation conditions for amylase enzyme from the three isolates of endophytic bacteria were optimum in media with a concentration of 1.5% starch, in media with a pH of 6, and optimum in the temperature range of 30ºC.
Key words: Endophytic bacteria; amylase; starch; pH; temperature

INTRODUCTION

Amylase is a starch-degrading enzyme with wide-scale applications (Rana et al., 2013; Saini et al., 2017). It is an extracellular enzyme produced inside the cell and released to the surrounding substrate. Furthermore, it plays a role in the hydrolysis of starch into simpler sugars such as maltose, glucose, and dextrin (Rana et al., 2013), and it contributes about 25-30% of the world's total enzymes (Park et al., 2018). Amylase enzymes are found in microbes, plants, and animals, and they are dominantly applied in the industrial field. Endophytic bacteria are a potential source to obtain bioactive compounds, including enzymes. Therefore, the exploration of efficient and high-producing amylase is still ongoing, even though many studies and implementations have reported the isolation and crystallization of the enzyme. However, not many utilized endophytic bacteria that have the uniqueness and specifications of their host plants. Endophytic bacteria with amylolytic abilities and specific niches in their host plants are an excellent opportunity to develop biotechnology objects.

Mangroves are plants distributed in the intertidal zone of tropical and subtropical areas. They have high salt content and good adaptability to a flooded environment (Morales-Covarrubias et al., 2019). The potential of bacteria associated with mangrove plants has been reported in several studies. Bioprospection of endophytic bacteria isolated from mangrove leaves were the activities of antimicrobials, amylase, cellulase, lipase, pectinase, protease, inulinase, L-asparaginase, and invertase enzymes. Other plant growth-promoting activities include the production of ammonia and acetoin, nitrogen fixation, phosphate solvent, and output of indole-

acetic acid (IAA) (Castro et al., 2014; Deivanai et al., 2014; Maulani et al., 2019; Prihanto et al., 2018; Rori et al., 2020).

Numerous studies on endophytic bacteria as a source of enzyme production were previously conducted to understand the functional role of these species in the host plant. Endophytes contribute to plant defense by producing enzymes and secondary metabolites associated with host organelles. Subsequently, they grow in their host plants and conduct extracellular metabolism by producing secondary metabolites and enzymes from absorbed nutrients (Khan et al., 2017).

The determining factor for enzymes in the industrial sector is their production and application level. Based on this, different studies have been conducted to increase the production of enzymes with high activity. One of the approaches used to improve the activities of the enzyme was to determine the optimum conditions. Furthermore, environmental factors such as substrate concentration, pH, temperature, time, co-substrate, and the type and concentration of other combination compounds should be optimized to increase enzyme activity. High amylase enzyme production can be influenced by factors that support the process. Therefore, the activity is influenced by many factors, namely pH, temperature, substrate concentration, enzyme concentration, and the presence or absence of activators and inhibitors (Rana et al., 2013).

It is necessary to select strains that can produce high and stable amylase to support enzymes using endophytic bacteria of mangrove plants. High amylase enzyme production requires optimal supporting factors for its production. The optimum conditions of each factor supporting amylase production should be determined to apply the enzymatic product on a large industrial scale. Therefore, this study aims to determine the optimum conditions for endophytic bacterial fermentation, including substrate concentration, pH, and fermentation temperature, for the production of amylase from endophytic bacteria of mangrove plants

METHODS

Isolation of Endophytic Bacteria

Rhizophora apiculata leaves were collected from Tongkaina, Bunaken, Manado, North Sulawesi. Healthy plant leaves samples were selected and processed within 24 hours for bacterial isolation. The leaves of *Rhizophora apiculata* as samples were washed with running tap water and surface sterilized by soaking it in 1.3% sodium hypochlorite for 2 min, 70% alcohol for 2 min and rinsed with sterile distilled water three times. As much as 10 grams of leaves were mashed using sterile mortar and pestle, and then put into 90 mL of 0.9% NaCl aseptically. Isolation of endophytic bacteria began with serial dilutions of 10^{-2}–10^{-6} and continued with the spread plate method on Tryptic Soy Agar (TSA) media (Rori et al., 2020). Furthermore, the bacterial colonies that showed different morphology were taken. The bacterial colonies that grew were purified by the 4-way streak plate method on Nutrient Agar (NA) media to obtain pure cultures or single isolates.

Qualitative and Quantitative Screening for amylase

After subculturing, all purified isolates were tested for their amylolytic ability on solid media. The amylolytic test for qualitative screening was conducted by growing bacteria on 0.5% starch medium (starch, 20; peptone, 10; yeast extract, 4;

NaCl, 0.5; $MgSO_4$, 0.5; $CaCl_2$ 0.2 g/L) after incubation for 24 hours. Lugol's culture clock was dropped to cover the surface of the media, and isolates that are positive for the amylolytic activity will produce a clear (halo) zone (Hankin and Anagnostakis, 1975). Positive results were measured as the ratio of the diameter of the halo zone to the colony.

For quantitative screening, the isolates were subcultured in starch liquid broth (g/L): starch, 20; peptone, 10; yeast extract, 4; NaCl, 0.5; $MgSO_4$, 0.5; $CaCl_2$ 0.2, and incubated at 30°C, 150 rpm for 24 hours. The crude extracellular enzyme (supernatant) was obtained after centrifugation at 3,500 rpm for 30 min at 4°C. To determine the amylase activity of each endophyte bacterial isolate was used according to DNS method (Miller, 1959).

Optimization of Fermentation of Amylolytic Endophytic Bacteria Isolates

The three isolates of endophytic bacteria and the highest ratio of halo to colony diameters as well as quantitative screening were selected to determine and optimize amylase enzyme activity. It starts with the pre-culture stage by inoculating each endophytic bacterial isolate into 10 mL sterile liquid culture media. The incubation with 150 rpm agitation at room temperature for 24 hours was then performed. The culture stage was inoculated by putting 10% bacterial suspension into 100 mL sterile liquid culture media. Furthermore, it was incubated with agitation at 150 rpm for 24 hours. Sampling was conducted every 3 hours, and about 2 mL of the samples were tested for amylase activity. The filtrate was separated from the biomass by centrifugation at a rotation speed of 3500 rpm at 4°C for 30 minutes (modification of Elmansy et al., 2018). Additionaly, it was filtered using a 0.45 m filter membrane, and the measurements were made after centrifugation (1 mL) and ultrafiltration (1 mL).

Samples at each hour of the collection were then analyzed for amylase enzyme activity, and the optimization of each parameter was conducted in stages. This includes using the results of the previous parameter optimization. Subsequently, the optimum concentration of starch determination was carried out by varying the substrate in the production medium of pH 7, which was incubated at 30°C with agitation speed of 150 rpm for 24 hours. The variation of substrate concentration used is 0.5% concentration; 1%, 1.5%, 2%, and 2.5% starch. Meanwhile, optimization of pH production was conducted in the production medium with the optimum substrate concentration obtained from the previous test, namely 1.5%. Variations in the pH of the media used were pH 3, 4, 5, 6, 7, 8, and 9. Additionally, optimization of the fermentation temperature was executed at 30, 37, 45, and 55°C. All parameters were repeated three times, and the amylase enzyme activity was analyzed.

Determination of Amylase Enzyme Activity

Amylase activity was determined using the DNS method and the liberated reducing sugar was determined using the DNS method (Miller, 1959). A total of 0.5 mL of enzyme solution was added to 0.5 mL of substrate (2% starch dissolved in 0.05 M acetate buffer solution, pH 5), then incubated at 40°C for 10 minutes. After that, 2 mL of DNS reagent was added, and heated at 100°C for 5 minutes, then cooled with running water. After changing colour, added 1 mL of 40% (w/v)

potassium sodium tartrate to each tube, and 20 mL of distilled water to measure the absorbance at a wavelength of 540 nm. As a correction factor, 0.5 mL of enzyme solution was used without the addition of substrate, and 0.5 mL of the substrate without the addition of enzyme solution. Concentration was converted to standard glucose. Glucose standard curves are made from glucose concentrations of 10 – 100 ppm. Standard curves were made using absorbance data and concentration data.

RESULTS AND DISCUSSION

Isolation and Screening of Amylase Activity of Isolates of Endophytic Bacteria

The leaves samples of *Rhizophora apiculata* were subjected to serial dilution and then preceded for spread plating method in TSA media to observe bacterial isolates. Based on colony morphology, each distinct morphological character was considered different bacterial species and was subjected to the streak plate method for pure colony isolation. As a result, 18 other endophytic bacteria isolates were isolated from the *R. apiculata* sample leaves. The isolates were subcultured and maintained in NA media for a future test.

All isolated isolates of endophytic bacteria *R. apiculata* were tested qualitatively for their ability to produce amylase using a solid medium of 0.5% starch and incubated for 24 hours. The results showed that from a total of 18 isolated endophytic *R. apiculata* bacteria, 6 isolates showed the ability to produce extracellular amylase (**Table 1**). In addition, the potential of bacteria for amylase production grown on 0.5% starch media was determined based on the ratio between the diameter of the clear zone and the bacterial colony.

Table 1. The diameter ratio of qualitative screening and the value of amylase enzyme activity from six amylase positive isolates

Isolates	Diameter ratio	Amylase activity (U/mL)
EB.4	1.06 ± 0.10	2.93 ± 0.01
EB.5	1.32 ± 0.11	3.38 ± 0.39
EB.8	1.07 ± 0.12	0.71 ± 0.12
EB.10	2.89 ± 0.65	3.06 ± 0.47
EB.11	1.07 ± 0.12	1.95 ± 0.75
EB.12	2.04 ± 0.16	3.73 ± 0.52

The secondary screening was based on a quantitative analysis of amylase in starch broth, and the assay was performed by the DNSA method. The isolates EB.12 showed the highest activity (3.73 ± 0.52 U/mL), followed by EB.5 (3.38 ± 0.39) and EB.10 (3.06 ± 0.47). Meanwhile, the lowest activity was found in EB.8 (0.71 ± 0.12), and based on quantitative analysis, three amylolytic bacterial isolates of EB.12, EB.5, and EB.10 were selected for further investigations.

Optimum Starch Substrate Concentration, pH of Culture Media and Temperature of Fermentation for Amylase Production

The results obtained showed that the amylase enzyme activity of the three isolates of endophytic bacteria was optimum in media with a concentration of 1.5%

starch (**Figure 1**). For each isolate, EB5, EB10 and EB12 showed amylase activity of 3.26 ± 0.30, 3.45 and 2.92 U/mL at 1.5% starch concentration, respectively.

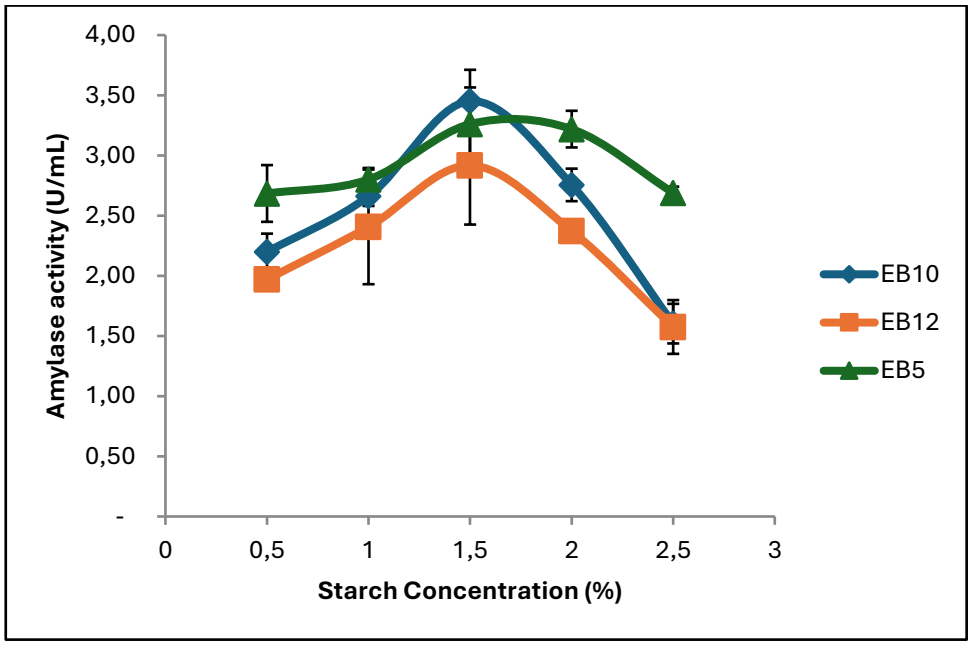

Figure 1. The optimum starch substrate concentration for amylase production

The pH variations used are pH 4, 5, 6, 7, 8, and 9, and the results of determining the optimum pH of culture media for amylase production can be seen in **Figure 2**. The results obtained showed that the amylase enzyme activity of the three isolates was optimum in media with a pH of 6-7. EB5 isolate showed amylase activity 6.53 ± 0.26 U/mL at pH 6 and 6.30 ± 0.10 U/mL at pH 7. EB10 isolate showed amylase activity 6.75 ± 0.15 U/mL and 6.48 ± 0.01 U/mL at pH 6 and 7, successively. Likewise, EB12 isolates, showed amylase activity 6.77 ± 0.16 and 6.34 ± 0.15 U/mL at pH 6 and 7.

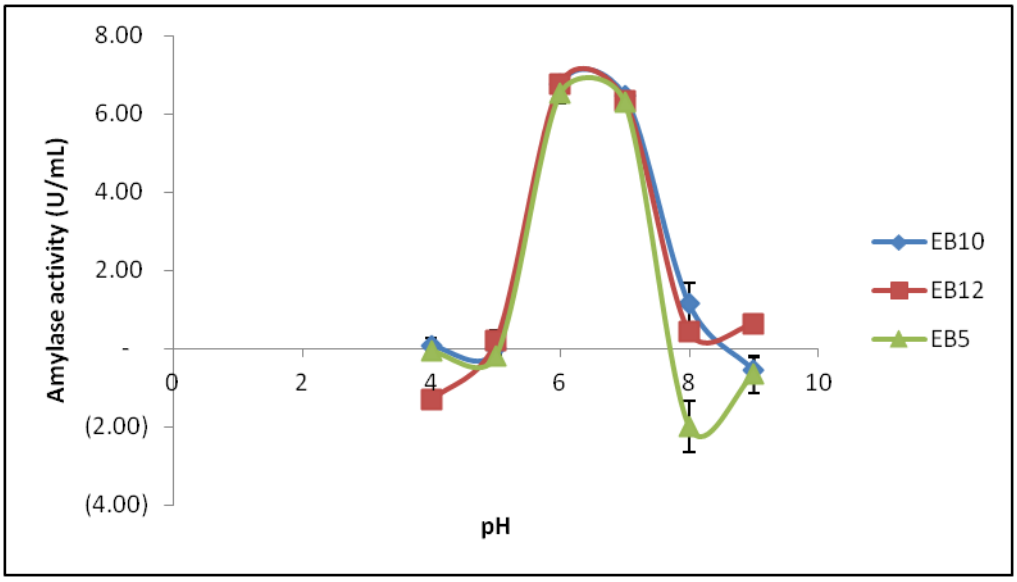

Figure 2. The optimum pH of culture media for amylase production

The optimum temperature was carried out by measuring the activity of the amylase enzyme in hydrolyzing starch substrates into glucose. The treatment for determining the optimum temperature was carried out with variations of 30,37,45, and 55 ºC. In addition, the three endophytic bacterial cultures (EB5, EB10, and EB12) were incubated according to the treatment temperature for 24 hours. The results of determining the optimum temperature of the amylase enzyme can be seen in **Figure 3**.

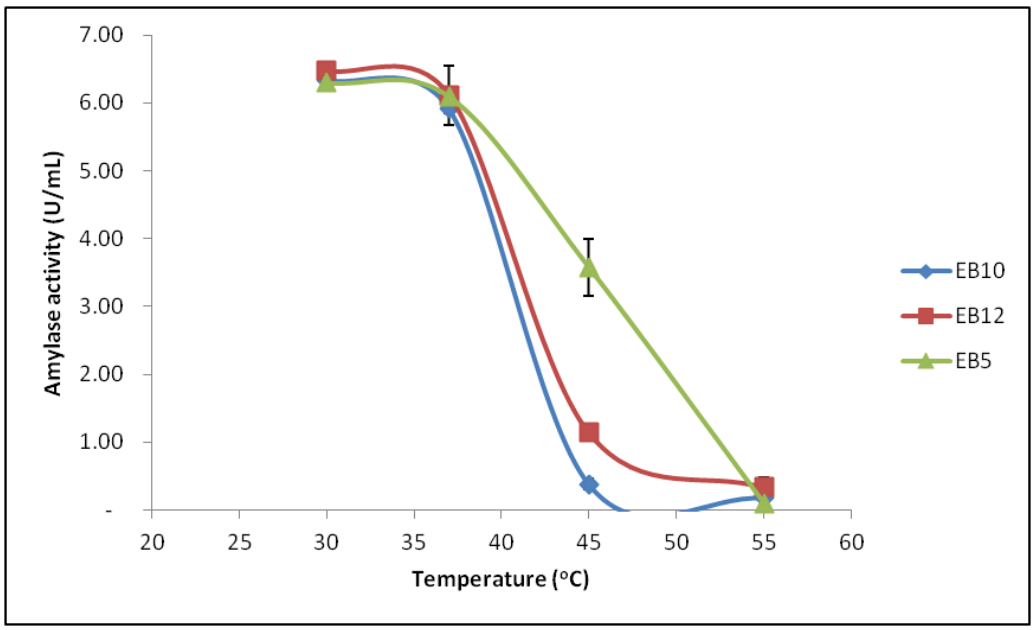

Figure 3. The optimum temperature of fermentation of culture for amylase production

The results obtained showed that the amylase enzyme activity of the three isolates tested was optimum at a temperature range of 30-37ºC. Isolate EB.5 with a yield of 6.3 ± 0.1 U/mL at 30ºC was not significantly different from 37ºC with enzyme activity of 6.1 ± 0.04 U/mL. Isolate EB.12 showed amylase activity 6.48 ± 0.01 U/mL at 30ºC and 6.11 ± 0.44 U/mL at 37ºC. Meanwhile, EB.10 showed amylase activity of 6.34 ± 0.15 and 5.91 ± 0.06 U/mL at 30ºC and 37ºC, respectively. At the incubation temperature of 45ºC and 55ºC, there was a decrease in amylase activity for the three endophytic isolates.

Based on the colony and Gram staining morphology, the most frequently found shape of the endophytic bacteria of *R. apiculata* was bacilli and Gram-positive. Starch is a nutritional component for the induction of amylolytic enzymes (Vishnu et al., 2014). The concentration as a substrate is important since that of nutrients can affect the growth rate and the culture product. The 1.5% starch concentration showed the highest amylase enzyme activity, 2.5% starch substrate showed lower amylase enzyme activity than substrate concentrations of 1 and 0.5%. When the substrate is broken down into simpler molecules, the amount becomes high and then

can act as an inhibitor. Low concentrations will be a limiting factor, while it will be an inhibiting factor at high concentrations. High substrate concentration can be an obstacle that slows down the hydrolysis process.

Enzymes have maximum activity at a certain pH range called optimum pH. The procedure for determining the optimum pH of the culture media for amylase production from the three selected endophytic bacterial isolates was similar to the amylase activity test for determining the substrate concentration. The optimum pH conditions for the three isolates of *R. apiculata* endophytic bacteria were required by the amylase to form the right substrate enzyme complex and produce the full product.

Each bacterium has a pH of enzyme production with the highest activity, and the pH value of bacterial growth media has a significant role in inducing bacterial morphology and enzyme secretion changes. The pH value also affects the absorption of nutrients and the physiological activity of bacteria to affects the growth of biomass and the formation of its products. The optimum growth pH refers to the extracellular pH of the environment. In contrast, the intracellular pH should be relatively neutral to prevent the breakdown of more acidic or alkaline macromolecules in cells. Enzyme activity varies with changes in pH because H+ ions present in solution affect the catalytic part of the enzyme. It causes a difference in the confirmation structure of the enzyme protein, to decrease the activity. The optimum pH value of the media for the growth of *R. apiculata* endophytic bacteria is 6-7. When the pH of the medium was increased from pH 8 to pH 9, the amylase enzyme activity decreased.

Enzyme activity is related to the structure and changes in its design that can cause changes in enzyme activity. Enzymes have a tertiary structure that is sensitive to pH, and in general, the denaturation can occur at low or high pH values. Changes in pH can cause damage to the secondary and tertiary structures. It can also cause changes in the catalytic structure on the enzyme's surface to affect the active part of the enzyme and form an enzyme-substrate complex. Subsequently, the decrease in activity at an inappropriate pH can occur due to changes in the load of the enzyme and its substrate. This happens due to changes in the ionic properties in the carboxyl and amino groups. Changes in the charge on the enzyme affects the activity, and at low pH values the enzyme undergoes protonation and loses its negative charge.

Similarly, at high pH, the substrate will ionize and lose its positive charge. The pH value is related to hydrogen ions which significantly affect enzyme activity. Enzymes can be in an active state when the active amine site is in a suitable ionized form. A pH value that is too alkaline or acidic can cause the enzyme to be denatured and become inactive. The temperature factor also dramatically affects the work of enzymes. This is because they consist of an amino acid composition with a working system that influences environmental temperature. The substrate concentration factor also needs to be considered in the production of bacterial amylase enzymes (Rana et al., 2013).

Temperature is one of the factors that affect enzyme activity. The changes can cause the folding of enzyme protein molecules allowing the active site to hydrolyze the substrate. Furhermore, the temperature can affect the rate and the total number of growth of microorganisms. Each type of bacteria has a specific growth temperature range, and the optimum temperature allows the fastest growth to occur

in a short time. The temperature of 30°C showed the highest amylase enzyme activity value compared to the others because it can catalyze the bacterial metabolic reaction.

CONCLUSION

Fermentation conditions for amylase enzyme from the three isolates of endophytic bacteria of *R. apiculata* were optimum in media with a concentration of 1.5% starch, in media with a pH of 6, and optimum in the temperature range of 30°C.

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