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Enhanced Amylase Activity by Modulating Abiotic Factors and Enzyme Stability in the Thermophilic Bacterial Isolates

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Abstract: Amylase is one of the enzymes that is widely used in bioprocess technology, amylase from different microbial sources also varies in form, so it is suitable for its specific application. The purpose of this study was to determine the optimum temperature, pH, agitation, and stability of the amylase enzyme from two thermophilic bacterial isolates, TUA-07 and TUA-09. This study used a descriptive and an experimental method with a factorial Completely Randomized Design (CRD) with two factors, namely temperature and pH, followed by gradual agitation. The results showed that the optimum temperature, pH, and agitation for isolate TUA-07 was 80°C-pH 6, with the agitation of 125 rpm, while for isolate TUA-09 it was 50°C-pH 7 with the agitation of 125 rpm. Enzyme stability for isolate TUA-07 the amylase activity is 80% stable for 10 h, while for isolate TUA-09 the amylase activity is 80% stable for 8 h.

Keywords: Agitation, Enzymes, Optimization, Stability, Thermophilic Bacteria

Introduction

Amylase is a frequently used enzyme in bioprocess technology because it can hydrolyze starch molecules into polymers composed of glucose units (Saini *et al*., 2017). Amylase can break down the α-1, 4-glycosidic linkages in starch leading to the manufacture of limited amounts of dextrins (Nasution *et al.*, 2023). Amylase from different microbial sources also varies in form, so it is suitable for its specific application (Mehta and Satyanarayana, 2016).

Currently, amylase is one of the most popular enzymes, including in the field of biotechnology. Although enzymes can come from various sources, such as plants, animals, and microorganisms, enzymes from microorganism sources usually very often meet industrial demands, especially types of bacteria and fungi (Kumari *et al.*, 2019). In addition to the role of the amylase enzyme in the field of biotechnology, amylase produced by microbes has the potential to be used in the pharmaceutical and chemical industries and the use of amylase has expanded in other fields, such as medicine, analytical chemistry, and its application in starch saccharification as well as in the textile, food, manufacturing beer as well as distilleries (Martin *et al*., 2019).

The global market for enzymes in industrial applications should grow from $6.4 billion to $8.7 billion (Aehle, 2007). This is due to the increase in sales of microbial enzymes globally because enzymes derived from microorganisms have optimal thermostability and activity at high temperatures (Chandra *et al*., 2020). In 2018 there was an increase in enzyme sales of 9.2%, this increase in enzyme sales is predicted to continue to increase until 2023. One type of enzyme that has been applied in various industrial fields is amylase (Mehta and Satyanarayana, 2016).

Several studies on thermophilic bacteria and thermostable enzymes have been done, among them are obtaining optimum conditions for amylase production at 60°C with pH 7 and agitation of 150 rpm (Arzita and Agustien, 2013). Apart from that, (Indriati *et al*., 2015) stated that obtained optimum conditions at 60°C and pH 8.5, While (Indriati *et al*., 2018) stated that, obtained optimum conditions for amylase production at 50°C and pH 7.5 and (Ardhi *et al.*, 2020) stated that obtain optimum conditions at a temperature of 55°C with a pH of 6 and agitation of 200 rpm.

This study used bacterial isolates from the collection of biotechnology laboratory UPT Sumber biological resources which were isolated from three hot springs in South Solok, namely Sapan Aia Angek, Sapan Maluluang, and Balun in 2021. Thirty-one isolates were obtained from the study, six of which were potentially protease-producing, four potentially amylase-producing and 21 potentially producing. In this study, the isolates that have the highest Amylolithic Index (IA) values will be the bacterial isolates TUA-07 and TUA-09 (Andalas University thermophilic). For industrially

distributed applications, it is necessary to optimize the production of enzymes for this isolation by testing several abiotic factors, including temperature, pH, and agitation.

Materials and Methods

Growth Curve

Incubated at 50°C (inoculum), agitated at 150 rpm for 24 h. After that, 5 mL of inoculum into 95 mL of amylase production medium in a 250 mL Erlenmeyer. Sampling 1 mL of bacterial culture, then measuring turbidity using a spectrophotometer Thermo Scientific GENEYSY 150 UV-vis at a wavelength of 600 nm. The Optical Density (OD) measurement will show the growth curve. The OD value indicates whether bacteria are growing rapidly or slowly in a medium. At every 1 h interval, sampling was carried out in bacterial culture as in the previous step. The footage was stopped after a decrease in the growth of the bacterial isolates.

Iodine Method Amylase Activity Test

Prepared two test tubes, labeled control and test. In each tube, 1 mL of cassava starch substrate was added and incubated for 5 min at 50°C. Then, add 0.5 mL of distilled water to the control tube and 0.5 mL of enzyme to the test tube, and incubate for 30 min at 50°C, followed by 30 min at 125°C to stop the enzyme process. After 5 min, both tubes were cooled, and 1 mL of iodine-potassium iodide solution (Merck 109261) and 8 mL of distilled water were added to each tube. The absorbance was measured using a spectrophotometer with a wavelength of 540 nm (Ariandi, 2016).

Determination of Optimum Temperature and pH

Variation of pH medium 6, 7, 8 as factor A, pH changes can be achieved by using 1M HCl buffer to reduce the pH and 1 m NaOH buffer to raise the pH and incubated at 50, 60, 70 and 80°C as factor B by utilizing a temperature-adjustable incubator respectively. Then, agitated at 150 rpm the Kharisma Scientific Gemmy VRN-360 version of the shaker for a certain time (idiophase) of each bacterial isolate, and then, the enzyme activity assay was carried out.

Optimum Agitation Determination

Varyed at 100 rpm agitation; 125, 150, 175, and 200 rpm used the Kharisma Scientific Gemmy VRN-360 version of the shaker with medium conditions according to the results of the optimum treatment of temperature and pH. Then, it was sampled for a certain time (idiophase) from each bacterial isolate, and the enzyme activity assay was carried out.

Amylase Stability Testing

Testing the stability of the enzyme was carried out by incubating the crude extract of the enzyme obtained from the optimum treatment, the enzyme was incubated in an incubator at a temperature of 60°C. Then it was sampled at 1 h intervals until the amylase activity decreased.

Statistical Analysis

Data analysis on temperature and pH optimization was carried out statistically with a factorial Completely Randomized Design (CRD) with 3 replications, followed by Duncan's test. Then proceed with the observation parameters. The parameters analyzed were temperature, pH, agitation optimization, and amylase stability test by testing amylase activity.

Results

Growth Curve and Amylase Activity Curve

For the growth curve and amylase activity test, the temperature used is 50°C, pH 7, and agitation 150. The growth curve and amylase activity curve are used as the basis for treatment for further actions. The growth curve values were measured with Optical Density (OD) using a spectroscopic photometer and we also performed amylase activity tests simultaneously with the iodine method. The growth curve of the bacterial isolate TUA-07 shows a very short lag phase. This is because the medium used for inoculum is the same as the production medium so the bacteria do not take long to adapt. According to (Gharavi *et al*., 2021) one of the factors that affects the length of the adaptation phase is the medium, bacteria that are placed in the same medium as the previous medium require a short adaptation time.

Furthermore, the exponential phase is when cells in microorganisms are in a stable state and can divide multiple times. The graph shows a significant exponential phase occurring from the 1st to the 14th h. After that the bacteria began to enter the death phase in Fig. 1. It can be seen that after the 14th h, the number of bacterial cells began to decrease, this indicates that the number of dead bacterial cells increased due to the reduced food or nutrients needed by bacteria to live (Klumpp and Hwa, 2014).

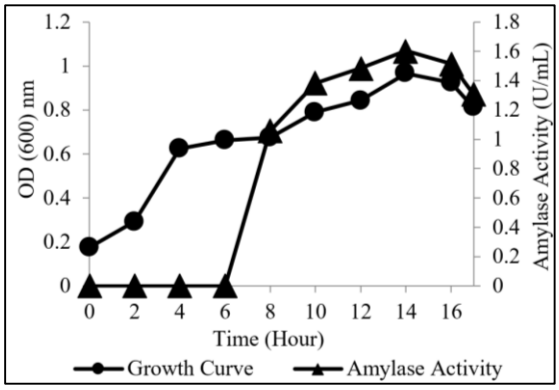

Fig. 1: Growth curve and amylase activity of isolate TUA-07

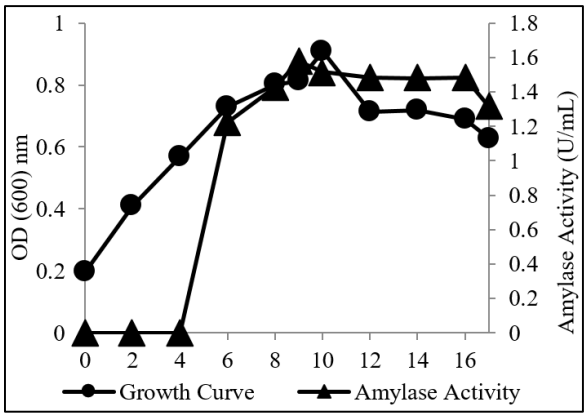

Fig. 2: Growth curve and amylase activity of isolate TUA-09

Figure 2 shows the growth curve and the activity test curve of the TUA-09 amylase isolate using the same media and parameters as TUA-07 isolates, i.e., temperature 50°C, pH 7, and agitation 150 rpm. Furthermore, the bacterial isolate TUA-09 has a very short lag phase. According to (Loutfi *et al.,* 2020), the short adaptation phase occurs because these bacteria are grown on the same media when making inoculums, this causes a short period of microbial adjustment to the new environment. then continued with the exponential phase at the 1st to 10th h. The ability of bacteria to multiply cells in the medium is indicated by the turbidity that forms in the medium. Turbidity occurs because bacterial cells grow, multiply, and secrete enzymes into the medium (Hatiboruah *et al*., 2020). Furthermore, the bacterial cells experienced a death phase seen at the 11-17th h. The next hour shows a decrease in the number of bacterial cells which means the bacteria are experiencing a death phase. The death phase occurs when the amount of substrate decreases below the concentration needed to maintain cell survival, so that the cell lysis and dies (Klumpp and Hwa, 2014).

Determination of Temperature and pH for Amylase Production

Table 1 shows the results of the analysis of temperature and pH treatments using a Completely Randomized Design (CRD). In this treatment, the samples were sampled according to the idiophase time on the growth curve and the previous amylase activity test, with the medium conditions adjusted to the temperature and pH parameters, while the agitation used was 150 rpm.

Table 1 shows that the treatment of the temperature factor showed the best results at 60°C, the pH of all treatments did not show significantly different results. Meanwhile, for all treatment combinations, the bacterial isolate TUA-07 averaged the highest amylase production, namely 2.049 U/mL at 80°C and pH 6.

Table 1: Average amylase production at different incubation temperatures and medium pH on bacterial isolate TUA-07

	pH			
Temp (°C)	pH 6	pH 7	pH 8	Average (Temp)
50	1.459e	1.793c	2.008ab	1.754B
60	1.767cd	1.787c	1.941b	1.831A
70	1.363f	1.375f	1.351f	1.363C
80	2.049a	1.692d	1.495e	1.745B
pH average	1.659A	1.661A	1.698A	

Note: The numbers in each row and column followed by the same uppercase and lowercase letters show results that are not significantly different for each single factor and interaction factor in the DNMRT test at the 5% level

Table 2: Average amylase production at different incubation temperatures and medium pH on bacterial isolate TUA- 09

	pH			
(Temp) (°C)	pH 6	pH 7	pH 8	Average Temp
50	1.565c	1.884a	1.713b	1.720A
60	1.844a	1.532c	1.838a	1.737A
70	1.409d	1.373d	1.379d	1.387C
80	1.534c	1.829a	1.645b	1.669B
pH average	1.588B	1.654A	1.643A	

Table 3: Average amylase production for different agitation

Agitation (rpm)	TUA-07	TUA-09
100	1.146	1.425
125	2.789	2.924
150	2.049	1.713
175	2.542	2.747
200	1.181	1.140

Table 2 shows the analysis of temperature and pH parameters of the TUA-09 insulation with the same treatment as the TUA-07 insulation. The treatment of bacterial isolate TUA-09 concerning incubation temperature showed that the highest amylase activity was at 50°C and 60°C, for the pH medium treatment the amylase activity was highest at pH 7 and 8, while for all combination treatments, the amylase activity showed that the incubation temperature was 50°C with pH 7; 60°C with pH 6; 60°C with pH 8 and 80°C with pH 7 did not show significant differences in the four treatments, but the highest amylase activity was shown at 50°C pH 7 is 1.884 U/mL.

Determination of Optimum Agitation for Amylase Production

Table 3 is an optimization for agitation using the treatment obtained from the best results at the previous stage, where the TUA-07 insulation is optimal at a temperature of 80°C with a pH of 6, whereas TUA-09 insulation is optimal at 50°C with a pH of 7. Table 3 shows that both isolates produced the highest amylase activity at

125 rpm agitation. This shows that agitation affects enzyme production. Agitation helps the process of oxygen transfer; the agitation system causes air bubbles to become smaller so that the outer surface where oxygen transfer occurs becomes larger and the residence time of air bubbles in the medium is longer (Zhou *et al.,* 2018).

Table 3 also shows that the agitation that has a low activity value is agitation at 200 rpm, this is because very fast shaking will form foam in the medium which can affect bacteria in enzyme production so that enzyme production becomes low (Grahame *et al*., 2015).

Conditions of Amylase Activity Before and After Optimization

Table 4 shows that the optimization of bacteria affects amylase activity. The bacterial isolate TUA-07 showed an increase in amylase activity up to 73.98% of the initial enzyme activity, while the bacterial isolate TUA-09 showed an increase in amylase up to 85.18% after optimization of the bacterial isolate. This proves that abiotic factors such as temperature, pH, and agitation have an influence on bacteria in enzyme production by the statement of (Shu *et al*., 2016) that increased enzyme production can be achieved by engineering the composition of the medium, medium pH, temperature, carbon source, and nitrogen source.

Amylase Stability Test

After obtaining optimal conditions for both isolates, a further stability test is carried out to determine how long the enzyme is stable. Based on the previous treatment obtained, the TUA-07 isolate is optimal at a temperature of 80°C, pH 6, with agitation of 125 rpm, whereas the TUA-09 isolate is optimal at a temperature of 50 °C, pH 7, with agitation of 125 rpm. Figure 3 shows stability test results on isolate TUA-07 showed that the enzyme activity at the 10th h had begun to decrease by 80% from the initial activity to the 15th h, namely 69% of the initial activity while in isolate TUA-09 at the 8th h, the activity amylase fell to 80% until at 15th h it fell to 52% of initial activity. This shows that the resulting enzyme has a fairly high stability. According to (Mitra *et al*., 2021) the high value of microorganisms that produce extracellular enzymes lies in their extreme thermostability where the thermostability of amylase is used in starch bioprocessing.

Table 4: Conditions of amylase activity before and after optimization

	Amylase activity (U/mL)		Increased amylase
Isolate	Before	After	activity (%)
TUA-07	1.603	2.789	73.98
TUA-09	1.579	2.924	85.18

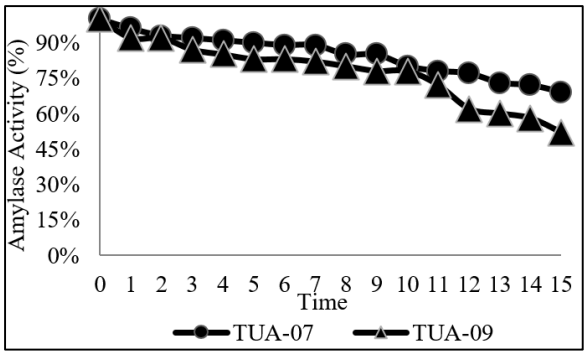

Fig. 3: Graph of amylase stability test results

Discussion

Growth curve and amylase activity of isolates TUA-07 shows the highest amylase activity at the 14th h, namely 1.603 U/mL, this indicates that the enzyme harvest time can be carried out at the 14th h. Research conducted by (Arzita and Agustien, 2013), *Bacillus* sp. PA-05 optimal activity at 14th h. The incubation time with the highest amylase activity is used as the optimum incubation time for amylase production (Berikashvili *et al.,* 2018).

Figure 1 it can be seen that after reaching the optimum activity at the 14th h the enzymes began to decrease. This is because after achieving optimum activity the glucose levels resulting from the hydrolysis of starch into glucose by enzymes are sufficient for the needs of the bacteria so that the bacteria do not need to produce enzymes by hydrolyzing starch (Shah *et al.,* 2014).

Meanwhile, in Fig. 2 it can be seen that the highest amylase activity was at the 9th h, namely 1.579 U/mL. This indicated that the bacterial isolate TUA-09 could be harvested at the 9th h, it's not the same as bacterial isolate TUA-07 which was harvested at the 14th h. According to (Loutfi *et al*., 2020), the largest amount of amylase was produced during the 10th h of incubation. This difference is related to the ability of the bacteria in each isolate to create primary and secondary metabolites.

Previous research showed that optimum conditions for amylase production were obtained at 60°C with a pH of 7 and agitation of 150 rpm (Arzita and Agustien, 2013). Apart from that (Indriati *et al*., 2015) stated that obtained optimum conditions at a temperature of 60°C and a pH of 8.5, and (Ardhi *et al.,* 2020) obtained optimum conditions at a temperature of 55°C with a pH of 6. Each enzyme has an optimum pH where at that pH its three-dimensional structure is most conducive for binding to substrates. According to Waldenström (1975) varies in the condition of the ion substrate and enzymes result in decreased enzyme activity when pH varies. These changes can occur in amino acid residues that function to maintain the tertiary and quaternary structures of active enzymes. Changes in temperature

and pH have an increasing effect on the growth of microorganisms and the production of amylase (Bisswanger, 2017). pH greatly influences enzymatic reactions because it can affect ionic groups of enzymes, thereby affecting the active site of the enzyme and conformation (Waldenström, 1975).

Table 2 shows that temperature has two different effects, firstly temperature can increase enzyme activity and vice versa can denature enzymes (Sakuma *et al.*, 2021). According to Nigam (2013), stated increasing the temperature will enhance the speed of the enzyme-catalyzed reaction, but only within a specific temperature range.

Indriati *et al*. (2018) discovered that the thermophilic had the maximum amylase activity at 50°C. Another study (Divakaran *et al.*, 2011) found that the optimal temperature for amylase activity was 55°C. This was due to protein denaturation that occurs when the temperature increases, if denaturation occurs, the active part of the enzyme will be disrupted and the reaction rate will also decrease. As for the pH of the medium, if it is not at the optimal pH, it will interfere with the activity of the bacterial enzymes (Waldenström, 1975). Under these conditions, the substrate will also undergo conformation so that the reactive group will be inhibited from binding to the active site of the enzyme (Klumpp and Hwa, 2014).

Table 3 is the continuation of optimization after obtaining the optimum conditions for temperature and pH where the TUA-07 insulation is optimal at a temperature of 80°C with a pH of 6, whereas TUA-09 insulation is optimal at 50°C with a pH of 7. Table 3 shows that both isolates produced the highest amylase activity at 125 rpm agitation. It shows that improved abiotic factors such as temperature, pH, and agitation have an influence on bacteria in enzyme production by the statement of (Shu *et al.,* 2016) that increased enzyme production can be achieved by engineering the composition of the medium, medium pH, temperature, carbon source, and nitrogen source.

After obtaining the specified optimal condition, the enzyme stability test as shown in Table 4 shows that both isolates can maintain their amylase activity after being treated according to their optimal condition where isolate TUA-07 the amylase activity is 80% stable for 10 h, while for isolate TUA-09 the amylase activity is 80% stable for 8 h. Several studies also experienced an increase in amylase activity after optimization including (Sharif *et al.*, 2023) whose research results showed that amylase activity increased by 31.1% after optimization at 55°C, pH 7.0. (Sharif *et al.,* 2023) showed that amylase activity increased by 85.5% at 60°C, pH 7.5, and (Sharif *et al.,* 2023) showed that amylase activity increased by 45.5% at 55°C, pH 7.0.

Martins *et al.* (2019) state that a decrease in enzyme activity of 20-30% from the initial value is considered a minimal limit for a decrease in enzyme activity that can be said to be stable and acceptable in many industrial or biological applications.

According to Sedijani *et al.* (2022), the thermostability possessed by enzymes derived from thermophilic microorganisms binds to the association of enzyme protein compounds with other molecules such as lipids, polysaccharides, and other proteins which causes the formation of a compound that has a mechanism that allows it to remain stable. Stable, in addition, there is an increase in hydrogen bonds and salt bridges in the proteins of thermophilic enzymes and differences in the types and composition of amino acids that make up thermophilic enzyme proteins compared to mesophilic enzyme proteins.

Stability is an important property that enzymes must possess in their application as biocatalysts. Enzyme stability can be defined as the stability of enzyme activity during storage and use of the enzyme, as well as stability to compounds that are damaging such as certain solvents (acids, bases), and under the influence of temperature and extreme pH. There are two main principles for obtaining enzymes that have high stability: Using enzymes that have extreme stability and trying to increase the stability of enzymes that are naturally lacking or unstable (Cao *et al.*, 2021).

Conclusion

Based on the results of the above study it can be concluded that the optimum temperature, pH, and agitation for isolate TUA-07 is 80°C at pH 6 and agitation of 125 rpm while for isolate TUA-09 it is 50°C at pH 7 and agitation of 125 rpm. Enzyme stability for isolate TUA-07 the amylase activity is 80% stable for 10 h, while for isolate TUA-09 the amylase activity is 80% stable for 8 h.

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Author's Contributions

Anthoni Agustien: Conceived the original idea, wrote the manuscript, designed the study, and reviewed and approved the manuscript.

Denny Bendrianis: Data analysis, experimental development.

Feskaharny Alamsyah: Designed research methodology and analyzed.

Mufidhatul Muqarramah: Materials and equipment engagement, literature search.

Mifthahul Jannah: Materials and equipment engagement.

Ethics

This article is completely original and has never been seen before. The relevant author declares that the work has been read and accepted by all other authors and that there are no ethical inconsistencies.

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