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Thermozyme Amylase from *Enterobacter* sp Extremophiles in Bioethanol Production

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Abstract: Bioethanol is an alternative fuel to replace fossil fuels due to the reduction of fossil fuels. Bioethanol is produced from starch and converted to sugar by thermozyme amylase. Thermozyme amylase produced by thermophile bacteria can be obtained from Pariangan hot springs which have a temperature of 55 °C, pH of 9.2, and have a high level of bacterial diversity. The aim of this study was to obtained isolates of thermozyme amylase-producing bacteria that have the potential for bioethanol production. The research method were bacteria isolation, amylase stability test, temperature and pH optimization, and bioethanol production. The result showed that *Enterobacter* sp has been isolated from Pariangan hot springs is stable up to 5 hours of incubation, temperature and pH optimum was 85 °C, pH 8.5, and fructose as a carbon source. Thermozyme amylase converts starch into sugar under optimum conditions with a yield of 9.8% bioethanol.

Keywords: amylase; bioethanol; extremophiles; *Enterobacter* sp; Pariangan Hot Spring

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Introduction

Amylase produced by bacteria or bacteria amylase is an enzyme that can hydrolyze starch to sugar (Megahati et al 2017). Amylase can convert starch to sugar in bioethanol production. Bioethanol is a biofuel that is renewable and environmentally friendly (Fatoni, & Zulfahair. 2012). Bioethanol is an alternative fuel to replace fossil fuels due to the reduction of fossil fuels (Svetlitchnyi et al., 2013). Bioethanol is used as a gasoline mixture to become biofuel. The composition of bioethanol in gasoline in the world has 5% in 2020 and increased by 20% in 2025.

Generally, bioethanol is produced from starch and converted to sugar by thermozyme amylase. As a thermozyme, amylase generally does not undergo denaturation and is active at high temperatures and is very stable at normal temperatures (Schiraldi, & Rosa, 2002). Thermozyme amylase is one of the three largest industrial group enzymes and accounts for approximately 65% of the world enzyme (Abdullah et al, 2014). Thermophile bacteria are commonly found in volcanic craters, craters on the ocean floor, and hot springs. Some hot springs in Indonesia generally have a pH of 7 or even below 7 or are acidic. In contrast to the Pariangan hot springs which have a temperature of 47 ^{o}C-51 °C and a pH of 9.2. This causes Pariangan hot springs to have a high level of bacteria diversity. The aim of this study was to obtained isolates of thermozyme amylase-producing bacteria that have the potential for bioethanol production. Bioethanol as a biofuel is needed as an alternative fuel to reduce the use of fossil fuels. Bioethanol can be obtained by utilizing the potency of thermophile amylase-producing bacteria which will be carried out in this study. The success of this research is a finding/innovation that can be applied in the development and development of science and technology.

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Method

Isolation and screening of bacteria

A hot water sample was poured into the medium NA ((20 g/l)) and incubated for 24 hours at 50 °C. Bacteria growing on the NA medium were transferred into the selective medium (10 g/l starch and 15 g/l agar) and incubated at 50 °C for 24 hours. Growing bacteria colonies are selected by pouring iodine solution around the bacteria colony.

Production and amylase activity

Bacteria were planted in 25 ml of basal medium pH 7.5 (3 g/l KH_2PO_4, 3g/l K_2HPO_4, 5 g/l K_2HPO_4, 5 g/l NaCl, 10 g/l starch) and were agitated at a speed of 150 rpm for 24 hours at 50 °C. 5% of bacterial culture was transferred to 50 ml of the new basal medium pH 7.5 and growth was followed by absorbance measurements at 540 nm. The growing bacteria culture was centrifuged at a speed of 5000 rpm for 5 minutes. The cell pellets were resuspended with 3 ml sucrose solution 0,3 M ; 0.1 M Tris HCL pH 8.5 ; and 0.2 mg/ml lysozime. The suspension was shaken with vortex for 1 minute and incubated for 30 minutes in ice. The suspension was centrifuged for 20 minutes at a speed of 15000 rpm at 4 °C. The supernatant produced is crude amylase enzyme whose activity will be tested. Amylase activity assay was determined according to the Samogy-Nelson methods (Nelson, 1974).

Amylase stability test

The amylase produced by the three bacterial isolates was incubated at 50 °C with varying incubation times (0-8 hours) and the amylase activity was measured. Bacteria that have the most stable amylase will be used in this study.

Morphological characterization and biochemical properties of bacteria isolates

Bacteria characterization was carried out by observing the bacteria's cell shape and Gram staining. Biochemical properties are carried out by performing several biochemical tests such as Catalase, Oxidase, TSIA, Urea, Citrate, Indole, Lactose, Glucose, Sucrose, Mannitol, MR, VP, OF, Arabinose, Nitrate, and Gelatin (Cowan & Steel's, 1974).

Temperature Optimization

Bacteria were planted in 50 ml of basal medium pH 7.5 (3 g/l KH_2PO_4, 3g/l K_2HPO_4, 5 g/l K_2HPO_4, 5 g/l NaCl, 10 g/l starch) and were agitated at a speed of 150 rpm for 24 hours at 50 °C. 5% bacterial culture was transferred to 100 ml of a new basal medium pH 7.5 and agitated at a speed of 150 rpm for 10 hours at various temperatures (60 °C-95 °C). The growing bacteria culture was centrifuged at a speed of 5000 rpm for 5 minutes, the supernatant containing amylase was tested for its thermozyme amylase activity.

pH Optimization

Bacteria were planted in 50 ml of basal medium pH 7.5 (3 g/l KH_2PO_4, 3 g/l K_2HPO_4, 5 g/l K_2HPO_4, 5 g/l NaCl, 10 g/l starch) and were agitated at a speed of 150 rpm for 24 hours at 50 °C. The 5% bacteria culture was transferred to 100 ml of a new basal medium with varying pH (7.0-9.5) and agitated at a speed of 150 rpm for 10 hours at the optimum temperature (the result of temperature optimization at stage 1). The growing bacteria culture was centrifuged at a speed of 5000 rpm for 5 minutes, the supernatant containing amylase was tested for its thermozyme amylase activity.

Carbon Sources

Source of carbon (fructose, sucrose, lactose, and glucose) with a concentration of 1% added to the production medium pH 8.5 and shaker at 150 rpm with a temperature of 50°C for 24 hours. The 5% bacteria culture was transferred to 100 ml of a new basal medium with varying pH (8.5) and agitated at a speed of 150 rpm for 10 hours at the optimum temperature (the result of temperature optimization at stage 1). The growing bacteria culture was centrifuged at a speed of 5000 rpm for 5 minutes, the supernatant containing amylase was tested for its thermozyme amylase activity.

Bioethanol production

Cassava has been crushed into a cooker tank and the liquefaction process is carried out at a temperature of 80°C-85°C by adding 5 ml of thermozyme amylase. The saccharification is carried out at a temperature of 55 °C and inserted 2.5 ml of glucoamylase and then stirred evenly. The saccharification is carried out at 37°C and fermentation are carried out by inserting 20 grams of Urea fertilizer and 5 grams of NPK fertilizer into the saccharified liquid. After stirring evenly, add 15 grams of *Saccharomyces cerevisiae*. Cover the fermentor tightly for 7 days and the fermentation process will occur anaerobically. Distillation is carried out using a fractional distillator (multi-level column) with the reflux column system technique. The distillation process is carried out at a temperature of 78 °C. Ethanol vapor in the distillator flow to the condenser so that it is condensed into bioethanol liquid.

Result and Discussion

Isolation and screening of bacteria

Three isolates of thermozyme amylase-producing bacteria have been obtained from 16 isolates that have

been successfully isolated in the Pariangan hot spring. This is shown by the formation of clear zones around bacteria growth (Figure 1).

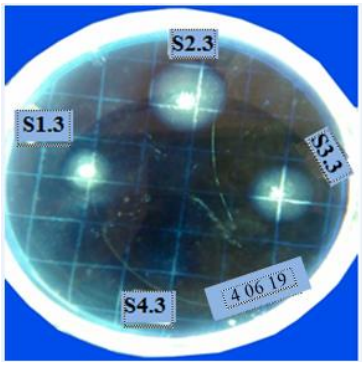


Figure 1. Clear zones around bacteria growth

The clear zone is formed as a result of the hydrolysis of starch by thermozyme amylase produced by bacteria. The media surrounding S4.3 bacteria isolate that does not produce thermozyme amylase will be blue when iodine solution drops. This shows that the starch in the medium is not degraded to simple sugars which means that bacteria do not produce thermozyme amylase (Megahati, 2018).

Production and amylase activity

The results of the thermozyme amylase activity test for the three bacteria isolates showed that the S2.3 bacteria isolate had the highest specific activity, namely 465.8 U/mg (Figure 2). The difference in amylase activity was due to the fact that the S2.3 bacteria isolates were better able to degrade starch than the S1.3 and S3.3 isolates.

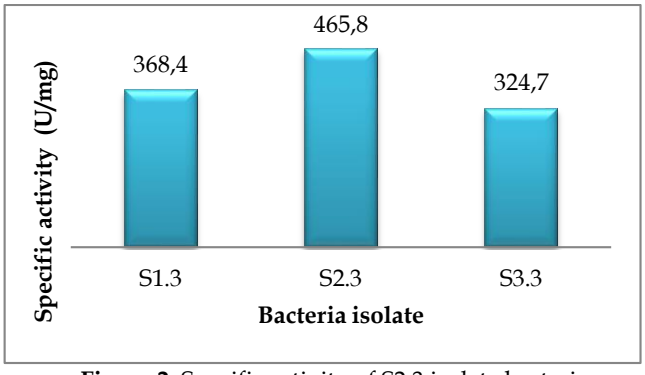
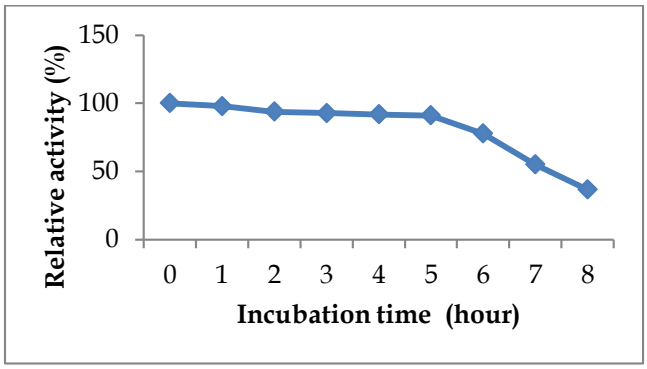


Figure 2. Specific activity of S2.3 isolate bacteria

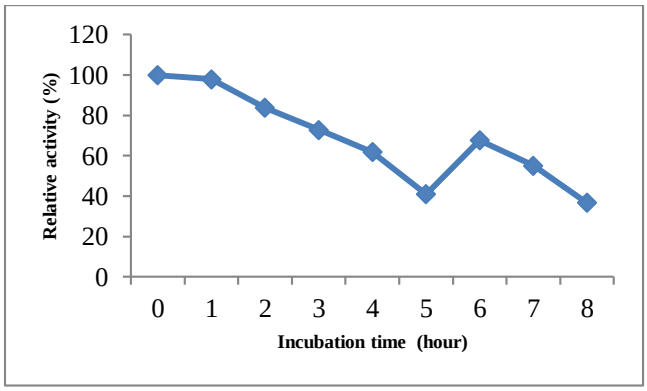
The difference in enzyme activity in each isolate is caused by the amount and activity of the enzyme secreted from each isolate (Agustien, 2005). Screening and testing for amylase activity were also conducted by Arfah et al, (2015) who obtained R-SAII-1b isolates from hot springs Lejja (South Sulawesi) with an activity of 0.0711 U/mg protein.

Amylase stability test

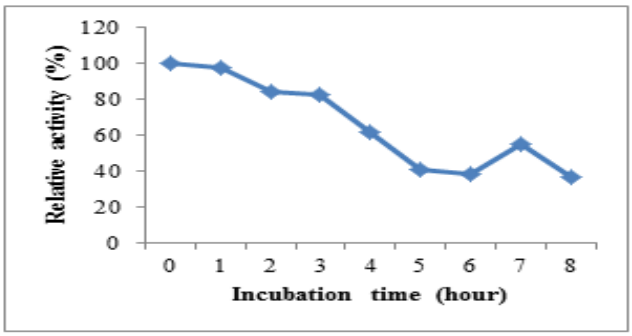
The amylase stability test on the three thermophile bacteria isolates showed that the amylase produced by the S2.3 bacteria isolate was more stable than the amylase produced by the S1.3 and S3.3 bacteria isolates (Figure 3). Amylase produced by the S2.3 bacteria isolates was 100% stable until the fifth hour of incubation (temperature 50 °C) while at the sixth to eight hours the amylase lost its stability.



a) Amylase stability of bacterial isolates S1.3



b) Amylase stability of bacterial isolates S1.3



c) Amylase stability of bacteria isolates S3.3

Figure 3. Amylase stability of bacteria isolate S2.3, S1.3, and S3.3

Amylase produced by bacteria isolates S1.3 and S3.3 is unstable, this can be seen from the fluctuating amylase activity. This is thought to be due to the influence of chaperonin, chaperonin is a functional

protein that plays a role in retaining the three-dimensional structure of proteins from denaturation of extreme environmental temperatures (Kumar & Nussinov, 2001). The amylase stability of *Bacillus* sp AB68A was 60% stable for 60 minutes of incubation at 50 °C (Aygan et al., 2008). The stability of proteins or enzymes is influenced by different factors from each taxon, organism, and even the same organism. Protein stability will vary in each organism even though these organisms come from the same environment (Kumwenda et al., 2013). Likewise, the S2.3 bacterial isolate was more stable than the amylase from other bacteria isolates even though it came from the same hot spring. Therefore S2.3 bacteria isolate will be used for research in bioethanol production.

Morphological characterization and biochemical test of bacteria isolates

Based on the results of morphological characterization and biochemical test of bacteria isolates shown that S2.3 isolate belonging to *Enterobacter* sp (Table 1). According to Cowan & Steel's (1974) *Enterobacter* sp is a group of Gram-negative rod-shaped bacteria, is aerobic and facultative anaerobic, capable of producing gas, on a positive motility test. *Enterobacter* sp from Pacet hot springs, Mojokerto, Indonesia can produce amylase with high activity (Sari, 2016).

Table 1. Morphology and biochemical properties test results

No	Test of morphology and biochemical properties	Results
		S2.3 isolate
1	Colony characteristic	Yellowness colored, rounded shape, flat smooth, and flat elevation
2	Gram staining	Gram-negative
3	Endospores	Positive
4	Cell shape	Bacillus
5	Catalase	+
6	TSIA	Red/yellow
7	Oxidase	+
8	Indole	+
9	Urea	-
10	Citrate	+
11	Lactose	+
12	Glucose	-
13	Sucrose	-
14	Mannitol	+
15	MR	+
16	VP	+
17	OF	-
18	Arabinose	+
19	Nitrate	-
20	Gelatine	-
21	Xylose	-
	Genus proposed	Enterobacter sp

Enterobacter sp is found in various places, such as in soil, hot springs, and seawater sediments. Enterobacter is rod-shaped, Gram-negative, anaerobic facultatively, and contains peritrichous flagella. This is oxidase-negative and catalase-positive (Streit , 2004).

Bacteria Growth Curve

The optimum time for thermozyme amylase activity and *Enterobacter* sp growth was obtained through a bacteria growth curve. Bacteria growth increased at eight hours followed by an increase in specific amylase activity of 27.64 U/mg (Figure 4). At the tenth hour of bacterial death followed by a decrease in amylase activity.

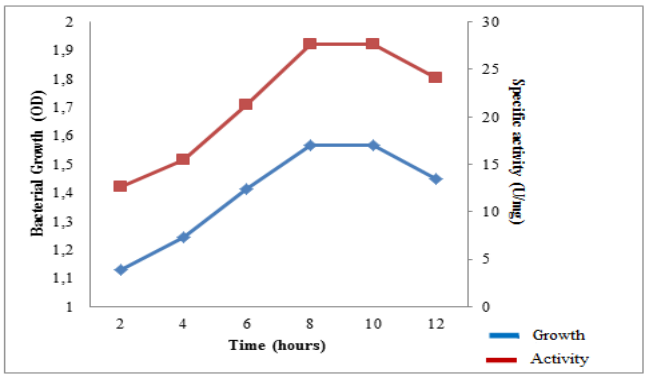


Figure 4. Time of bacterial growth and S2.3 isolate amylase production

Optimum growth and amylase activity of *Anobaxycillus* sp. IB-A occurs at 48 hours (stationary) after which the bacterial isolate goes to the death phase (Hauli et al., 2013). Increasing the incubation time decreases amylase production. This is due to reduced nutrients, accumulated toxicity in the medium, amylase proteolysis, and microorganisms (Yang & Liu, 2004).

Temperature optimization

The results of temperature optimization on amylase activity showed an increase in amylase specific activity at 85 °C which is 487.6 U/mg because it is the temperature suitable for the growth of bacteria for amylase production (Figure 5). An increase in incubation temperature above 85 °C will cause a decrease in amylase activity.

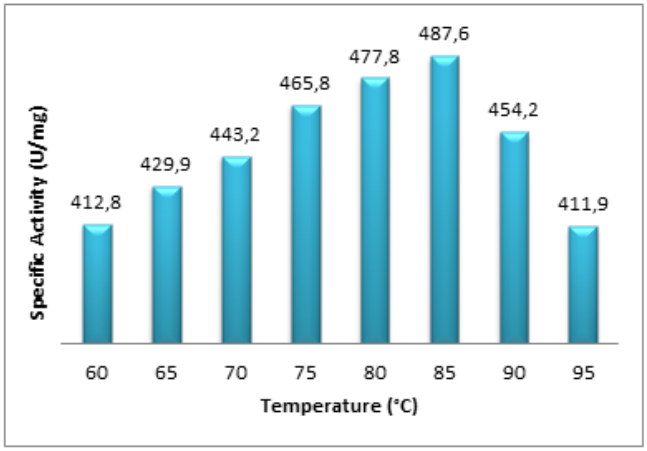


Figure 5. The effect of temperature to amylase production

This is caused by the amylase denaturation which causes amylase conformation change. Activity enzymes increase with increasing temperature of the growth medium to achieve optimum enzyme activity (Pelczar, 1988). The effect of temperature on amylase production is related to growth bacteria (Lehninger, 1982).

pH Optimization

Increasing the pH will increase the amylase activity up to reach the optimum pH. The pH of the production medium above pH 8.5 is caused by decreased amylase activity (Figure 6). Decreased amylase production above pH 8.5 caused by a change in the environmental conditions of the enzyme, where the pH an alkaline enzyme environment will cause an increase in concentration OH^- in the enzyme environment (Asgher et al., 2007).

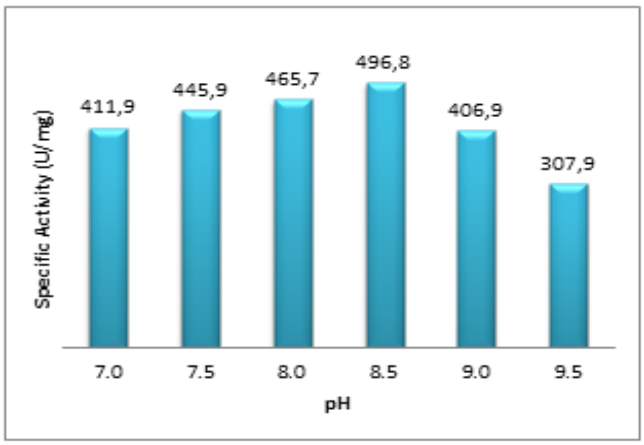


Figure 6. The effect of pH to amylase production

Each bacteria has a growth medium with the right pH because the pH of the media plays a role in morphological changes and enzyme secretion (Megahati, 2018). Each bacteria has an optimum growth media pH for the growth and production of the enzyme with the highest activity (Nwagu & Okolo, 2011).

Carbon Sources

Carbon sources (fructose) can increase amylase production compared to other carbon sources (Figure 7). According to (Mei & Chen, 2007; Sudarhsan et al., 2007) fructose is a good source of carbon in increasing amylase production. Fructose and glucose are very effective in stimulating bacterial growth and respiration (Choubane et al., 2015). Meanwhile, according to Hasan & Hameed, (2001), lactose can increase the activity and production of amylase compared to glucose, fructose, and sucrose. Glucose is efficient in producing amylase from *Bacillus thermooleovoran* (Belal et al., 2015). Carbon sources have an important role in providing nutrients and energy sources to bacteria.

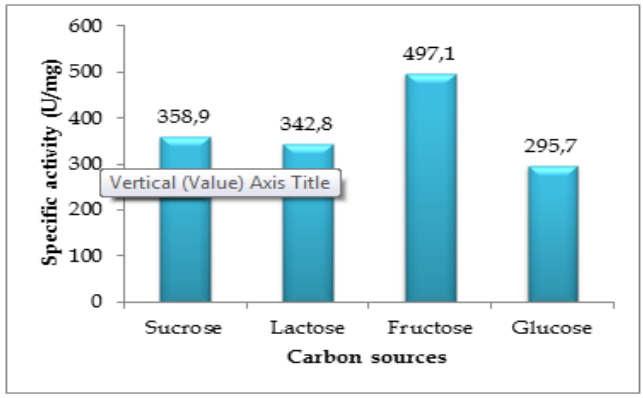


Figure 7. Effect of carbon sources on thermozyme amylase production

The addition of carbon sources to the production medium plays a role in enriching the formation of amino acids and gases from carbon sources (Hasan & Hameed, 2001).

Bioethanol production

Generally, bioethanol is produced from the basic ingredients of starch (Cassava) and converted to sugar by amylase. Biological conversion of biomass into bioethanol by utilizing the potential of extremophiles is preferred because it will save production costs. Bioethanol distillation using a fractional distillator (multi-level column) with the reflux column system technique produces bioethanol with a concentration of 9.8%. Utilizing the potential of amylase in converting starch from potato waste for bioethanol production and obtaining bioethanol at a concentration of 9.6% (Belal et al., 2015). This shows that the amylase produced by *Enterobacter* sp has the potential to produce bioethanol. Bioethanol as a biofuel is needed in the world, especially in Indonesia as an alternative fuel to reduce the use of fossil fuels (Svetlitchnyi et al., 2013). If the use of fossil

fuels is not controlled, it can cause the depletion of petroleum supplies.

Conclusion

Thermozyme amylase is stable up to 5 hours of incubation, temperature, and pH optimum was 85°C, 8.5, and fructose as carbon sources. Thermozyme amylase converts starch into sugar under optimum conditions with a yield of 9.8% bioethanol.

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