Thermostable amylase activity produced by thermophilic bacteria isolated from Pulu Hotspring, Central Sulawesi

P Satrimafitrah*, A R Razak, J Hardi, D J Puspitasari, I Yelenggete, and Rafik

Department of Chemistry, Universitas Tadulako
Jl. Soekarno Hatta Km.9, Kampus Bumi Tadulako Tondo Palu,

*E-mail: pasjan@untad.ac.id

Abstract. Amylases are important enzymes used in a wide range of industries, such as foods, cosmetics, and pharmaceutics. They are classified based on their mechanism of actions in hydrolyzing starch. Amylases can be isolated from soils, plants, fungi, and bacteria, including thermophilic bacteria. The present study was focused on the determination of best temperature and pH of thermophilic amylase produced by thermophilic bacteria, isolated from Pulu Hotspring. As much as 30 isolates showed amylolytic activity in medium enriched with 1% starch. Of 3 isolates with the highest amylolytic index, PL 16 isolate with amylolytic index of 4.04 mm were studied further to find out its activity in amylose degradation. Enzyme activity was measured with the DNS method using maltose as standard. Temperature and pH measurement were varied in the range of 50, 60, 70, 80, 90, 100°C and 5, 6, 7, 8, 9, respectively. Optimum amylase activity was at a temperature of 90°C and pH 8. These results suggested that thermostable crude amylase from Pulu hotspring has a potential future for industrial application.

1. Introduction
Amylases are enzymes that able to degrade starch into smaller molecule such as glucose, and maltose [1]. These enzymes are classified into α-amylases, β-amylase, glucoamylase and pullulanase, based on their action in hydrolyzing starch [2]. The most common one is Alpha-amylase (EC.3.2.1.1), an extracellular enzyme that degrades starch into di- and trisaccharide, can be produced from animals, plants, and microorganisms such as bacteria [3]. The utility of amylase produced from bacteria has advantages in low cost but large and less time to produce, easily modified and optimized [4].

Amylase plays important role in the industrial processes such as food, paper, textiles, fuel alcohol, detergents and pharmaceuticals. The most common application of amylase is in production of glucose or fructose syrups where starch is hydrolyzed during liquefaction process [5]. As such a process is performed in high temperature, it requires amylase with specific thermostability and optimized pH [6]. Therefore, it is important to screen potential microorganisms that produce thermostable amylase with high activity in order to discover novel amylases for industrial application [7].

Central Sulawesi has several potential hot springs, such as Pulu, Uwelera, Uwedeka, and Bora [8]. The latter has been reported to have potential thermostable enzymes, i.e. α-amylase [9] and Chitosanase [10]. Pulu Hotspring has high temperature range of 60 - 70°C which makes it potential for screening of thermophilic bacteria that produce amylases. To date, information of properties of amylases originated from hot springs in Indonesia, particularly in Central Sulawesi remain little. Thus,
it is crucial to study amylases from local hot spring to better know its likely future implementation in industries.

2. Experiment procedure

2.1. Screening of thermophilic bacteria producing amylase

Water and sediment samples from Pulu hot spring were collected, homogenized, and serially diluted up to $10^{-9}$. Diluted samples then cultured in NA medium, $60^\circ C$ for 24 hours. Selected 30 colonies then further cultured in NA medium contained 1% starch in the same condition as previous incubation. Bacterial culture in petri dishes dripped with iodine solution (2% I$_2$ and 0.2% KI). Clear zones formed around the colony indicate amylase activity.

Amylolytic index values can be calculated using the formula [11]:

$$\text{Amylolytic Index} (AI) = \frac{\text{diameter of clear zone (cm)}}{\text{diameter of colony (cm)}}$$ (1)

With an assumption of the higher amylolytic index the higher enzyme activity

2.2. Production of crude amylase

Amylase enzymes were produced according to Asgher et al [12] using 1% starch in an MSM medium with the composition of 0.08% KH$_2$PO$_4$, 1% NaCl, 0.04% (NH$_4$)$_2$SO$_4$, 1% yeast extract and 1% starch in 100 mL distilled water. Starter culture was incubated at 60°C, stirred at 180 rpm for 24 h then transferred into fresh MSM media containing 1% starch and incubated further. After 24 hours, culture was centrifuged at 8000 rpm at 4°C then supernatant was collected as crude amylase.

2.3. Amylase activity assay and total dissolved protein content

Amylase activity was determined using the Bernfeld method [13]. A total of 2 mL of the reaction mixture consisting of 1 mL of 1% starch solution in 0.05 M phosphate buffer pH 7 and 1 mL of crude enzymes were incubated for 15 minutes at 60°C. Two mL of 3,5-dinitrosalycylic acid reagent was added to each tube to stop the reaction. Control samples were prepared by adding 3,5-dinitrosalycylic acid reagents before the addition of crude enzymes. Blank samples were made by adding 3,5-dinitrosalycylic acid reagents and adding substrates. All tubes were placed in boiling water for 15 minutes and were cooled at room temperature. The amount of reducing sugar is measured on a spectrophotometer with a wavelength of 540 nm. One unit of amylase activity is defined as the amount of enzyme that produces 1 µmol reducing sugars in the form of maltose per minute. Amount of total dissolved protein was determined using Warburg-Christian methods [14].

2.4. Determination of optimum temperature and pH

Optimum temperature and pH was determined by measuring amylase activity at 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C while optimum pH was ranging from 5 – 10 both in a 0.05 M phosphate buffer for 15 min.

3. Results and discussion

Of 50 thermophilic bacteria isolated from Pulu hot spring, 30 of them showed amylolytic activity in a 1% starch agar medium. The amylolytic index value showed 3 isolates with the highest amylolytic index values, namely PL 2, PL 15, and PL 16 (as shown in figure 1) with amylolytic index values of 2.31 mm, 2.42 mm, and 4.04 mm, respectively.

Amylolytic index is influenced by the diameter of the clear zone to the diameter of the colony. Bacterial colonies on solid media are not always rounded, in this study it was obtained, there are those that are round and not grained. So that measurements of bacterial colony diameters and clear zones are carried out on 3 different sides which will produce an average value of bacterial colony diameters and clear zones, as shown in figure 2. The amount of amylolytic index (enzymatic index) is influenced by
the speed of microbial growth in solid media and the ability to produce and the effectiveness of enzymes in solid media [15]. In our study, as seen in figure 2, PL 16 showed consistency as a colony with the highest amylolytic index thus we studied it further to know its enzymatic activity.

![Figure 1. Clear zone formation by PL16 (upper colony)](image)

**Figure 1.** Clear zone formation by PL16 (upper colony)

![Figure 2. Chitinolytic Index of isolated termophilic bacteria.](image)

**Figure 2.** Chitinolytic Index of isolated termophilic bacteria.

Isolate PL 16 has the enzymatic activity of 0.0274 U/mL with the total protein content of 5.13 mg/mL when treated under the same condition as in Pulu hotspring (pH 7 and temperature of 60°C). To find out its optimum working temperature we treat under several temperature variations of 50°C, 60°C, 70°C, and 80°C, 90°C, and 100°C at pH 7 for 15 minutes. In figure 3, Activity of amylase was increased from the temperature of 50 to 90°C and started to decrease at 100 °C indicating that our crude amylase work best at 90°C with the activity of 0.431 U/mL and confirming its suitability in the industrial sector [16]. Amylase activity produced by thermophilic bacteria isolated from Pulu has higher optimum temperature compared to crude amylase of the GK3 isolate from Gurukinayan Karo hot water with an optimum activity of 0.176 U/mL at 60°C [17]. Crude amylase isolated from thermophilic bacteria of Lejja hot spring has activity of 0.165 U/mL at 60°C and pH 6 [18]. While crude amylase from Bora hot spring has activity of 2 U/mL at 50°C [9].
The pH of the environment in which the enzyme works has an effect on enzyme activity. Many enzymes are sensitive to changes in environmental pH. Each enzyme has its optimum pH for its activity [19]. Based on figure 4, it can be seen that the amylolytic activity of PL 16 isolates increased from pH 5 to pH 8 and underwent a very large decrease at pH 9. This shows that pH 8 is the optimum pH of amylolytic activity of PL 16 isolates with an activity of 0.431 U/mL. A large decrease in enzyme activity occurred at pH 9, from 0.421 U/mL to 0.037 U/mL. This is likely caused by damage to the enzyme protein due to the influence of a high pH resulting in decreased enzyme activity. According to Palmer [19], changes in pH can cause the cessation of enzyme activity due to denaturation processes in the three-dimensional structure of enzymes.

Changes in charge on enzyme molecules can affect activity, both by changing the structure and by changing the charge on amino acid residues that function to bind the substrate. Presume a negatively charged enzyme reacts with a positively charged substrate to form an enzyme-substrate complex, then at a low pH value, the enzyme will be protonated and lose its negative charge. The same thing at high pH substrate will ionize and lose its positive charge [20]. Changes in H⁺ ions present in the enzyme solution give an effect on the catalytic site and conformation of enzyme. An extremely small or large value of pH causes the activity to decline [21].
4. Conclusions
Our present research suggested that crude amylase from Pulu hotspring possessing a promising potential to be applied in industries due to its high optimum temperature. However, further work such as purification is required to understand characters of enzyme more properly.

Acknowledgements
The authors would like to express special gratitude to the Faculty of Sciences, Tadulako University for supporting funds and facilities and to David Tamutuan for his technical assistance in experiments.

References
[1] Yang C H and Liu W H 2004 Enzyme Microb Technol. 35 254–60
[2] Goesaert H, Slade L, Levine H and Delcour J A 2009 J Cereal Sci. 50 345–52
[3] Pandey A, Nigam P, Soccol C R, Soccol V T, Singh D and Mohan R 2000 Biotechnology and applied biochemistry 31 135-152
[4] Souza P M de and Magalhães P de O 2010 Brazilian J. Microbiol. 41 850–61
[5] Nielsen J E and Borchert T V 2000 Biochim Biophys Acta. 1543:253-274
[6] Abdel-Fattah, Y R, Soliman, N A, El-Toukhy N M, El-Gendi H and Ahmed R S 2012 Journal of Chemistry 2013:1-11
[7] Gupta R, Gigras P, Mohapatra H, Goswami V K and Chauhan B 2003 Process Biochemistry 38:1599–1616
[8] Idral A and Mansoer W R 2015 Proc. World Geothermal Congress Melbourne Australia pp 1–5
[9] Gazali F M and Suwastika I N 2018 Journal of Physics: Conf. Series 979 012001
[10] Razak A R, Satrimafitrah P, Hardi J, Khoridah E N, Asmarni, M Gita and Dahyana 2019 Journal of Physics: Conf. Series 1242 012015
[11] Jamilah I, Meryandini A, Rusmana I, Suwanto A and Mubarak R N 2009 Microbiol. Indonesia 3 67–71
[12] Asgher M, Asad M J, Rahman S U and Legge R L 2007 J. Food Eng. 79 950–5
[13] Bernfeld P 1955 Methods Enzymol. 1 149–58
[14] Warburg O and Christian W 1941 Biochem Z 310: 384-421
[15] Florencio C, Couri S and Farinas C S 2012 Enzyme Research 2012
[16] Goyal N, Gupta J K and Soni S K 2005 Enzyme Microb. Technol. 37 723–34
[17] Sutiamiharja N 2008 Isolasi Bakteri dan Uji Aktivitas Amilase kasar Termofilik dari Sumber Air Panas Gurukinayan Karo Sumatera Utara (Master's thesis).
[18] Rugaiyah A A, Ahmad A, Djide M N, Anis M and Zakir M 2015 American Journal of Biomedical and Life Sciences 3 115-119
[19] Palmer T 1985 Understanding Enzyme Ellishorwood Publisher
[20] Lowry O H, Rosebrough N J, Farr A L and Randall R J 1951 J Biol Chem 193:265-275
[21] Asad W, Asif M and Rasool A S 2011 Pak. J. Bot. 43(2):1045-1052.