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Effect of temperature on the activity of crude protease enzymes of *Bacillus pumilus* from Anchovy isolates *(Stolephorus* sp.)

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ABSTRACT

Proteolytic bacteria that are potential to produce protease enzymes are widely used in food processing which can be isolated from various parts of fish and from various processes, which can be isolated from fish entrails, fresh fish and processed fish such as anchovy, and fish canning process waste. The study of proteolytic bacteria in anchovy has great biotechnology potential to be investigated because bacteria that can grow in anchovy are bacteria that can live in extreme environments. Given the great potential of the *Bacillus pumilus* bacteria, research can be carried out on enzyme activity from bacteria that grow in the isolation of anchovy. The purpose of this study was to obtain *Bacillus pumilus* protease enzyme activity from anchovy isolates which included temperature. The study used the experimental method, which is called Complete Random Design (CRD) with different temperature treatments including *Bacillus pumilus* 20 °C, 30 °C, 40 °C, and 50 °C. Based on the results of the study, it was found that the crude extract of the protease enzyme anchovy isolate in *Bacillus pumilus* was 50 °C at 1.2589u/mL. The results of the analysis show that the same temperature gives an influence between *Bacillus pumilus* on enzyme activity carried out to determine the optimum condition of the enzyme in degrading the substrate.

Key words : *Activity, Protease enzyme, Bacillus pumilus, Anchovy, Temperature*

Introduction

Enzymes play role in every biochemical reaction, from energy conversion, food metabolism, cell defense mechanisms, and communication between cells to conversion of hereditary traits. For this reason, enzymes have a high potential for biotechnology (Mótyán *et al.*, 2013), fields of industry (Younes and Rinaudo, 2015) and medical applications (Chou *et al.*, 1998). One of the enzymes that is widely used for commercial purposes is the protease enzyme. Protease enzyme is a derivative enzyme that catalyzes the hydrolysis of peptide bonds in proteins to convert proteins into amino acids (Kim *et al.*, 2011). This enzyme can be produced from microorganisms (Razzaq *et al*., 2019), especially *Bacillus sp* (Alnahdi, 2012) that have been isolated from anchovies (*Stolephorussp*) (Kim and Kim, 2005), that can live in

extreme environments, high salt content or halophilic and high temperature or thermophilic (Ismail *et al.,* 2018).

The ability of organism to produce enzyme is influenced by several factors and special conditions that cause enzymes to work optimally and efficiently (Kumar and Takagi, 1999) one of which is temperature (Rahman *et al.,* 2005). At low temperatures, enzymatic reactions take place slowly, rising temperatures will accelerate the reaction, until the optimum temperature is reached and the enzymatic reaction reaches its maximum (Wuryanti, 2004). An increase in temperature past the optimum temperature will cause the enzymes to be denatured and decrease the speed of the enzymatic reaction (Johnvesly and Naik, 2001). Therefore, this study conducted a study on the effect of temperature on the activity of crude enzyme protease extracts from anchovy isolates.

Materials and Methods

Ingredients and tools

The main ingredients used in this study are pure culture of Bacillus pumilus and Bacillus firmus obtained from anchovy isolates (*Stolephorus* sp.). The chemicals used in this study include: distilled water, ammonium sulfate ((NH_4) $2SO_4$), trichloroacetic acid (TCA), casein and tyrosine.The tools used are 25 mL, 50 mL and 100 mL measuring cups, spatulas, 50, 100, 200 and 500 mL beaker glass, micropipettes, water heaters, centrifuges, spectrophotometers, and thermometers.

Production of crude enzyme extracts (El-Tanboly and Al-Sayed, 2003)

There are several stages in determining the production of crude enzyme extracts using the method of Grata *et al.,* (2010). Proteolytic bacterial isolates that have been obtained are taken 1 ose and then inoculated into enriched media (1 g peptone, 0.6 g beef extract, 1000 mL distilled water and 10 g skim milk) with the addition of 15% NaCl. The isolate was then incubated for 10 hours at 37 °C, this isolation method was used as a starter. The amount of starter used which is 2.5 mL was inoculated into the production medium and incubated for 1 day at a temperature of 37 °C. Furthermore, after the incubation period, the isolate was centrifuged at a speed of 15,000 rpm at 4 °C so that it would form the enzyme supernatant liquid. Then after getting the supernatant the enzyme was stored in a storage medium at 4 °C ro be used in testing the activity of the protease enzyme.

Testing of protease activity

The protease activity was determined according to the modified Bergmeyer (1983) method, using Hammersten casein as (2% casein in 0.05 M phosphate buffer solution pH 7.0). 0.5 mL of the enzyme solution was added to 0.5 mL of a 0.05 M solution of phosphate buffer pH 7 and incubated at 37 °C for 5 minutes. Next 0.5 mL substrate was added. The reaction mixture was re-incubated at 37 °C for 10 minutes. The reaction is stopped by adding 1 mL of 0.4 M Trichloroacetic acid (TCA). Furthermore the supernatant was separated from the precipitation by using Whatman filter paper no. 1 as much as 0.5 mL filtrate added with 2.5 mL 0.5 M sodium carbonate, preincubated for 10 minutes, then added with 0.5 mL FolinCiocalteau reagent and reincubated for 30 minutes. Absorbance was measured using a spectrophotometer at a wavelength of 578 nm.

Determination of the optimum temperature of the protease enzyme (El-Sayed, 2001)

The method used is the method of El-Sayed (2001), in which the variation used is the temperature variation used are 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C. From some of the temperatures above the bacteria are taken at a minimum to maximum temperature that is 20 °C, 30 °C, 40 °C, and 50 °C.

Results and Discussion

Bacillus pumillus Growth Curve Analysis

On the growth curve of *Bacillus pumilus* bacteria, it can be analyzed using direct count treatment using Haemocytometer. The results of the analysis by the Haemocytometer Method can be seen in Figure 1.

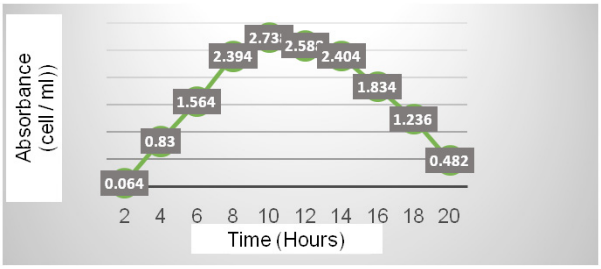

Fig. 1. Results of Optical Density Analysis using the Haemocytometer Method

From the method of determining the growth curve above, the best results are obtained using the Haemocytometer method. From this data, the log phase was obtained, at 10th hour the absorbance was 2,738 cells/mL. To get the best protease activity, the bacterium *Bacillus pumilus* is grown until the 10th hour at maximum growth. The adaptation phase cannot be experienced by isolation method because of the use of a starter with the same medium so that the bacteria have adapted and immediately entered the log phase. Based on the growth curve in Picture 1 it can be seen that the stationary phase of *Bacullus pumilus* isolation method on the optimization medium occurred starting at 48th hour.

The microbial growth phase consists of 3 phases (Llorens *et al.*, 2010). The first phase is the slow phase which is often called the adaptation phase. This phase is characterized by slow microbial growth. The second phase is the exponential phase. This phase is characterized by the rapid growth of fermented product cells. The third phase is the stationary phase. The stationary phase is characterized by a steady cell growth or as a starting point for growth accompanied by primary products.

Results of Analysis of Protease Activity in Temperature Treatments

Analysis carried out at this temperature treatment uses four different temperatures which consist of 20 °C, 30 °C, 40 °C, and 50 °C. Data from the analysis of temperature treatment can be seen in Table 1 and (Figure 2).

Table 1. *Bacillus pumilus* Protease Activity in Temperature Treatment

Temperature	Activity Protease (U/mL) ± STD
20 °C	4.164 ± 0.184
30 °C	4.507 ± 0.062
40 °C	4.627 ± 0.089
50 °C	4.593 ± 0.072

From the observation, the enzyme activity at 20 °C is quite small because the temperature is too low. The optimum enzyme activity is at the temperature of 40 °C which is equal to 4,627 units/mL. This *Bacillus pumilus* bacterium is able to produce protease enzymes with a maximum temperature of 40 °C, so it can be said that these bacteria are thermophilic (Ismail *et al.*, 2018) bacteria that are able to live at 40 °C. Grata *et al.*, (2010) also reported the protease activity of Bacillus mycoides A 134 using casein as the

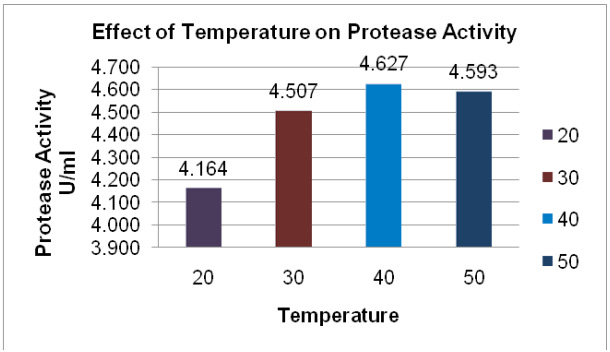

Fig. 2. Effect of Temperature on Protease Activity

medium with the highest protease activity value at a temperature of 60 °C (11.44 µmol). Dewi (2006), reported that the maximum activity of Bacillus sp. in producing protease enzymes is at 50 °C. This optimum temperature difference is thought to be due to differences in the species of *Bacillus* tested.

The test on the effect of temperature on enzyme activity is carried out in order to find out the optimum conditions of the enzyme to degrade the substrate. Each enzyme has a maximum activity at a certain temperature, enzyme activity will be able to continue to increase with increasing temperature until the optimum temperature (Pelczar and Chan, 1986). Further increase in temperature results in reduced activity and ultimately destruction of enzymes. An increase in temperature above the optimum temperature will cause in decreasing enzyme activity (Johnvesly and Naik, 2001).

References

Alnahdi, H.S. 2012. Isolation and screening of extracellular proteases produced by new isolated *Bacillus* sp. *J. of Applied Pharmaceutical Science*. 2 (09) : 071-074.

Bergmeyer, H.U. 1983. *Method of Enzymatic Analysis 2*. Verlag Chemie. Weinstein.

Chou, J.J., Matsuo, H., Duan, H. and Wagner, G. 1998. Solution structure of the RAIDD CARD and model for CARD/CARD interaction in caspase-2 and caspase-9 recruitment. *Cell*. 94 : 171–180.

Dewi, W.K. 2006. Pemurniandan Pencirian Protease dariIsolat Bakteri W-1 yang Dihasilkanoleh Tauco Hitam. Msc, Bogor Agricultural University, Bogor, Indenosia.

El-Sayed, S.T. 2001. Purification and Characterization of Rhapanin, A Neutral Protease, from Raphanussativus Leaves. *J. Bio. Sci*. 4 (5) : 564-568.

Grata, K., Nabrdalik, M. and Latala, A. 2010. Evaluation of Proteolytic Activity of *Bacillus mycoides strains*.

Proceedings of ECOpole. 4 (2) : 253-256.

Ismail S.N.F., Shah, A., Edbeib, M.F., Adamu, A., Aliyu, F., Wahab, A.R. and Huyop, F. 2018. Dehalogenase producing bacteria from extreme environment: A review. *Malaysian Journal of Microbiology.* 14 (5) : 424-434.

Johnvesly, B.Naik, G.R. 2001. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. *Process Biochem.* 37 : 139-144.

Kim, W., Bae, S., Park, K., Lee, S., Choi, W., Han, S. and Koh, Y. 2011. Biochemical characterization of digestive enzymes in the black soldier fly, *Hermetiaillucens* (Diptera: Stratiomyidae). *J. of Asia Pacific Entomology.* 14(1) : 11-14.

Kim, W.J and Kim, S.M. 2005. Purification and characterization of *Bacillus subtilis* JM-3 protease from anchovy sauce. *Journal of Food Biochemistry.* 29 (5) : 591 – 610.

Kumar, C.G. and Takagi, H. 1999. Microbial alkaline proteases from a bioindustrial view point. *Biotechnol Adv*. 17 : 561-594.

Llorens, N.J., Tormo, A. and Martínez-GarcíA, E. 2010. Stationary phase in gram-negative bacteria. *Rev. FEMS Microbiol.* 34 : 476–495.

Mótyán, J.A., Tóth, F. and Tõzsér, J. 2013. Research Applications of Proteolytic Enzymes in Molecular Biology. *Biomolecules.* 3 (4) : 923–942.

Pelczar, M.J. and Chan, E.C.S. 1986. *Dasar-dasar Mikrobiologi.* Jakarta: UI Press.

Rahman, R.N.Z.R., Geok, L.P., Basri, M. and Salleh, A.B. 2005. Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. *Bioresource Technol.* 96 : 429-436.

Razzaq, A., Shamsi, S., Ali, A., Ali, Q., Sajjad, M., Malik, A. and Ashraf, M. 2019. Microbial Proteases Applications. *Front. Bioeng. Biotechnol.* 7 : 110.

Wuryanti, W. 2004. Isolasidan Determination of the Specific Activation of Bromelin Enzyme from Pineapple (*Ananascomosus* L). *JKSA.* 7 (3) : 83-87.

Younes, I. and Rinaudo, M. 2015. Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs*. 13 : 1133–1174.