INITIAL CHARACTERIZATION OF ALKALINE PROTEASE FROM *PSEUDOMONAS SP* ISOLATED FROM CHICKEN FECES (POSTER)

Yuyun Firdausi Nuzulah*[1] and Suharti[2]
1) Chemistry Department, Faculty of Mathematics and Natural Sciences, State University of Malang, Malang, Indonesia (faradeiss@aim.com)
2) Chemistry Department, Faculty of Mathematics and Natural Sciences, State University of Malang, Malang, Indonesia (s.suharti@gmail.com)

Received 20 October 2018
Accepted 20 December 2018

Abstract

Proteases are ubiquitous enzymes and it can be found in all living organisms. Extracellular proteases have high economic value, where the enzymes can be applied to various industrial sectors. In order to find an alternative source of protease, a search for microbe producing extracellular protease has been done using a selective medium. An extracellular producing bacterium that is identified as Pseudomonas sp has been isolated from chicken feces, that is Gallus gallus bankiva, collected from residential areas at Lowokwaru, Malang, Indonesia. The analysis shows that the bacterium produces extracellular protease with optimum activity after incubation of 66 hours at 35^{o}C. Initial characterization of the extracellular protease shows that the extracellular protease produced by Pseudomonas sp has an optimum activity at pH 12 and an optimum temperature at 39^{o}C. At these conditions, the crude extract enzyme has a specific activity of 0,522 U/mg. The high pH resistance allows its application to variety of industrial processes that require high pH operation. Therefore, further studies need to be done to get better understanding of enzyme properties.

Keywords: *Alkaline protease, Chicken feces, Gallus gallus bankiva, Pseudomonas sp*

Introduction

Protease is an important enzyme in industrial process, including detergent, tanneries, waste treatment, medical industry etc. Requirement of alkali tolerant enzyme on industrial process, due to its high pH environment, rising numerous research in finding new alkaline protease sources. Alkaline protease producing microbes have been screened from various sources including soil (Aftab, 2006; Dubey, 2010; Khan, 2011; Rao, 2007), water (Abou-Elela, 2011; Damare, 2006; Ibrahim, 2007; Smita, 2012), and waste material (Fuad, 2004).

Bacillus sp is the important bacterial sources of comercial alkaline protease production (Khan, 2011). This bacteria strain has been studied in various research (Abou-Elela, 2011; Chan, 2004; Fuad, 2004; Khan, 2011; Rao, 2007) for best both physical and chemical properties, dealing with industrial requirement of the enzyme. In this study an alkaliphilic bacteria were isolated from *Gallus gallus bankiva* feces, due to its eating stone behavior (Jacob, 2011). The bacteria were later identified as *Pseudomonas sp*. The protease was employed for characterization of optimum

temperature and pH, as well as specific activity. The protease properties expected to be compatible with industrial requirement and can be produced commercially in Indonesia.

Method and Experimental Details

Screening of Extracellular Protease Producing Microbe

Gallus gallus bankiva feces samples were collected from residential areas at Lowokwaru, Malang. The sample was first diluted 10 times then continued to 10^{18} times. The 10^{18} times dilution sample was spread into screening media, consist of 1% (w/v) bacto tryptone, 1% NaCl, 0,5% yeast extract, 0,14% agarose and 1% skimmed milk on destilied water, followed incubation at 37^{o}C. After 72 hours incubation screening cultures were studied for the colonies produce transparant zone. The colonies was then transferred into new screening media, and selected for one single colony for protease assay. The selected single colony was then inoculated into new agar plate. The microbe species of the selected colony then determined by phenotype properties assay, held in Biochemistry Laboratory of Medical Faculty, Airlangga University.

Protease Assay

The selected single colony was employed for protease production. One full loop of culture was transferred into 10 mL aqueous media (screening media without agarose) followed by 20 hours incubation at 37^{o}C in water bath, with 100 rpm aeration. One mL of the preculture was then transferred into 100 mL aqueous media, called production culture, for protease assay.

The protease assay method used in this study was the modified method of Khan (2011). Crude enzyme was obtained by the centrifugation of production culture at 12.500 rpm for 6 minutes. One mL of crude enzyme was then transferred into tube containing 4 mL buffer (K_2HPO-KH_2PO_4, pH 8), followed by 2 mL casein 2% (incubated at reaction temperature before) addition. The reaction mixture was incubated at 37^{o}C for 20 minutes, by the addition of casein. This is the enzymatic reaction temperature of the protease assayed. To stop the reaction 3 mL TCA 20% was added, then homogenized with vortex for 1 minute. The reaction mixture was incubated at 4^{o}C for 15 minutes then centrifuged at 3.000 rpm for 20 minutes. The filtrate (2 mL) was added into 4 mL NaOH followed by addition of 1 mL Folin Reagent. The homogenous coloured solution was then measured for the absorbance at 650 nm. One unit of protease activity is defined as "the quantity of enzyme, which liberates amino acids and non precipitated peptides equivalent to 1 µg/mL of tyrosine per minute under assay conditions".

The protease assay was held every 24 hours start form 0 hours, for optimum protease production assay.

Initial Characterization of Crude Protease

The optimum temperature determination was held by varying the enzymatic reaction temperature; at 37^{o}C, 39^{o}C, 40^{o}C, 41^{o}C, and 43^{o}C. The optimum pH assayed by varying buffer solution used in the reaction, that is pH 7, 8, 9, 10, 11, 12 and 13. The buffer was made from KH_2PO_4, K_2HPO_4, and K_3PO_4.

Specific activity determined by measuring optimum protease activity and total protein content of the crude enzyme. Total protein content assayed by Lowry Method, using 1,2 and 12 times diluted crude enzyme. Crude enzyme (1,2 mL) were added into 6 mL Lowry Reagent, then mixed well and waited for 10 minutes. Folin Reagent (0,3 mL) then added into the mixture, and waited for 30 minutes after it

mixed well. The coloured solution measured for absorbance at 500 nm. The standar curve held by using BSA solution.

Screening and Fenotipe Assay of Extracellular Protease Producing Microbe

Result and Discussion

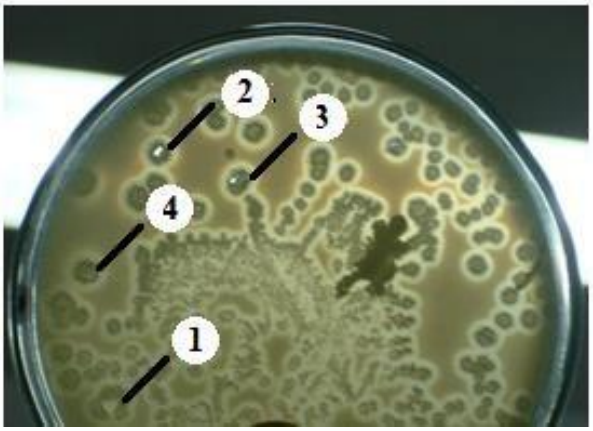

Figure 1. Screening culture of 10^{18} times diluted feces sample; colony number 4 (as labelled in the picture) was selected to be potential protease producer for further assay.

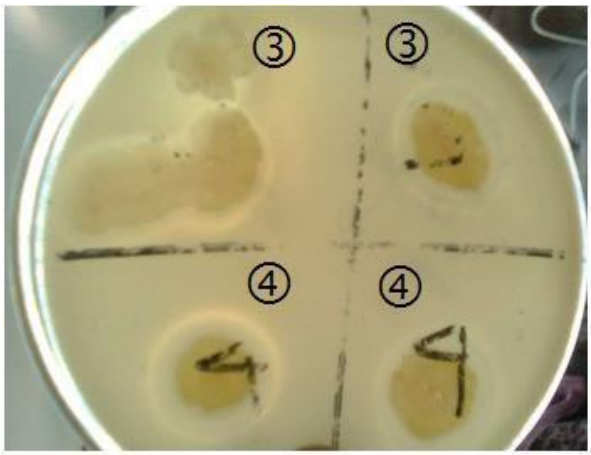

Figure 2. New culture of number 3 and 4 colonies, show that number 4 produce larger transparent zone

The pH value of 10 times diluted feces sample was 8, showed the alkaline environment of the microbe living in chicken feces. The screening result (Fig. 1) showed that cultured microbe from chicken feces produce extracellular proteses, indicated by transparent zone formed arround the colonies. The transparent zone best appeared after 72 hours incubation. The colonies was then selected based on the zone formed, which is the largest transparent zone indicating best microbe ability in producing protease. From the colony selection it was collected 8 colonies produce the largest zone, 4 of them were shown at Fig. 1 (for number 1-4 colonies). The new culture of colony number 1-8 showed that number 4 produce largest transparent zone (Fig. 2).

Colony labeled number 4 then selected for further protease assay.

The bacterial fenotipe assay showed that microbes in colony number 4 are *Psedomonas sp* bacteria. The phenotype assay result showed at Table 1.

Table 1. Conventional phenotype test of Colony Number 4

Test	Result
MacConkey	(-)
Triple Sugar Iron	(-) all sugar (-) gas (-) H_2S
Citrate Test	(-)
Urease Test	(-)
Indole Test	(-)
Motility Test	(+)

Optimum Protease Production

Proteolyc activity of the production culture was determined every 24 hours, start from 0 hour. The colored solution absorbance as the result of protease assay used to express enzyme activity. It is plotted with bacteria growth time to study protease activity change by the production time (Fig. 7). Five days assay showed that protease production of the bacteria was optimum at 66 hours, showed the highest absorbance value.

Optimum pH and Temperatur

As well as protein, enzyme structure stabilization affected by physical and chemical environment like temperature and pH. Once temperature increasing average energy of reactant molecule, causing lower activation energy of the reaction, thus increasing the reaction rate. Temperature, in contrary, held enzyme thermal denaturation, which is structural changes of the enzyme reduce bond strength of the enzyme-substrat binding, and reduce enzyme activity (Garret 1999). Due to the effect of temperature, enzyme has its optimum temperature, that is the best temperature for the catalytic process occured. Optimum temperature of protese in this study is 39^{o}C, with the activity value 2,41 U/mL (Fig. 3). The protease activity shown the best temperature resistance at 39^{o}C-40^{o}C, with 5,56% activity reduction. The reaction temperatures out of the range will increase reduction of the enzyme activity (Fig. 4).

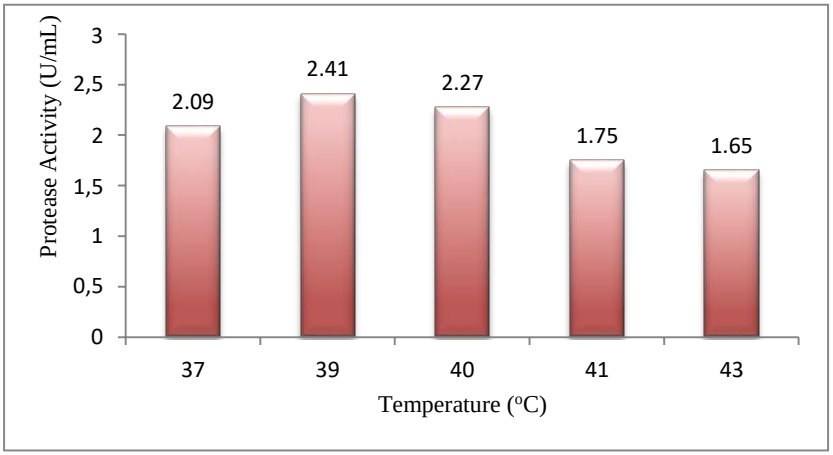

Figure 3. Effect of temperature on protease activity. The optimum temperature is 39°C, showed highest activity value.

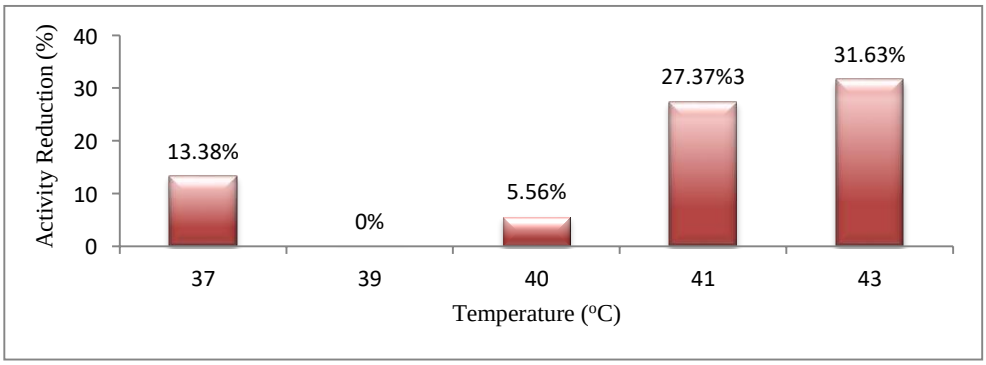

Figure 4. Protease activity reduction on various temperature, referred to the optimum temperature activity. Least activity reduction is on 40°C, show the best temperature resistance range of the enzyme.

As well as temperature, environment pH affecting enzyme stability, that is on electrical charge of the enzyme (Jakubowski, 2010). The presence of H^+ increase positive charge of the enzyme, as well as OH^- in contrary. At one point the positive/negative charge of the enzyme will be saturate by the increasing of H^+/OH^-. Enzymes reduce the exceed charge density by self-unfolding, to gain more stability. Environment pH affecting enzyme's structure, thus affecting enzyme activity. The optimum pH of the protease in this study is 12, with the activity value 3,42 U/mL (Fig. 5). The study of pH changes effect to protease activity showed that on its optimum pH the enzyme is quiet affected by pH change. Enzyme lost its activity of 25,28% by the change one degree of the pH (Fig. 6). Considering activity reduction at pH 10, the enzyme is suggested to be used at the pH range 10-13, which it will gain its optimum activity at pH 12. The optimum pH of the protease in this study fit with industrial requirement, therefor the enzyme expected to developed further for another properties.

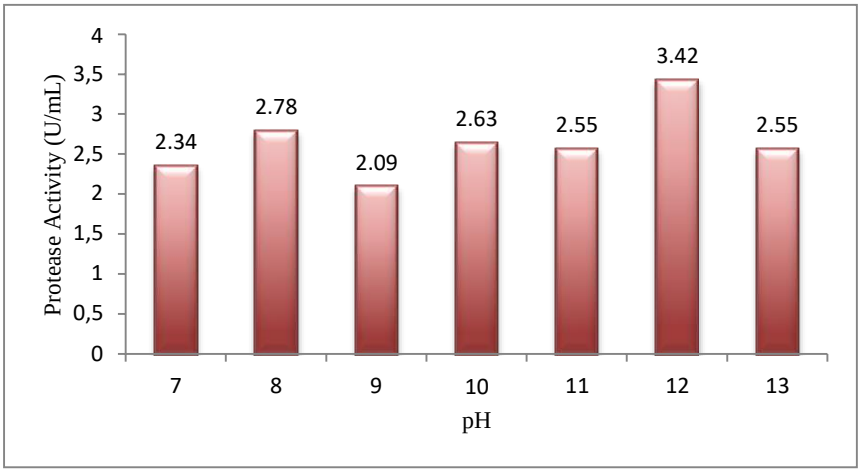

Figure 5. Effect of pH on protease activity. The optimum pH reaction is 12.

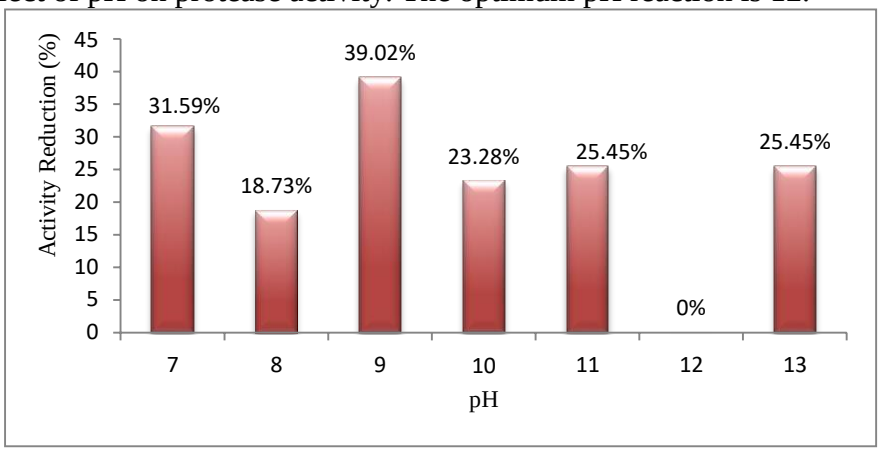

Figure 6. Protease activity reduction on various pH, the activity reduction is relatively constant at pH 10-13.

Specific Activity of the Protease

Specific activity is important parameter in enzyme study. It is implicitly show organism tendency in producing studied enzyme. Great specific activity means great organism ability in producing enzyme. The study showed that the protease of *Pseudomonas sp* has optimum activity of 3,42 U/mL, with specific activity of crude enzyme is 0,522 U/mg. The protease specific activity is however less than previous research on another *Pseudomonas sp* species; that is 1,36 U/mg (Chan, 2004), 7 U/mg (Najafi, 2005), and 12,15 U/mg (Nathan, 2005). Enzyme purification should be held in order to gain more protease specific activity, as well as another optimization method. For industrial application, the bacteria protease production ability can be increased by genetic manipulation, include site directed mutagenesis and random mutagenesis.

Conclusion

The study result show that microbes living in *Gallus gallus bankiva* feces produce extracellular protease, with the optimum enzyme production was at 66 hours of the growth. The microbe selected for further protease study was *Pseudomonas sp* bacteria.

The initial characterization held to the crude protease showed that the enzyme has

optimum temperature of 39^{o}C, optimum pH of 12, and specific activity of 0,522 U/mg.

Overall initial study of the protease show enzyme probability to be applied in industrial process. However, other enzyme properties should be determined for further understanding of enzyme nature, due to other requirements of industrial application.

Acknowledgements

The equipment and infrastructure facility of this study was supported by Chemistry Department and Faculty of Mathematics and Natural Sciences, State University of Malang.

References

Abou-Elela, G. M., Ibrahim, H. A. H., Hassan, S. W., Abd-Elnaby, H., and El-Toukhy, N. M. K. (2011), *Alkaline Protease Production by Alkaliphilic Marine Bacteria Isolated from Marsa-Matrouh (Egypt) with Special Emphasis on Bacillus Cereus Purified Protease*. African Journal of Biotechnology. vol 10(22). 4631 –4642.

Aftab, S., Ahmed, A., Saeed, S., and Rasool, S. A. (2006), *Screening, Isolation and Characterization of Alkaline Protease Producing Bacteria From Soil*. Pakistan Journal of Biological Sciences. vol 9(11). 2122 – 2126.

Chan, S. W., Ong, G. I., and Nathan, S. (2004), *Neutralizing Chimeric Mouse-Human Antobodies Against Burkholderia Pseudomallei Protasae: Expression, Purification, and Characterization*. Journal Of Biochemistry and Molecular Biology. vol 37(5): 556 – 564.

Damare, S., Raghukumar, C., Muraleedharan, U. D., and Raghukumar, S. (2006), *Deep-Sea Fungi As a Source of Alkaline and Cold-Tolerant Proteases*. Enzyme and Microbial Technology. vol 39(2). 172 – 181.

Dubey, R., Adhikary, S., Kumar, J., and Sinha, N. (2010), *Isolation, Production, Purification, Assay and Characterization of Alkaline Protease Enzyme From Aspergillus Niger And Its Compatibility With Commercial Detergents*. Developmental Microbiology and Molecular Biology. vol 1(1). 75 – 94.

Fuad, A. M., Rahmawati, R., and Mubarik, N. R. (2004), *Produksi dan Karakterisasi Parsial Protease Alkali Termostabil Bacillus Thermoglucosidasius AF-01*. Jurnal Mikrobiologi Indonesia. vol 9(1). 29 – 35.

Garret, R and Grisham, C. (1999), Biochemistry, *2nd Edition*. http://www.web.virginia.edu/Heidi/1thru25. pdf

Ibrahim, A. S. S., El-Shayeb, N. M. A., and Mabrouk, S. S. (2007), *Isolation and Identification of Alkaline Protease Producing Alkaliphilic Bacteria from an Egyptian Soda Lake*. Journal of Applied Sciences Research. vol 3(11). 1363 – 1368.

Jacob, J., Pescatore, T., and Cantor, A. (2011), *Avian Digestive System*. Lexington: University of Kentucky.

Jakubowski, H. (2010), *Online Biochemistry: Protein Structure: Thermodynamics and IMFs in Protein Stability*. http://employees.csbsju.edu/hjakubowski/cl asses/ch331/protstructure/olhydrophobprot.html

Khan, M. A., Ahmad, N., Zafar, A. U., Nasir, I. A., and Qadir, M. A. (2011), *Isolation and Screening of Alkaline Protease Producing Bacteria and*

Physio-Chemical Characterization of The Enzyme. African Journal Of Biotechnology. vol. 10(30). 6203 – 6212.

Najafi, M. F., Deobagkar, D., and Deobagkar, D. (2005), *Potential Application of Protease Isolated From Pseudomonas Aeruginosa*. Electronic Journal of Biotechnology. vol 8(2). 197 – 203.

Nathan, S., Leong, Y. P., and Sa'ariwijaya, M. S. F. (2005), *Burkholderia Pseudomallei Secretes Metallo- and Serine Protease in Culture*. Malaysian Journal of Biochemistry And Molecular Biology. vol 11(1): 13 – 19.

Rao, K. and Narasu, M. L. (2007), *Alkaline Protease From Bacillus Firmus 778*. African Journal of Biotechnology. vol 6(21). 2493 – 2496

Smita, G. S., Ray, P., and Mohapatra, S. (2012), *Quantification and Optimisation of Bacterial Isolates for Production of Alkaline Protease*. Asian Journal of Experimental Biological Science. vol 3(1). 180 – 186.

Appendix

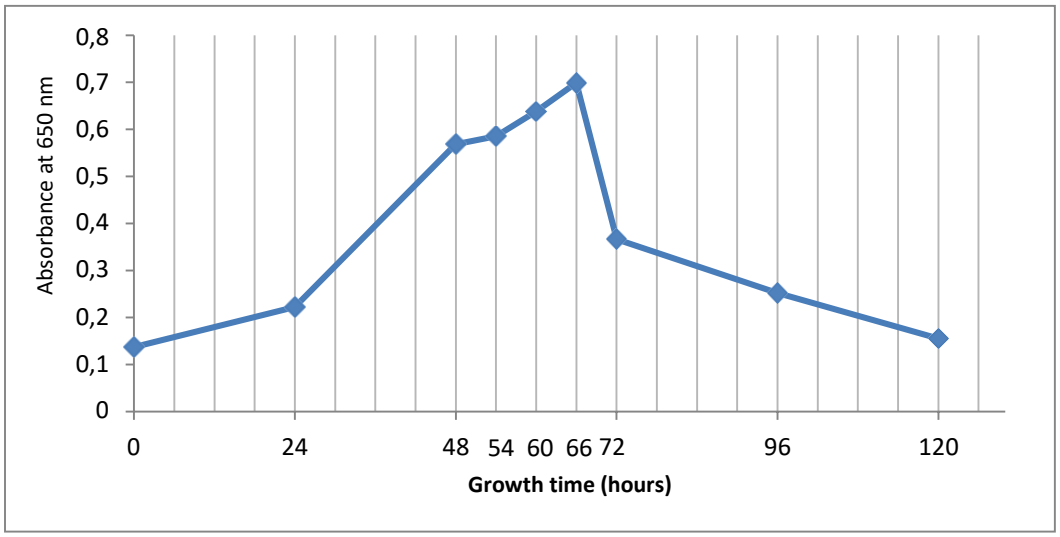

Figure 7. Protease activity produced by bacteria on growth state. Coloured solution absorbance, as the result of activity measurement, showed the highest protease activity production was on 66 hours of growth.