

## PROTEASE ACTIVITY OF THERMOPHILIC BACTERIA FROM LEJJA HOT SPRINGS IN SOPPENG SOUTH SULAWESI

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### Abstract

This research aimed to observe quantitatively the effect of temperature and pH on protease activities from thermophilic bacteria collected from Lejja—Hot springs. Spectrophotometry on the casein substrate was used to test the proteolytic activity of the crude protease. Mixtures of enzyme and casein were incubated at various temperatures and pH for 20 minutes. The absorbance of tyrosine from protein hydrolysis was determined by spectrophotometry on  $\lambda$  280 nm. Temperature and pH impacted on protease activity were determined at temperature (60; 65; 70; 75; 80; 85 and 90°C) and pH (6.0; 6.5; 7.0; 7.5; 8, 0; 8,5 and 9,0). Results showed that treatment of temperature variations and pH had a significant effect on protease activity. Crude extract of *Bacillus licheniformis* protease showed that the highest activity at 80°C and pH 7.5 was 0.1303 unit/ml/minute. *Bacillus stearoformis* showed the highest enzymatic activity of the protease at 85°C, and pH 7.5 was 0.1226 Unit/ ml/minute. In comparison, *Bacillus coagulans* reached optimum activity at 75°C, and pH 7.5 was 0.2052 Unit/ml/minute. Isolates of *Bacillus licheniformis*, *B. coagulans* and *B. stearoformis* are bacteria that produce thermostable protease enzymes that can be developed as a source of genes and as a producer of the enzyme itself.

**Keywords** : protease, thermophilic bacteria, casein, Lejja

### INTRODUCTION

Enzymes are catalysts of choice expected to reduce the impact of pollution and waste of energy because the reactions do not require high energy, are specific, and are non-toxic (Liu & Kokare, 2017). Protease is one of the essential enzyme groups and has high economic value because of its extensive application (Ward, 2011; Ward et al., 2009). This enzyme function is hydrolyzed peptide bonds in proteins into oligopeptides and amino acids. The protease user industries include the detergent, leather, textile, food, protein hydrolyzate, dairy, pharmaceutical, food, beer, film processing industries, and molecular biology and waste handling practices. (Banerjee & Ray, 2017; dos Santos Aguilar & Sato, 2018; López-Otín & Bond, 2008; Maurer, 2004; Razzaq et al., 2019; Ward et al., 2009). Proteases that

are most often used in the industrial sector are thermostable because they can produce much better and more efficient products. Considering the process of using them, they can reduce the possibility of microbial contamination at high temperatures. In addition, it can save cooling costs on large-scale fermentation so that work using high temperatures has more advantage in the industrial field (Banerjee & Ray, 2017; Haki, 2003).

Proteases can be isolated from various organisms, namely animals, plants, fungi and bacteria, either from extracellular and intracellular (Sunil Kumar et al., 2018; Rawlings & Salvesen, 2013; Ward, 2011). Microorganisms are the most widely used source of enzymes compared to plants and animals. As the source of enzymes, microorganisms are more profitable than others because microorganism cells are

relatively easy to grow. Furthermore, the growth rate is relatively faster, and the scale of cell production is easier to increase. Its higher productivity through regulating growth conditions and genetic engineering conditions during production does not depend on changes of seasons, and it requires a shorter time for the production process (Razzaq et al., 2019; P. Singh & Kumar, 2018; R. S. Singh et al., 2019; Ward et al., 2009). To find out that these microorganisms produce enzymes, it is necessary to know the enzymes' activity they produce. An enzyme is said to be active if the enzyme can carry out its catalytic activity (Banerjee & Ray, 2017; Ward et al., 2009).

Indonesia has very abundant natural resources, one of the potential natural resources is hot springs which can be used either as tourist destinations or sources of thermophilic bacteria (Amin et al., 2017; Gupta et al., 2002; Haki, 2003; Rachma et al., 2009). Mohammad et al. (2017) provided that the sources of thermophilic bacteria include hot springs, volcanic craters, and deep-sea trenches and volcanoes under the sea. As a country with rich hot springs, it certainly has great potential as a medium for the growth of thermophilic bacteria. However, until now, the exploration of these potential locations is still uneven. One of the hot springs in Indonesia is Lejja hot spring in Soppeng Regency South Sulawesi, which is currently used as one of the tourist attractions for local people and Indonesian and foreign tourists.

In order to find the source of proteases, a study was conducted to explore the potential of thermophilic bacteria from Lejja—Hot water spring in Soppeng Regency South Sulawesi to produce thermostable proteases. This research was preceded by the isolation of bacteria from the Lejja hot spring and the selection of superior isolates to test the potential activity of the protease enzymes they produce. The three isolates were *Bacillus licheniformis*, *Bacillus coagulans* and *Bacillus Steariformis* (Hafsan, 2018). This research was conducted to determine the optimum pH and temperature of

protease enzyme activity from local bacteria. The protease enzymes of bacteria, yeast or mould have different characters and specificity even though they are isolated from the same species. That the characteristics of proteolytic enzymes will show different activities in terms of pH and optimum temperature in hydrolyzing the substrate (Banerjee & Ray, 2017; dos Santos Aguilar & Sato, 2018; Ward et al., 2009), so it is important to reveal. The results obtained will of course be a reference for production in the context of utilizing these enzymes in various fields, as well as providing new nuances in the renewal and development of science and technology in Indonesia.

## METHODS

The study was conducted with a factorial experimental design consisting of 2 factors, namely temperature and pH using a completely randomized design with three replications for each treatment. The tools and materials used in this research include; Petri dishes, test tubes and tube racks, Erlenmeyer flasks, measuring flasks, Laminar Air Flow (LAF), autoclave, oven, funnel, water bath, measuring cup, cup glass, dropper pipette, volume and micropipette, spoit, icebox, ultracentrifuge, electric scale, inoculation needle (ose), bunsen, aluminium foil, cotton, refrigerator, thermometer, vortex, microscope, haemocytometer, spectrophotometer (Spectronic 20 D), pH meter, magnetic stirrer, incubator shaker, zone reader, alcohol, pure culture of thermophilic bacteria from Lejja hot springs, namely *B. licheniformis*, *B. coagulans* and *B. Steariformis*, medium LB, Medium skim milk agar, Medium V-P, gelatin, phosphate buffer, trichloroacetic acid (TCA), casein, tyrosin, NaOH, Folin, Aquades, NaOH, and HCl.

Data collection includes determination of the growth standard curve, production and isolation of protease, and measurement of protease activity. Growth standard curves are made by measuring values of Optical Density (OD) fermentation medium. Bacteria were inoculated in 50 ml of media as much as two ose isolates. The suspended

bacterial isolates were incubated in a shaker incubator at a temperature of 60°C with 200 rpm agitation. The OD value is measured every two hours by Spectronic 20 at  $\lambda$  280 nm to obtain one series OD using the turbidimetric method. The standard growth curve is the relationship between the number of cells per millilitre and the OD value.

Protease production performed by a method adapted from (Olajuyigbe & Ajele, 2005; Suman & Ramesh, 2008; Tanzadeh et al., 2006) begins with making a starter by inoculating two ose isolates from the medium to slant into 50 ml of liquid medium then shaking it off at 60°C with 200 rpm agitation until the bacteria reach the logarithmic phase and stored as a starter during protease production. Then as much as 5 ml of starter culture medium is inoculated into a new liquid medium then shaken at 60°C for 24 hours with a shaker incubator at 200 rpm. The culture of incubated bacterial cells was separated from the medium by centrifugation at 6000 G for 20 minutes at 4°C. The supernatant was separated from the precipitate. This supernatant obtained was a crude extract of the protease enzyme, then tested for its activity. The protease activity was determined based on the ability of the crude extract protease to hydrolyze the peptide bonds on the casein substrate by 2% (w/v) for 20 minutes. For control, 0.5 ml of phosphate buffer pH seven was incubated with 2 ml of 2% casein solution at 60°C for five minutes. Then to the sample solution, 1 ml of crude enzyme extract was added and incubated at 60°C. The reaction was stopped by adding 3 ml of 10% TCA solution. The solution was stirred vigorously with the vortex and then incubated in the refrigerator for 30 minutes. The blank and sample solutions were centrifuged at 11000 rpm for 10 minutes. The absorbance of the

supernatant for each solution was measured at  $\lambda = 340$  nm. Whereas for the treatment of temperature variations and pH measurements of protease activity were carried out at variations in temperature (60; 65; 70; 75; 80; 85 and 90°C) and pH (6.0; 6.5; 7.0; 7.5; 8, 0; 8,5 and 9,0).

The effect of temperature and pH on protease activity will be analyzed using a different test of Analysis of Variance. The data obtained are described first. From the significant ANOVA results, it is followed by Honest Significant Difference.

## RESULTS AND DISCUSSION

Growth cycle is inevitable on each bacteria, and its duration on one cycle is various across of types bacteria, it is depend on strain, medium, temperature and age of culture. Determination of the growth curve is needed to estimate the correct growth time at the production stage so that an optimum amount of protease enzyme can be produced and has high activity (Pedro Fernandes & Carvalho, 2017; Sanchez & Demain, 2017). Bacterial growth was observed by measuring optical density (OD) of the production media at certain time intervals, until a constant OD value is achieved. The constant OD value indicates that the bacterial cell growth has reached a stationary phase, a phase where cell growth remains. The stationary phase appears on the growth curve with the formation of a horizontal line parallel to the x-axis. The start of the stationary phase is the perfect time to isolate the enzymes produced by bacteria (Indriati & Megahati, 2018). Three ose of each isolate were inoculated on the production medium, then agitated. Changes in the OD value of the production media were observed at each time interval of two hours for 42 hours. The growth curve of the isolates was made by plotting the incubation time against OD as figure 1.

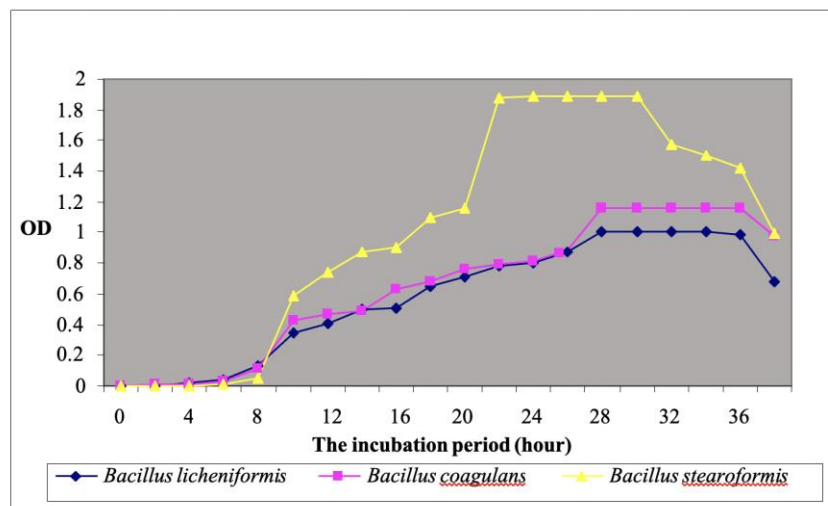


Figure 1. Growth curves of three thermophilic bacteria from the Lejja hot spring

A practical and relatively fast method for observing bacterial cell growth is to determine the concentration of bacterial cells in a solution through the turbidimetric method (Chavasit et al., 2018; Gooch, 2011). Based on the results of determining the maximum wavelength of tyrosine, the liquid medium suspension has a maximum optical density at  $\lambda_{maks}$  280 nm. Thus, bacterial growth is observed by measuring or OD of the production media at certain time intervals until a constant value at  $\lambda$  280 nm is reached. Isolates that have been rejuvenated, partially transferred to the production medium under aseptic conditions, then placed on shaker incubator. The use of this type of incubator is intended to regulate aeration and agitation in the medium. Chavasit et al. (2018) provided that aeration and agitation are essential factors in enzyme production, predominantly liquid media. However, it is necessary to avoid forming some foam during agitation. From the OD measurement data for each 2-hour interval, a graph is generated as in Figure 1.

In general, there are four phases of growing bacteria, as follows; lag phase, logarithmic phase, static phase and death phase (Marshall, 1917). Based on the graph in Figure 1, it can be seen that the 0 to 8 hours is the lag or adaptation phase of the

three isolates, which are shown a small OD value which means the number of cells at that time is also small. Surinder Kumar (2016) stated that in the lag phase, cell division is slow so that the number of cells is small, but there is an increase in the size of the cell two or three times from the previous one, especially in bacteria in the form of bacilli/rods. Adaptation of cells to new media is related to enzymes used for the metabolism of different nitrogen and carbon sources and the synthesis of vitamins or other co-factors. In addition to the media used, bacterial cells in the lag phase are susceptible to changes in temperature, where the lag phase is shortened if the temperature approaches the optimum temperature for cell growth.

*B. Licheniformis* and *B. coagulans* cells went on logarithmic phase at the 8<sup>th</sup> to 28<sup>th</sup> hour, while *B. stearoformis* enter this phase on the fourth hour up to 22<sup>th</sup>, wherein the cell increases until the division rate is maximum. The increase of OD value proved this. The speed of cell growth is due to the cells of the three bacteria, which have been able to metabolize N as a source of amino acid synthesis and C as source energy for the building block of protoplasm. If energy increase, so that cell growth become maximum. Other factors that affect the

growth rate are aeration and culture agitation. Aeration and agitation maintain physical and chemical conditions in the media so that the culture remains homogeneous and oxygen circulation is evenly distributed. It also plays a crucial role to increase oxygen solubility and accelerates the transfer of nutrients and oxygen into cells (Chavasit et al., 2018; Surinder Kumar, 2016).

After 22 hours, the OD values of *B. stearoformis* became constant, while cells of *B. licheniformis* and *B. coagulans* experiencing constant cell growth after passing the 28 hours. This phase is the stationary phase. In this phase concentration of the final product is high or begins to become toxic while substrate starts to run out, thus inhibits cell reproduction. Furthermore, the rate of reproduction will decrease until there is an equilibrium with the rate of cell death. The chart shows up to the 15 hours (*B. Steariformis*) and up to the 18 hours (*B. licheniformis* and *B. coagulans*). The OD value did not decrease, possibly because the dead bacterial cells added turbidity to the suspension so that the OD value remained constant. After passing through this phase, the phase of bacterial cell death is seen, marked by a drastic decrease in the OD value.

Half of the logarithmic phase and the beginning of the stationary phase were used as a reference for the length of incubation time inoculum and the incubation time for enzyme production because, at that time, toxin production and inhibition by-products did not occur. Based on the growth curve in Figure 1., it appears that the incubation time of *B. licheniformis* reached half of the logarithmic phase on the 18 hour and the initial stationary phase on the 28 hour. From those two data (half of the logarithmic phase and initial stationary phase), it can be

concluded that the inoculum incubation time is 18 hours, and the incubation time for enzyme production is 28 hours. Likewise for *B. coagulans* has an inoculum incubation time of 14 hours, and the incubation time for enzyme production is 28 hours. While *B. stearoformis* has an inoculum incubation time of 16 hours and the incubation time for enzyme production is 22 hours.

The protease activity of the three thermophilic bacteria from the Lejja hot spring measured in the combination of temperature and pH is presented in Figure 2. Based on the analysis of the effect of temperature and pH on the protease enzyme produced by the three superior isolates of thermophilic bacteria. It was revealed a very significant effect of temperature and pH on the protease enzyme produced by the three superior thermophilic bacterial isolates from the Lejja-Hot water spring. As with the analysis, it was proved that the interaction of temperature and pH was very significant so that to determine the combination treatment that produces the most optimum protease enzyme activity needs to be done further Honest test with results as follows; (1) protease enzyme activity of the isolate *Bacillus licheniformis* optimum on  $T_{80}P_{7.5}$ , (2) protease enzyme activity of the isolate *Bacillus stearoformis* optimum on  $T_{85}P_{7.5}$ , dan (3) protease enzyme activity of the isolate *Bacillus coagulans* optimum on  $S_{75}P_{7.5}$ . Based on further Honest test above and described on the graph, crude extract of the isolate protease enzyme *Bacillus licheniformis* showed the highest activity at 80°C and pH 7.5 by 0,1303 unit/ml/minute, isolate of *Bacillus stearoformis* showed highest protease enzyme activity at 75°C pH 7.5 by 0,2086 Unit/ml/minute. In comparison, *Bacillus coagulans* reached their highest protease enzyme activity at 85°C and pH 7.5 by 0,1226 Units/ml/minute.

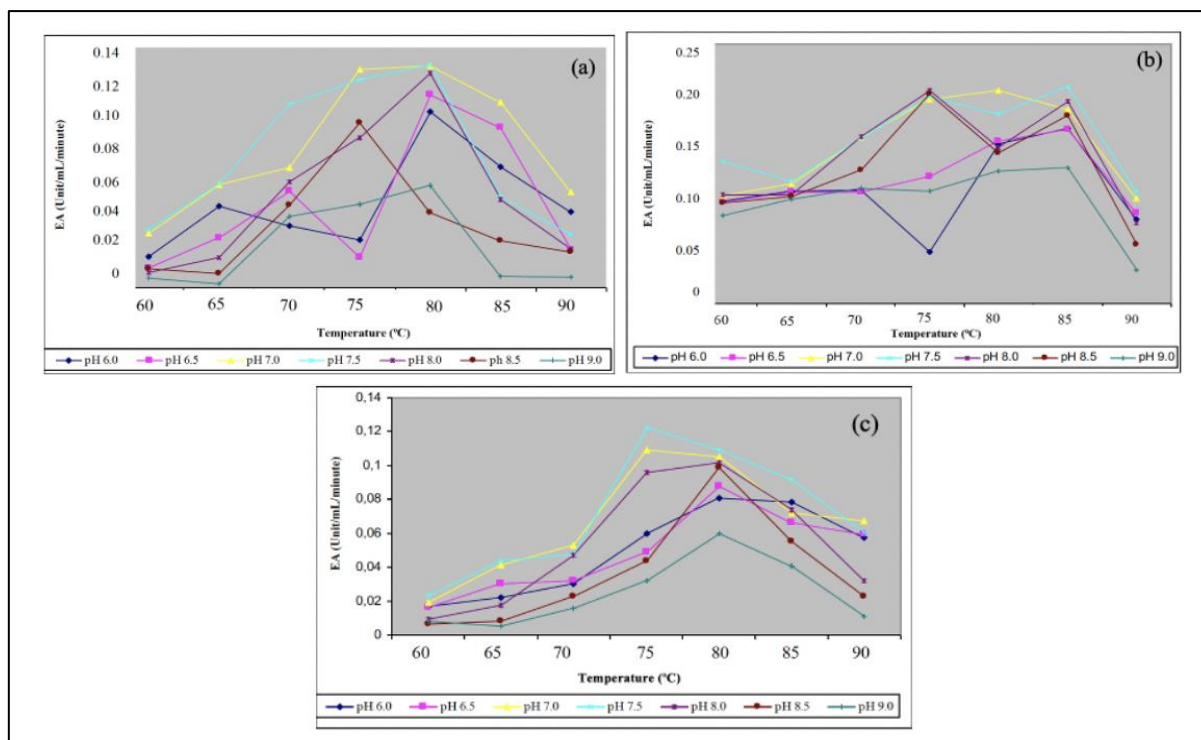


Figure 2. Protease Activity of *B. Licheniformis* (a), *B. stearoformis* (b) and *B. coagulans* (c) on various pH and temperatures

The crude extract of the protease enzyme produced by the three selected isolates was extracellular with casein as a casein inducer so that there is no need for bacterial cell wall destruction, and protease isolation can be done directly by centrifuging the media to separate the enzyme from other compounds in the medium based on molecular weight. Centrifugation of the production media is carried out at low temperatures to maintain the stability of the enzyme (Muñoz & Barceló, 2004; Punekar, 2018; Rawlings & Salvesen, 2013). Therefore, the protease isolation process was carried out using cold centrifugation at 4°C. Before centrifugation, a phosphate buffer solution with pH 7 was added to the production medium (10%). The purpose of adding a buffer is to maintain a stable pH condition of the production medium so that enzyme remains stable when isolated. PH 7 buffer is used because this pH is the natural pH of the physiological solution of living things. In addition, pH 7 is the optimum pH

of neutral protease (Rawlings & Salvesen, 2013; Ward, 2011; Ward et al., 2009). The results of centrifugation are in the form of precipitates and supernatants. The supernatant obtained still contains impurities from other components/compounds in the production media, so it is called crude enzyme extract (Mikdarullah & Nugraha, 2017; Nuritasari et al., 2017; Rachma et al., 2009).

Based on the analysis of variance, it was revealed that the treatment of temperature and pH variations had a very significant effect on the activity of the protease enzyme produced by the three superior thermophilic bacterial isolates from Lejja—Hot water spring. This significant indicates that temperature and pH have a considerable influence. The effect of temperature and pH shows that the two independent variables as Physico-chemical factors are crucial in optimizing the

prosthesis enzymes produced by the three isolates.

Based on the Honest test and graph in figure 2, it is seen that the highest protease activity *Bacillus licheniformis* reached 80°C and *Bacillus coagulans* reached its highest protease activity at 85°C. In comparison, optimum protease activity of *Bacillus stearoformis* reached 75°C. Those temperatures are optimum for the protease activity of the three isolates. These data also show that, in general, the temperature increase is accompanied by increased protease activity, and the increase continues until it reaches the optimum temperature. If this were exceeding the optimum temperature, protease activity would decrease, even decrease dramatically.

The increase in temperature causes an increase in the molecular kinetic energy of the enzyme and substrate (P. Fernandes & Carvalho, 2016; Sunil Kumar et al., 2018; Muñoz & Barceló, 2004). Puneekar (2018) stated that increased kinetic energy means accelerating vibrational movements and rotation of the enzyme and the substrate. The chance of a collision between the two is more excellent. Roy Choudhury (2019) agreed that kinetic energy increases the fraction of a molecule with sufficient energy to enter a transition state. At this point, there is an equal opportunity for reactant molecules to undergo reactions, form products or return to unreactive reactant molecules. The more of the frequency of collision of enzyme molecule with the substrate, the greater the chance of interaction between enzyme and substrate and the chance of product formation. At the optimum temperature, optimum enzyme activity is achieved, and the optimum product is produced.

Puneekar (2018) stated that every enzyme has optimum pH that leads to

maximum activity where pH, when the vital proton giving or receiving groups on the enzyme's catalytic site, is within the desired ionization stage. Alteration of pH does not significantly affect the ionic state of the enzyme and the substrate. On pH < 7.5, there is the accession of H<sup>+</sup>, which can bind to the enzyme's active site or another side of the enzyme that has a negative ion. This can block the interaction between enzyme and substrate or the interaction between functional groups of the enzyme itself, resulting in conformational changes on the enzyme's active site. If the enzyme's active site is incompatible with the substrate, the enzyme cannot bind to the substrate, which means that enzymatic reaction cannot occur (Kuriyan et al., 2021; Osho, 2019; Robinson, 2015; Roy Choudhury, 2019).

When optimum pH was reached, the conformation of the active site of the enzyme according to the substrate so that interactions can occur between the enzyme and the substrate appropriately (Sunil Kumar et al., 2018; Muñoz & Barceló, 2004). At this optimum pH condition, the highest enzymatic activity was obtained. While at pH > 7.5, the condition around enzyme has an excess of OH<sup>-</sup> or a lack of H<sup>+</sup> so that the functional groups on the substrate tend to be deprotonated. The deprotonation of the functional groups of the substrate causes no bond between the substrate and the enzyme's active site, which causes a decrease in enzyme activity (Kuriyan et al., 2021; Lopina, 2017; Puneekar, 2018).

Based on these findings, several suggestions are put forward: (1) generally genus of *Bacillus* produces more than one type of thermostable protease enzyme. Therefore it is necessary to do further research to ascertain the type of proteolytic enzymes from 3 species of *Bacillus* that have been isolated, considering that the enzyme tested in this study has not yet undergone the purification stage; (2) optimum enzyme

activity of protease at temperature and pH obtained from this research can be used as preliminary information for engineering conventional enzymes to be more heat resistant and with high productivity; (3) isolates of *B. licheniformis*, *B. coagulans* and *B. stearoformis* are bacteria that produce thermostable protease enzymes which have the potential to be developed as a source of genes and as a producer of the enzyme itself.

## CONCLUSION

Based on the research results that have been described, it can be concluded that there is a very significant effect of temperature and pH on the protease enzyme activity of the three superior thermophilic bacterial isolates from Lejja hot springs. Protease from *B. Licheniformis*, *B. stearoformis* and *B. coagulans* achieved optimum activity at the same pH but at different temperature ranges with relatively high activity and has the potential to be applied for various purposes.

## REFERENCES

- Amin, A., Ahmed, I., Salam, N., Kim, B.-Y., Singh, D., Zhi, X.-Y., Xiao, M., & Li, W.-J. (2017). Diversity and Distribution of Thermophilic Bacteria in Hot Springs of Pakistan. *Microbial Ecology*, 74(1), 116–127.
- Banerjee, G., & Ray, A. K. (2017). Impact of microbial proteases on biotechnological industries. *Biotechnology and Genetic Engineering Reviews*, 33(2), 119–143.
- Chavasit, V., Photi, J., Purttiponthane, S., & Saekoo, P. (2018). Use of Bacterial Growth Curve for Assessing Risk of Microbiological Pathogens in Food Products. In *Microbial Contamination and Food Degradation* (pp. 341–365). Elsevier.
- dos Santos Aguilar, J. G., & Sato, H. H. (2018). Microbial proteases: Production and application in obtaining protein hydrolysates. *Food Research International*, 103, 253–262.
- Fernandes, P., & Carvalho, F. (2016). Enzymes in Food Processing. In *Agro-Industrial Wastes as Feedstock for Enzyme Production: Apply and Exploit the Emerging and Valuable Use Options of Waste Biomass*.
- Fernandes, Pedro, & Carvalho, F. (2017). Microbial Enzymes for the Food Industry. In *Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications*.
- Gooch, J. W. (2011). Standard Bacterial Growth Curve. In *Encyclopedic Dictionary of Polymers* (pp. 925–925). Springer New York.
- Gupta, R., Beg, Q., & Lorenz, P. (2002). Bacterial alkaline proteases: Molecular approaches and industrial applications. In *Applied Microbiology and Biotechnology*, 59(1), pp. 15–32.
- Hafsan. (2018). *Isolasi dan Identifikasi Bakteri Termofilik Penghasil Protease dari Sumber Air Panas Lejja Soppeng Di Sulawesi Selatan (Research Report)*. Lembaga Penelitian dan Pengabdian kepada Masyarakat UIN Alauddin Makassar.
- Haki, G. (2003). Developments in industrially important thermostable enzymes: a review. *Bioresource Technology*, 89(1), 17–34.
- Indriati, G., & Megahati, R. R. P. (2018). Isolation of Thermophilic Bacteria and Optimizing the Medium Growth Conditions. *International Journal of Current Microbiology and Applied Sciences*, 7(1).
- Kumar, Sunil, Kumar, R., Pal, A., & Singh Chopra, D. (2018). Enzymes. In *Postharvest Physiology and Biochemistry of Fruits and Vegetables*.
- Kumar, Surinder. (2016). Essentials of Microbiology. In *Essentials of Microbiology*. Jaypee Brothers Medical Publishers (P) Ltd.
- Kuriyan, J., Konforti, B., & Wemmer, D. (2021). Principles of Enzyme Catalysis. In *The Molecules of Life*.
- Liu, X., & Kokare, C. (2017). Microbial Enzymes of Use in Industry. In *Biotechnology of Microbial Enzymes* (pp. 267–298). Elsevier.
- López-Otín, C., & Bond, J. S. (2008). Proteases: multifunctional enzymes in life and disease. *The Journal of Biological Chemistry*, 283(45), 30433–30437.



- Lopina, O. D. (2017). Enzyme Inhibitors and Activators. In *Enzyme Inhibitors and Activators*.
- Marshall, C. E. (1917). Microbiology : a text-book of microorganisms general and applied. In *Microbiology : a text-book of microorganisms general and applied*. P. Blakiston's son & co.
- Maurer, K. (2004). Detergent proteases. *Current Opinion in Biotechnology*, 15(4), 330–334.
- Mikdarullah, M., & Nugraha, A. (2017). Teknik Isolasi Bakteri Proteolitik Dari Sumber Air Panas Ciwidey, Bandung. *Buletin Teknik Litkayasa Akuakultur*, 15(1), 11.
- Mohammad, B. T., Al Daghistani, H. I., Jaouani, A., Abdel-Latif, S., & Kennes, C. (2017). Isolation and Characterization of Thermophilic Bacteria from Jordanian Hot Springs: *Bacillus licheniformis* and *Thermomonas hydrothermalis* Isolates as Potential Producers of Thermostable Enzymes. *International Journal of Microbiology*, 12(1), 21-29.
- Muñoz, R., & Barceló, A. R. (2004). Enzymes. In *Handbook of Food Analysis Second Edition: Physical Characterization and Nutrient Analysis* (Vol. 1).
- Nuritasari, D., Sarjono, P. R., & Aminin, A. L. N. (2017). Isolasi Bakteri Termofilik Sumber Air Panas Gedongsongo dengan Media Pengaya MB (Minimal Broth) dan TS (Taoge Sukrosa) serta Identifikasi Fenotip dan Genotip. *Jurnal Kimia Sains Dan Aplikasi*, 20(2), 84–91.
- Olajuyigbe, F. M., & Ajele, J. O. (2005). Production dynamics of extracellular protease from *Bacillus* species. *African Journal of Biotechnology*, 4(8).
- Osho, M. B. (2019). Industrial Enzyme Technology. In *Biotechnology*.
- Punekar, N. S. (2018). Enzymes: Catalysis, Kinetics and Mechanisms. In *Enzymes: Catalysis, Kinetics and Mechanisms*.
- Rachma, A., Sarjono, P. R., & Aminin, A. L. N. (2009). Isolasi Bakteri Termofilik Sumber Air Panas Gedongsongo dengan Media Pengaya Minimal YT (Yeast Tripton) serta Identifikasi Genotipik dan Fenotipik. *Jurnal Kimia Sains Dan Aplikasi*, 12(3), 66–71.
- Rawlings, N. D., & Salvesen, G. (2013). Handbook of Proteolytic Enzymes. In *Handbook of Proteolytic Enzymes*.
- Razzaq, A., Shamsi, S., Ali, A., Ali, Q., Sajjad, M., Malik, A., & Ashraf, M. (2019). Microbial Proteases Applications. *Frontiers in Bioengineering and Biotechnology*, 7.
- Robinson, P. K. (2015). Enzymes: principles and biotechnological applications. *Essays in Biochemistry*, 59, 1–41.
- Roy Choudhury, A. K. (2019). Introduction to enzymes. In *Sustainable Technologies for Fashion and Textiles*.
- Sanchez, S., & Demain, A. L. (2017). Useful Microbial Enzymes-An Introduction. In *Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications*.
- Singh, P., & Kumar, S. (2018). Microbial enzyme in food biotechnology. In *Enzymes in Food Biotechnology: Production, Applications, and Future Prospects*.
- Singh, R. S., Singh, T., & Pandey, A. (2019). Microbial enzymes-an overview. In *Biomass, Biofuels, Biochemicals: Advances in Enzyme Technology*.
- Suman, S., & Ramesh, K. (2008). An extracellular thermostable protease production from thermophilic *Bacillus* species isolated from soil. *Biosciences Biotechnology Research Asia*, 5(2).
- Tanzadeh, J., Yasouri, F. N., & Ghaemi, N. (2006). Isolation and identification of the thermophilic protease-producing bacteria from soil of north of Iran. *Biosciences Biotechnology Research Asia*, 3(2 A).
- Ward, O. P. (2011). Proteases. In *Comprehensive Biotechnology* (pp. 571–582). Elsevier.
- Ward, O. P., Rao, M. B., & Kulkarni, A. (2009). Proteases, Production. In *Encyclopedia of Microbiology* (pp. 495–511). Elsevier.