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Optimization of Temperature and pH for Protease Production from Some Bacterial Isolates of Mangrove Waters

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Abstract: Protease enzymes are widely used in biotechnology to produce amino acids and peptides from high molecular substrates, as well as in the leather industry for wastewater treatment, textiles, pharmaceuticals, cosmetics, leather processing, and poultry industry. Indonesia's demand for protease enzymes is increasing. This study aims to optimize the temperature and pH for the production of protease from certain bacterial isolates of mangrove waters. The study examined growth profiles, enzyme activity, and the effects of temperature and pH on enzyme production. The research method used is experimental. Protease activity is carried out using the Takami method. The results showed that the protease activity of bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 are optimum at 35°C. The optimum pH for protease production from the bacterial isolates PUA-10 and PUA-16 was at pH 7, while the bacterial isolates PUA-12 and PUA-14 are at pH 8. There is an increase in protease activity after optimization on the bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 that amounted to 23.84; 17.90; 21.12; and 24.58%, respectively. The research can determine the optimum temperature and pH for each isolate and improve the efficiency of protease production from some bacterial isolates of mangrove waters.

Keywords: Microorganism, Protease Activity, Abiotic Factor, Mangrove Ecosystem

Introduction

Enzymes have become indispensable in technology and industry, gradually replacing traditional chemical catalysts. As biocatalysts, enzymes accelerate biochemical reactions within and outside cells, playing a vital role in both food and non-food industrial processes (Elleuche *et al*., 2014). One particularly important enzyme group is proteases, which are widely utilized in the food, pharmaceutical, and chemical industries. These hydrolase enzymes break down proteins into simpler molecules, such as oligopeptides and amino acids, and are crucial in producing amino acids and peptides, wastewater treatment, textiles, pharmaceuticals, cosmetics, leather processing, and poultry farming (Agustina *et al*., 2019).

Proteases dominate the enzyme market, accounting for nearly 60% of the global enzyme market and 40% of all enzyme sales worldwide (Sharma *et al.*, 2019). Despite the growing demand for protease enzymes in Indonesia, the country remains dependent on imports due to limited domestic production capacity (Kurniasari *et al.*, 2022). Microorganisms, particularly bacteria, are preferred sources of commercial enzyme production due to their favorable physical and biochemical properties and ease of cultivation (Schwan *et al.*, 2011; Adrio and Demain, 2014).

Bacteria are integral to ecosystems, including mangrove ecosystems, where they significantly impact ecological processes (Hrenovic *et al*., 2003). Previous research, such as that by Yulma *et al*. (2018), has identified bacterial isolates from mangrove waters with potential industrial applications. Protease-producing bacterial genera include *Bacillus, Staphylococcus, Flavobacterium, Serratia, Alcaligenes, Vibrio, Brevibacterium, Pseudomonas*, and *Halobacterium*, with *Bacillus* strains being the most dominant (Gupta *et al.*, 2005).

To fully harness the potential of protease enzymes from mangrove bacteria, it is essential to optimize abiotic factors such as temperature and pH to enhance enzyme activity. This study, titled "Optimization of temperature and pH for protease production from some bacterial isolates of mangrove waters," aims to provide a foundation for industrial collaboration by optimizing conditions for high protease activity.

Although mangroves play a crucial ecological role, research on optimizing protease production from mangrove aquatic bacteria is significantly lacking,

especially in Indonesia. This study seeks to address this gap by examining the protease production capabilities of mangrove bacteria in Indonesia, aiming to offer valuable insights for both environmental and industrial uses.

Materials and Methods

Tools and Materials

The tools used in this study were Petri dishes, test tubes, Erlenmeyer flasks, thermometers, pH meters, cotton, gauze, tissues, measuring cups, pipettes, platform shakers, magnetic stirrers, Eppendorf tubes, Ose needles, hotplate stirrers, autoclaves, spirit lamps, spectrophotometers, analytical balances, beakers, incubators, and aluminum foil. The materials used in this study were isolates of potential proteolytic bacteria from mangrove waters in the Mandeh region, Pesisir Selatan, distilled water, NaOH, HCl, medium nutrient agar, NaCl, KH_2PO_4, $MgSO_4$, K_2HPO_4, Casein, Tris-HCl, TCA, Na_2CO_3 and Folin Ciocalteu.

Sterilization of Tools and Materials

All equipment and medium used were sterilized using an autoclave with a temperature of 121°C and a pressure of 15 LBS for 15 min.

Preparation of Medium Nutrient Agar (NA) and Protease Production Medium

Weigh NA as much as 20 g, put it into a beaker, then add sterile distilled water to a volume of 1000 mL. Then heated to boiling, the medium was poured into a sterile Erlenmeyer, tightly closed using cotton, and wrapped in aluminum foil. Finally, the medium was sterilized in an autoclave at 121°C with a pressure of 15 LBS for 15 min. Medium for producing protease enzymes by weighing: 3 g KH_2PO_4; 5 g $MgSO_4$; 3 g K_2HPO_4, 5 g NaCl, and 10 g casein, dissolved in 1000 mL of distilled water. Once homogeneous, the medium was sterilized using an autoclave at 121°C and 15 LBS pressure for 15 min (Agustien, 2010).

Provision of Bacterial Isolates

The bacterial isolates used in this study were four isolates from the collection of the biotechnology laboratory Universitas Andalas, Padang. The isolates were isolated from Mangrove waters in Mandeh Area, Pesisir Selatan district, with the codes PUA-10, PUA-12, PUA-14, and PUA-16. Rejuvenation of the four bacterial isolates was carried out by taking one Ose needle amount of pure culture each, then streaking it on NA medium, incubating for 24 h at room temperature, then inoculating it on slanted media and storing it at room temperature.

Bacterial Growth Profile

The growth profile of these bacteria was determined by providing 100 mL of protease production medium in a sterile 250 mL Erlenmeyer flask. Then inoculated, by utilizing 1-2 loops of the oblique culture of each isolate on the protease production medium and incubated at 30°C (inoculum), pH 7, and agitation of 150 rpm for 24 h. After that, pipette 5 mL of inoculum into 95 mL of protease production medium in a 250 mL Erlenmeyer flask. A sample of 1 mL of bacterial culture was measured for turbidity using a spectrophotometer at a wavelength of 600 nm. At every 2 h interval, sampling was carried out in bacterial culture as in the previous step. The measurements were stopped after a decrease in the growth of bacterial isolates.

Determination of Optimum Temperature for Protease Production

To determine the effect of temperature on bacteria, prepare some Erlenmeyer flasks containing 95 mL of media to produce protease, which has been sterilized. An inoculum was made for each potential isolate based on the results obtained. Pipette 5 mL of each inoculum onto 95 mL of protease production medium, then incubate at 23, 27, 31, 35, and 39°C respectively, pH 7, and agitation of 150 rpm for a defined time (idiophase) of each bacterial isolate. The bacterial culture was centrifuged at 6000 rpm at 30°C for 20 min so that the enzyme solution was obtained and the enzyme activity was determined.

Determination of Optimum pH for Protease Production

To determine the effect of bacterial pH, some Erlenmeyer flasks containing 95 mL of protease production medium were prepared. Then the pH of the medium was adjusted by adding 0.1 M HCl or 0.1 M NaOH to the solution and measured at several predetermined pH values (pH 5, 6, 7, 8, and 9). An inoculum was made for each potential isolate based on the results obtained from the determination of the optimum temperature for protease production and incubated at the optimum temperature with agitation at 150 rpm. The bacterial culture was centrifuged at 6000 rpm at 30°C for 20 min to obtain the enzyme solution and determination of enzyme activity.

Protease Enzyme Activity Assay

Protease activity Assay is carried out using the Takami method (Takami *et al*., 1989), where casein was used as a substrate. Starting with 1% casein substrate, the substrate solution was pipetted into a test tube (with as much as 0.5 mL), then as much as 0.5 mL of the available enzyme solution was added. Finally, 0.25 mL of 50 mM Tris-HCL buffer, pH 8.0 was added and incubated at 30°C

for 15 min. After that, 0.5 mL of TCA was added and incubated at 30°C for 20 min. Then the test tube was vortexed and centrifuged at 6000 rpm for 20 min. After that, 0.375 mL of the supernatant was taken and transferred to a new test tube, after which 1.25 mL Na_2CO_3 and 0.25 mL 1N Folin Ciocalteu were added. The incubation results were measured with a spectrophotometer at λ = 578 nm.

Enzyme activity can be determined by the formula:

$$UA = \frac{(Asp - Abl)}{(Ast - Abl)} \times \frac{1}{T} \times P$$

Information;

UA : Protease activity units (U/mL)
Asp : Sample absorbance
Abl : Blanko absorbance
Ast : Standard absorbance
P : Dilution
T : Incubation time (minutes)

Data Analysis

Data analysis for research was carried out on growth profiles, enzyme activity, and the effect of temperature and pH on enzyme production. Data is presented in the form of tables and graphs.

Results and Discussion

Growth Profile and Protease Activity

Growth profiles and protease activity of bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 are shown in Fig. (1).

Figure 1 shows the growth profile and protease activity of four bacterial isolates. Isolate PUA-12 had a 2-h adaptation phase, while PUA-10, PUA-14, and PUA-16 entered the logarithmic phase directly. According to Suberu *et al.* (2019), repeated growth in the same medium shortens the adaptation phase. Factors such as initial cell count, physiological conditions, and medium composition influence this phase.

The highest growth peaks occurred at the 16th h for PUA-10 and PUA-12 and at the 20th h for PUA-14 and PUA-16 (Fig. 1). Rapid growth during these hours boosts enzyme production due to abundant nutrients. However, as substrate levels decrease, proteolytic activity drops, affecting enzyme structure and efficiency (Marnolia *et al.*, 1930).

The stationary phase for PUA-12 lasted from 16-20 h, while PUA-10, PUA-14, and PUA-16 had brief stationary phases. Jaishankar and Srivastava (2017) attribute this to high metabolic activity. Observations every 2 h might miss the stationary phase, making it appear that bacteria quickly enter the death phase. PUA-10's death phase started at 18 h, while PUA-12, PUA-14 and PUA-16 began at 22 h. Differences in growth phase durations can result from variations in bacterial species and environmental conditions (Wijanarka *et al.*, 2016).

Bacterial growth profiles were measured by medium turbidity using a spectrophotometer, which assesses scattering caused by large molecules (Pagarra, 2020). Turbidity reflects bacterial growth, reproduction, and enzyme secretion (Marnolia *et al.*, 1930). Monitoring growth profiles helps identify the transition from logarithmic to stationary phases, indicating peak enzyme production. Enzyme production is linked to microbial growth (Blanco *et al.*, 2016).

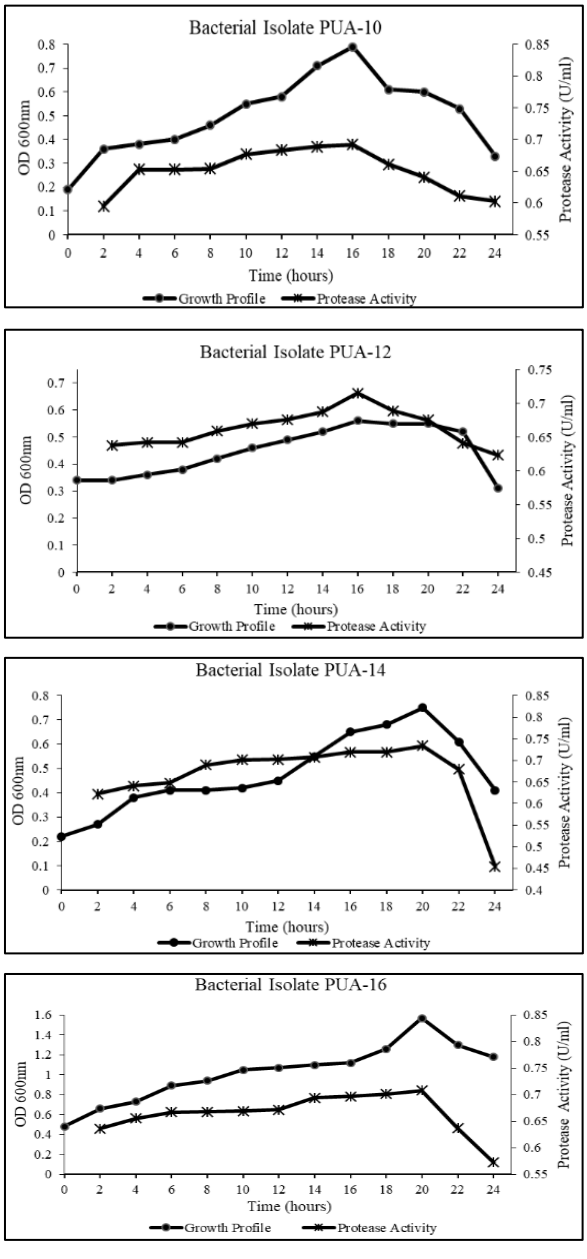


Fig. 1: Growth profiles and protease activity of bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 at 35°C, pH 7, and agitation at 150 rpm

Fitriana and Asri (2021) found protease activity in three bacterial isolates from soybean plant rhizospheres after 24 h of fermentation, coinciding with the end of the exponential growth phase. Uttatree *et al*. (2017) reported maximum protease activity from *Bacillus megaterium* during the stationary phase after 15 h.

Primary metabolites, like protease enzymes, are synthesized during the active exponential growth phase, which is optimal for microbial growth (Purkan *et al*., 2014). As nutrient availability declines, proteolytic activity slows due to nutrient limitations and toxic waste accumulation, inhibiting growth (Pepper *et al*., 2015).

Extracellular enzyme synthesis follows the growth curve pattern, with optimal production in the late exponential or initial stationary phases (Oktavia *et al*., 2018). Increased protease activity results from high metabolic activity during cell division and enzyme synthesis. Enzyme secretion depends on cell count and the microorganism's growth phase (Das *et al*., 2013). Different microbial types and the number and sequence of amino acids formed influence enzyme activity (Agustien, 2010).

Optimum Temperature for Protease Production

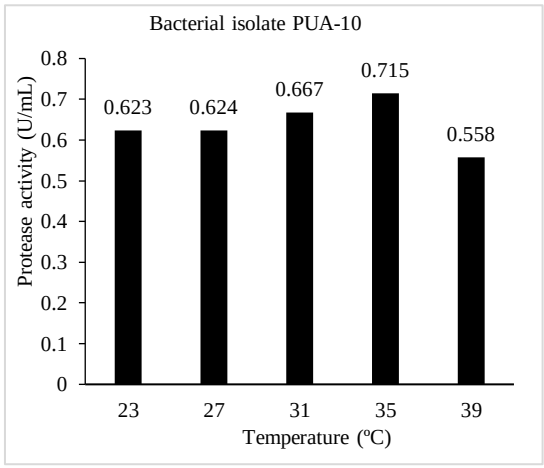
The optimum temperature for protease production from bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 is shown in Fig. (2).

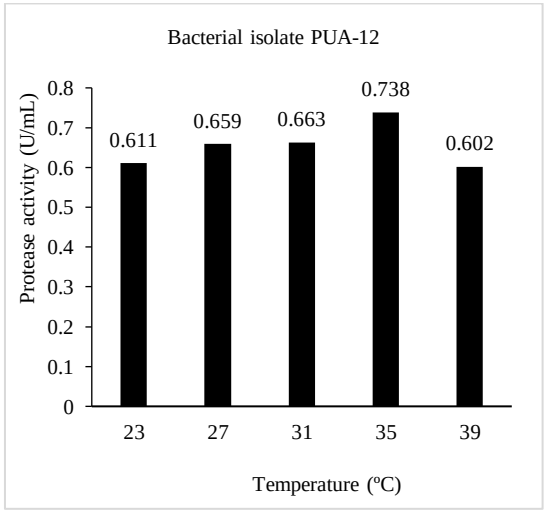
The optimum temperature for protease enzyme production was determined by varying the temperature: 23, 27, 31, 35, and 39°C. Sampling occurred at 16 h for bacterial isolates PUA-10 and PUA-12 and at 20 h for isolates PUA-14 and PUA-16, based on their peak protease activity shown in Fig. (1). Figure 2 indicates that the optimal temperature for protease activity in all four isolates (PUA-10, PUA-12, PUA-14, PUA-16) was 35°C, with activity values of 0.715, 0.738, 0.820 and 0.736 U/mL, respectively.

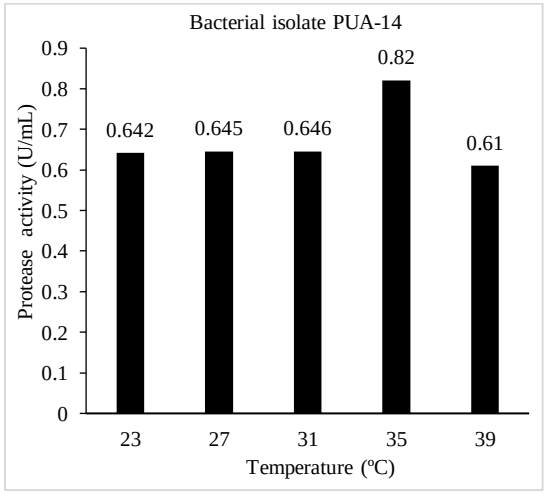
Temperature significantly influences bacterial growth and enzymatic activity. Higher temperatures increase enzyme activity up to an optimal point, beyond which activity decreases due to protein denaturation (Suriani *et al*., 2013). Susanti and Febriana (2018) noted that microbial growth rates increase slowly up to a certain temperature, after which they drop rapidly. Elias *et al*. (2014) also highlighted that temperature affects enzyme biosynthesis and inactivation rates.

Previous studies have shown that many bacteria, including *P. aeruginosa* PseA, *B. licheniformis*, *B. coagulans*, *B. cereus*, *P. aeruginosa* MCM B-327 and *P. chrysogenum* IHH5, have an optimum temperature of 35°C, while certain *Bacillus* species such as *B. amovivorus*, *B. proteolyticus* CFR 3001, *B. aquimaris* VITP4 and *B. subtilis* have an optimum temperature of 37°C (Sharma *et al*., 2019).

Temperature affects substrate-enzyme interactions by altering kinetic energy, which increases collision rates and facilitates complex formation, thus enhancing product yield. However, temperatures above the optimum can cause denaturation, reducing enzyme activity (Khusro, 2016; Noviyanti *et al*., 2012).







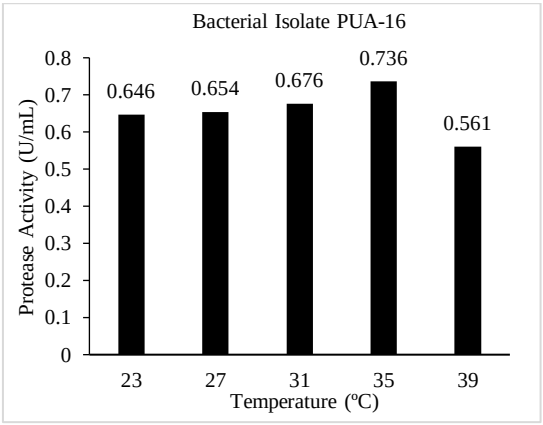


Fig. 2: Optimum temperature histogram for protease production of bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 at pH 7 and agitation at 150 rpm

Optimal temperatures are crucial for industrial applications as they reduce contamination risk, save time, cost, and energy, and lower the viscosity of fermentation solutions, thereby improving production efficiency (Laishram and Pennathur, 2016).

Optimum pH for Protease Production

The optimum pH of protease production from bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 is shown in Fig. (3).

pH significantly impacts enzyme activity. Optimal enzyme activity occurs under specific environmental conditions. In this study, isolates PUA-10 and PUA-16 showed the highest protease activity at pH 7, while PUA-12 and PUA-14 peaked at pH 8. Aznia *et al.* (2014) found similar results, with isolate M5-24 from Medang River hot springs exhibiting the highest protease activity at pH 8 and lowest at pH 10.

For isolates PUA-10 and PUA-16, activity decreased at pH 8 and for PUA-12 and PUA-14, it dropped at pH 9. Changes in pH can denature enzymes by disrupting the non-covalent interactions that stabilize their structure. H^+ ion concentration at different pH levels affects enzyme catalysis and activity, with each microorganism having an optimal pH. A suitable pH helps enzymes bind to substrates, significantly impacting microbial metabolic pathways. Altering pH affects the ionization of nutrient molecules, reducing their availability and overall metabolic activity (Khusro, 2016).

The optimum pH for protease production in isolates PUA-10 and PUA-16 is pH 7, indicating neutral proteases. Below this pH, production decreases due to metabolite accumulation leading to enzyme inactivation (Santhi, 2014). Proteases from isolates PUA-12 and PUA-14 are alkaline proteases, working best at pH 8. Microorganisms producing alkaline proteases show high activity at alkaline pH, making them suitable for industrial applications (Vanitha *et al.*, 2014).

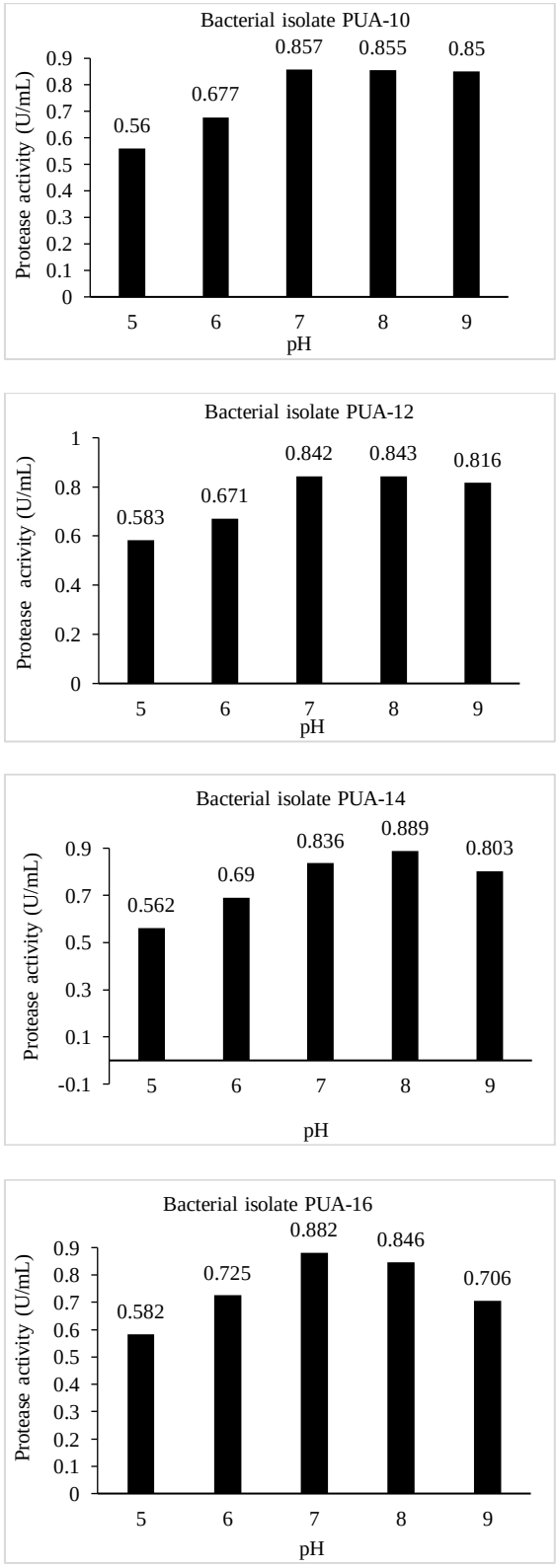


Fig. 3: Optimum pH histogram of protease production of bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 at 35°C and agitation at 150 rpm

Table 1: Production protease from four bacterial isolates in mangrove waters before and after optimization

Bacterial isolate	Protease activity (U/mL)		Increase in protease activity (%)
	Before optimization	After optimization	
PUA-10	0.692	0.857	23.84
PUA-12	0.715	0.843	17.90
PUA-14	0.734	0.889	21.12
PUA-16	0.708	0.882	24.58

Enzymes require specific conditions of temperature and pH due to their protein nature, which affects their shape and function. Optimal temperature aligns with the organism's normal conditions, while pH profoundly influences enzyme activity. Extremes in pH can deactivate enzymes, which generally operate most effectively within a limited pH range (Soeka and Sulistiani, 2017).

Protease Production Before and After Optimization

Observation results of production proteases from four bacterial isolates in mangrove waters before and after optimization are presented in Table (1).

Table (1) shows the increase in protease activity of the four bacterial isolates after optimizing temperature and pH. Isolate PUA-16 had the highest increase at 24.58%, followed by PUA-10 (23.84%), PUA-14 (21.12%) and PUA-12 (17.90%). Aznia *et al*. (2014) found that optimizing conditions for isolating M5-24 from Medang River hot springs increased protease activity by 1.75 times. Similarly, Subba Rao *et al*. (2008) reported a 1.5-fold increase in protease production for *Bacillus circulans* from soil samples in India. Systematic optimization can significantly boost enzyme production, highlighting the importance of optimizing pH and temperature for enhancing enzyme activity in bacterial isolates from Mandeh mangrove waters (Alamsjah *et al.*, 2024).

Enzyme activity can be influenced by pH, temperature, and incubation time, affecting the enzyme's tertiary structure. This structure is stabilized by hydrogen bonds, ionic attractions, hydrophobic interactions, and covalent bonds. Optimal pH can change ionization in the enzyme's ionic group, enhancing the active site's ability to bind and convert substrates effectively. Changes in the enzyme-substrate group also impact enzyme activity (Arya *et al.*, 2020).

Conclusion

The study has the potential to determine the optimum temperature and pH conditions for each bacterial isolate, leading to enhanced efficiency in the production of protease from various bacterial isolates of mangrove waters. The optimum temperature for the production of protease from bacterial isolates of mangrove waters (PUA-10, PUA-12, PUA-14, and PUA-16) is 35°C, the optimum pH for protease production from bacterial isolates PUA-10 and PUA-16 was at pH 7, while bacterial isolates PUA-12 and PUA-14 were at pH 8. Protease activity increased after optimization on bacterial isolates PUA-10, PUA-12, PUA-14, and PUA-16 by 23.84, 17.90, 21.12 and 24.58%, respectively.

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Author's Contributions

Feskaharny Alamsjah: Originated the study idea, participated in the design and planning of the experiment, offered guidance and supervision during the research, and reviewed and provided critical feedback on the manuscript.

Yorasakhi Ananta: Designed the experimental approach and methodology, conducted the experiment collected data, and drafted the initial manuscript.

Anthoni Agustien: Reviewed and edited the manuscript for clarity and coherence and supplied the essential resources and equipment for the study.

Ethics

No ethical concerns were identified during the course of this research. The authors affirm that there are no conflicts of interest associated with this study.

References

Adrio, J. L., & Demain, A. L. (2014). Microbial Enzymes: Tools for Biotechnological Processes. *Biomolecules*, *4*(1), 117–139. https://doi.org/10.3390/biom4010117

Agustien, A. (2010). *Protease Bakteri Termofilik*. Universitas Padjajaran.

Agustina, D. K., Sulistiana, D., & Anggraini, D. P. (2019). *Bioteknologi Mikroba: Tinjauan Umum dan Aplikasi* (1st Ed.). Serang Banten.

Alamsjah, F., Aswan, D. R., & Agustien, A. (2024). pH and Temperature Optimization of Several Bacterial Isolates from Mangrove Waters in the Mandeh Area to Produce Cellulase Enzyme. *OnLine Journal of Biological Sciences*, *24*(3), 367–373. https://doi.org/10.3844/ojbsci.2024.367.373

Arya, P., Trivedi, M., Ramani, N., Patel, K., & Rajput, K. N. (2020). Isolation, Production and Applications of Alkaline Protease from Hot Springs Bacterial Isolates. *Bioscience Biotechnology Research Communication*, *13*(1), 85–91. https://doi.org/10.21786/bbrc/13.1special issue/15

Aznia, A., Agustien, A., & Nasir, N. (2014). Optimasi Parsial Isolat Termofilik M5-24 dalam Produksi Protease. *Jurnal Biologi Universitas Andalas*, *3*(3), 238–243.

Blanco, P., Hernando-Amado, S., Reales-Calderon, J., Corona, F., Lira, F., Alcalde-Rico, M., Bernardini, A., Sanchez, M., & Martinez, J. (2016). Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants. *Microorganisms*, *4*(1), 14. https://doi.org/10.3390/microorganisms4010014

Das, A., Paul, T., Halder, S. K., Maity, C., Mohapatra, P. K. D., Pati, B. R., & Mondal, K. C. (2013). Study on Regulation of Growth and Biosynthesis of Cellulolytic Enzymes from Newly Isolated Aspergillus fumigatus ABK9. *Polish Journal of Microbiology*, *62*(1), 31–43. https://doi.org/10.33073/pjm-2013-004

Elias, M., Wieczorek, G., Rosenne, S., & Tawfik, D. S. (2014). The universality of enzymatic rate–temperature dependency. *Trends in Biochemical Sciences*, *39*(1), 1–7. https://doi.org/10.1016/j.tibs.2013.11.001

Elleuche, S., Schröder, C., Sahm, K., & Antranikian, G. (2014). Extremozymes — biocatalysts with unique properties from extremophilic microorganisms. *Current Opinion in Biotechnology*, *29*, 116–123. https://doi.org/10.1016/j.copbio.2014.04.003

Fitriana, N., & Asri, M. T. (2021). Aktivitas Proteolitik pada Enzim Protease dari Bakteri Rhizosphere Tanaman Kedelai (*Glycine max* L.) di Trenggalek. *LenteraBio : Berkala Ilmiah Biologi*, *11*(1), 144–152. https://doi.org/10.26740/lenterabio.v11n1.p144-152

Gupta, R., Chauhan, B., Ramnani, P., & Singh, R. (2005). Bacterial alkaline proteases: recent trends and industrial Applications. In T. Satnarayan & B. Johri (Eds.), *Microbial Diversity: Current Perspectives and Potential Application* (pp. 769–789). IK International.

Hrenovic, J., Vilicic, D., & Stilinovic, B. (2003). Influence of nutrients and salinity on heterotrophic and coliform bacteria in the shallow, karstic Zrmanja Estuary (eastern Adriatic Sea). *Ekoloji Çevre Dergisi*, *12*(46), 29–37.

Jaishankar, J., & Srivastava, P. (2017). Molecular Basis of Stationary Phase Survival and Applications. *Frontiers in Microbiology*, *8*, 2000. https://doi.org/10.3389/fmicb.2017.02000

Khusro, A. (2016). One Factor at A Time-based optimization of protease from poultry associated Bacillus licheniformis. *Journal of Applied Pharmaceutical Science*, *6*(3), 088–095. https://doi.org/10.7324/japs.2016.60315

Kurniasari, I., Sulistyaningtyas, A. R., & Darmawati, S. (2022). Isolasi Bakteri Proteolitik Hasil Fermentasi Inasua Ikan Bawal. *Prosiding Seminar Nasional UNIMUS*, 1285–1296.

Laishram, S., & Pennathur, G. (2016). Purification and Characterization of a Membrane-Unbound Highly Thermostable Metalloprotease from Aeromonas Caviae. *Arabian Journal for Science and Engineering*, *41*(6), 2107–2116. https://doi.org/10.1007/s13369-015-1849-9

Marnolia, A., Haryani, Y., & Puspita, F. (1930). UJI Aktivitas Enzim Protease Dari *Isolat Bacillus* sp. Endofit Tanaman Kelapa SAWIT (Elaeis quinensis). *Photon: Jurnal Sain Dan Kesehatan*, *6*(02), 1–5. https://doi.org/10.37859/jp.v6i02.429

Noviyanti, T., Ardiningsih, P., & Rahmalia, W. (2012). Pengaruh Temperatur Terhadap Aktivitas Enzim Protease Dari Daun Sansakng (Pycnarrhena Cauliflora Diels). *Jurnal Kimia Khatulistiwa*, *1*(1), 31–34.

Oktavia, Y., Lestari, S. D., Lestari, S., & Jannah, M. (2018). Optimasi Waktu Inkubasi Produksi Protease Dan Amilase Isolat Bakteri Asal Terasi Ikan Teri Stolephorus SP. *Jurnal Ilmu Dan Teknologi Kelautan Tropis*, *10*(3), 719–725. https://doi.org/10.29244/jitkt.v10i3.18840

Pagarra, H. (2020). Isolation and Optimization of Endophytic Bacteria from Roots of Karst Area Ecosystems Producing Protease Enzymes. *Journal of Research on the Lepidoptera*, *51*(2), 431–439. https://doi.org/10.36872/lepi/v51i2/301110

Pepper, I. L., Gerba, C. P., & Gentry, T. J. (2015). Chapter 1- Introduction to Environmental Microbiology. In *Environmental Microbiology*, 3rd Ed., pp: 3-8. Academic Press. https://doi.org/10.1016/B978-0-12-394626-3.00001-6

Purkan, P., Azizah, B., & Sumarsih, S. (2014). Eksplorasi Bakteri Kitinolitik dari Sampah Organik: Isolasi dan Karaktrisasi Enzim Kitinase. *Molekul*, *9*(2), 128–135. https://doi.org/10.20884/1.jm.2014.9.2.160

Santhi, R. (2014). Microbial production of protease by Bacillus cereus using cassava waste water. *European Journal of Experimental Biology*, *4*(2), 19–24.

Schwan, R. F., Rodarte, M. P., Dias, D. R., & Vilela, D. M. (2011). Proteolytic activities of bacteria, yeasts and filamentous fungi isolated from coffee fruit (*Coffea arabica* L.). *Acta Scientiarum. Agronomy*, *33*(3), 457–464. https://doi.org/10.4025/actasciagron.v33i3.6734

Sharma, M., Gat, Y., Arya, S., Kumar, V., Panghal, A., & Kumar, A. (2019). A Review on Microbial Alkaline Protease: An Essential Tool for Various Industrial Approaches. *Industrial Biotechnology*, *15*(2), 69–78. https://doi.org/10.1089/ind.2018.0032

Soeka, Y. S., & Sulistiani, S. (2017). Karakterisasi Enzim Protease Dari Bakteri Stenotrophomonas sp. ASAL GUNUNG BROMO, JAWA TIMUR. *Berita Biologi*, *16*(2), 203–211. https://doi.org/10.14203/beritabiologi.v16i2.2940

Subba Rao, Ch., Madhavendra, S. S., Sreenivas Rao, R., Hobbs, P. J., & Prakasham, R. S. (2008). Studies on Improving the Immobilized Bead Reusability and Alkaline Protease Production by Isolated Immobilized Bacillus circulans (MTCC 6811) Using Overall Evaluation Criteria. *Applied Biochemistry and Biotechnology*, *150*(1), 65–83. https://doi.org/10.1007/s12010-008-8147-x

Suberu, Y., Akande, I., Samuel, T., Lawal, A., & Olaniran, A. (2019). Optimization of protease production in indigenous Bacillus species isolated from soil samples in Lagos, Nigeria using response surface methodology. *Biocatalysis and Agricultural Biotechnology, 18*, 101011. https://doi.org/10.1016/j.bcab.2019.01.049

Suriani, S., Soemarno, & Suharjono. (2013). Pengaruh suhu & pH terhadap laju pertumbuhan lima isolat bakteri anggota genus Pseudomonas yang diisolasi dari ekosistem sungai tercemar deterjen di sekitar kampus Universitas Brawijaya. *Indonesian Journal of Environment and Sustainable Development*, *4*(1), 58–62.

Susanti, R., & Fibriana, F. (2018). *Teknologi Enzim*. Penerbit Andi.

Takami, H., Akiba, T., & Horikoshi, K. (1989). Production of extremely thermostable alkaline protease from Bacillus sp. no. AH-101. *Applied Microbiology and Biotechnology*, *30*(2), 120–124. https://doi.org/10.1007/bf00263997

Uttatree, S., Kobtrakool, K., Ketsuk, A., Kaenngam, W., Thakolprajak, P., & Charoenpanich, J. (2017). A novel metal-tolerant, solvent and surfactant stable protease from a new strain of Bacillus megaterium. *Biocatalysis and Agricultural Biotechnology*, *12*, 228–235. https://doi.org/10.1016/j.bcab.2017.10.012

Vanitha, N., Rajan, S., & Murugesan, A. G. (2014). Optimization and production of alkaline protease enzyme from Bacillus subtilis 168 isolated from food industry waste. *International Journal of Current Microbiology and Applied Sciences*, *3*(6), 36–44.

Wijanarka, W., Kusdiyantini, E., & Parman, S. (2016). Screening Cellulolytic Bacteria from the Digestive Tract Snail (Achatina fulica) and Test the Ability of Cellulase Activity. *Biosaintifika: Journal of Biology & Biology Education*, *8*(3), 386–392. https://doi.org/10.15294/biosaintifika.v8i3.7263

Yulma, Y., Ihsan, B., Sunarti, S., Malasari, E., Wahyuni, N., & Mursyban, M. (2017). Identifikasi Bakteri Pada Serasah Daun Mangrove yang Terdekomposisi di Kawasan Konservasi Mangrove dan Bekantan (KKMB) Kota Tarakan. *Journal of Tropical Biodiversity and Biotechnology*, *2*(1), 28–33. https://doi.org/10.22146/jtbb.27173