Physical Education, Health and Social Sciences

https://journal-of-social-education.org

E-ISSN: 2958-5996

P-ISSN: 2958-5988

Molecular Analysis, De-Hairing Activity and Optimization of Protease Producing Bacteria Isolated from Soil

Nasir Bilal [1], Aamir Aziz [2], Muhammad Saleem [3], Rahman Wali [4]

[1] Sarhad Instititute of Allied Health Sciences , Sarhad University Peshawar Email: bilal.siahs@suit.edu.pk

[2,3,4] Sarhad University of Science and Information technology, Peshawar Email: aamir.biotech@suit.edu.pk msmalikpak11111@gmail.com Rehmanwali250@gmail.com

DOI: https://doi.org/10.63163/jpehss.v4i1.1102

Abstract:

The growing demand for environmentally friendly products has encouraged industries to replace chemical formulations with microbial alternatives. Among microbial enzymes, protease is one of the most commercially valuable due to its wide range of industrial applications. This study focused on the isolation, identification, and optimization of protease-producing bacteria from soil samples collected from a garden and a dairy farm at Agriculture University, Peshawar. Soil samples were serially diluted and cultured on nutrient agar. Screening for protease production was performed using skim milk agar, where clear zones indicated enzymatic activity. Out of six isolates obtained, four were from garden soil (HG-1, HG-2, HG-3, HG-4) and two from dairy soil (HD-1, HD-2). Soil pH was 8 in the garden and 7.5 in the dairy farm. Morphological and Gram staining results showed four Gram-positive and two Gram-negative isolates. The most potent protease producers were selected for molecular identification through 16S rRNA sequencing. HG-1 was identified as Acinetobacter baumannii, while HD-1 was confirmed as Pseudomonas aeruginosa. Crude enzyme extracts were partially purified and subjected to optimization studies. Maximum protease production was observed after 24 hours of incubation at pH 8 and 37 °C. The enzymes demonstrated effective feather degradation, achieving complete dehairing within seven days.

Introduction

Enzymes are biological catalysts that regulate and accelerate nearly all biochemical reactions in living systems. Structurally, they are mostly globular proteins whose activity depends on their specific three dimensional conformation determined by amino acid sequence (Xu et al., 2021). The term “enzyme” was introduced by Wilhelm Kühne in 1877, derived from the Greek word meaning “in yeast,” reflecting early observations of fermentation (Heckmann & Paradisi, 2020). Historically, enzymes have been used in food production such as bread, vinegar, and fermented beverages, long before their biochemical nature was understood (Kaur et al., 2019).

Proteases represent one of the most commercially significant enzyme groups, accounting for nearly 60 percent of total industrial enzyme consumption (Mamo & Assefa, 2018). These enzymes hydrolyze peptide bonds and participate in essential biological processes including cell signaling, apoptosis, and post translational modifications (Madzharova et al., 2019). Because of their broad substrate specificity and catalytic efficiency, proteases are widely applied in detergents, leather processing, pharmaceuticals, food industries, and waste management (Gurumallesh et al., 2019; Solanki et al., 2021).

Although proteases can be derived from plants and animals, microbial sources are preferred due to rapid growth, ease of genetic manipulation, and high yield production under controlled conditions (Toghueo & Boyom, 2021). In earlier decades, most enzymes were extracted from plant and animal tissues, but microbial biotechnology has since replaced these sources due to efficiency and scalability (Mahato et al., 2019). Among microorganisms, bacteria are the dominant producers of industrial proteases, particularly species of Bacillus, which secrete stable extracellular enzymes active across a wide pH and temperature range (Pham et al., 2021). Alkaline proteases, active between pH 7 and 10, are especially valuable in detergent and leather industries (Mustefa et al., 2021).

Other genera such as Pseudomonas, Proteus, and Acinetobacter have also demonstrated significant proteolytic activity, particularly in protein rich environments like tannery soils (Arora et al., 2020). Given the limited exploration of protease producing bacteria in District Peshawar, especially from dairy and garden soils, this region presents promising potential for isolating robust strains adapted to semi arid conditions. The diverse soil composition of the area may support metabolically versatile bacteria capable of efficient enzyme production.

Study Area and sample collection

This study was conducted in District Peshawar, Khyber Pakhtunkhwa. Twenty gram soil samples were collected from a depth of 9 cm from garden and dairy farm sites at Agriculture University, Peshawar. A soil corer was used for sampling, and the middle layer soil was aseptically transferred into sterile zip lock polythene bags using a sterile spatula. Soil temperature and pH were recorded at the time of collection. The samples were then transported to the Microbiology Laboratory, Institute of Allied and Health Sciences, Sarhad University, Peshawar, for further analysis.

Serial dilution of samples

Following soil collection, serial dilution was carried out to reduce microbial load. One gram of each soil sample was mixed with 10 mL of sterile distilled water and vortexed for five minutes to obtain a uniform suspension. From this mixture, 1 mL was transferred into a test tube containing 9 mL of sterile distilled water to prepare a tenfold dilution. The dilution process was repeated up to six fold. From the fifth and sixth dilution tubes, 0.1 mL of suspension was spread onto nutrient agar plates and incubated at 37 °C for 24 hours. After incubation, distinct colonies were observed. Well isolated colonies were selected using a sterile loop and sub-cultured onto nutrient agar slants to obtain pure cultures for further characterization (Benguenab & Chibani, 2021).

Screening of the isolated bacteria for protease activity

The isolated bacteria were tested for their ability to produce protease enzyme. The pure bacterial colonies were cultured on skim milk agar media. All the plates were incubated for 48 hours at 37 °C. After this, the plates were analyzed and the zones of hydrolysis were measured. A distinct zone encircling bacterial colonies demonstrates that protease is being produced (Asha and Palanisway, 2018).

Identification of the isolated protease producing bacteria

Further biochemical characterization was performed on the bacterial isolates that had the highest levels of protease activity with a clear zone. Gram staining and biochemical tests, such as catalase, citrate, urea hydrolysis, indole and oxidase test were performed (Hossain et al., 2021; Shekhawat et al., 2019).

Molecular characterization of the isolated protease producing bacteria

The isolates with highest enzyme activity were further selected for molecular characterization. Molecular identification was carried out through 16s rRNA sequencing. The

DNA of the bacterial isolates was extracted and sent to Korea Macrogen sequencing company. The sequenced 16s rRNA products were used to search the NCBI genebank database using the BLAST alignment search tool. The first ten sequences were selected based on their maximum identity score and aligned using the multiple alignment tool Clustal W (Shekhawat et al., 2019).

Phylogenetic analysis

After characterization, MEGAX software was used to plot the phylogenetic tree by comparing the identified bacterial isolates with other organisms showing resemblance (Cravalho et al., 2021).

Partial purification of proteases enzymes

A selected protease producing isolate was cultured in nutrient broth at 37 °C for 24 hours under shaking conditions. The culture was centrifuged to obtain the enzyme containing fraction. Proteins were precipitated using 70 percent ammonium sulphate, kept on ice for complete precipitation, and centrifuged again. The resulting pellet was dissolved in buffer, and enzyme activity was measured at 540 nm using a spectrophotometer. The final preparation was considered partially purified protease (Wajahat et al., 2022).

Optimization of several culture conditions for protease producing bacteria

Optimization of Incubation time

The isolated bacteria were inoculated in nutrient broth and subjected to incubated period of 24, 48 and 72 hours and the enzyme activity was assessed using spectrophotometer (Shekhawat et al., 2019).

Optimization of pH

The protease producing bacteria was inoculated in nutrient broth and their protease producing activity was assessed at pH 6, 7 and 8 using spectrophotometer (Shekhawat et al., 2019).

Optimization of Temperature

The protease producing activity of the isolated bacteria was assessed at various temperatures, including 30 °C, 37 °C and 45 °C (Shekhawat et al., 2019).

De-hairing activity of the isolated protease producing bacteria

Chicken feathers collected from a local slaughterhouse were washed, oven dried, and incubated at 37 °C with crude enzyme solution. Feathers treated with distilled water served as a control. De-hairing activity was assessed after 2, 4, and 7 days by gentle scraping (Akhter et al., 2020).

Results

Screening of the isolated bacteria for protease activity

Four bacterial isolates were obtained from garden soil and two from dairy farm soil. The recorded soil pH was 8 for the garden and 7.5 for the dairy farm. Garden isolates were designated HG-1, HG-2, HG-3, and HG-4, while dairy isolates were labeled HD-1 and HD-2.

On nutrient agar, HG-1 produced round, smooth, white colonies. HG-2, HG-3, and HG-4 formed smooth white colonies with rod shaped morphology. HD-1 showed opaque, flat colonies, whereas HD-2 produced rod shaped, golden yellow colonies. All six isolates exhibited clear zones of hydrolysis on skim milk agar, confirming protease production. The diameter of the proteolytic zones was measured in milli-meters to assess enzyme activity.

Table.1. Size of the proteolytic zones of the bacteria isolated from garden and dairy farm

S. No.	Bacterial isolate code	Proteolytic zone (mm)
1.	HG-1	16 mm
2.	HG-2	14 mm

3.	HG-3	14 mm
4.	HG-4	14 mm
5.	HD-1	15 mm
6.	HD-2	13 mm

Identification of the isolated protease producing bacteria

The microscopic features of all the six protease producing isolates from the selected habitats are listed in table 2. Out of the six protease producers, four were gram positive, while two of them were gram negative.

Table 2. Microscopic characterization of protease producers

Features	**HG-1**	**HG-2**	**HG-3**	**HG-4**	**HD-1**	**HD-2**
Cell shape	Coccobacillus	Rod	Rod	Rod	Rod	Spherical
Motility	Non-motile	Motile	Motile	Motile	Motile	Non-motile
Gram nature	Gram Negative	Gram positive	Gram positive	Gram positive	Gram Negative	Gram Positive

Table 3. Biochemical analysis of protease producing bacterial isolates

Biochemical Tests	**Isolated protease producing bacteria**					
	HG-1	**HG-2**	**HG-3**	**HG-4**	**HD-1**	**HD-2**
Catalase	-ve	+ve	+ve	+ve	+ve	+ve
Citrate	+ve	+ve	-ve	-ve	+ve	------
Urea hydrolysis	-ve	-ve	-ve	+ve	+ve	+ve
Indole	-ve	-ve	-ve	-ve	-ve	-ve
Oxidase	+ve	+ve	+ve	+ve	+ve	-ve
Similarity of bacteria	Acinetobacter sp.	Bacillus sp.	Bacillus sp.	Bacillus sp.	Pseudomonas sp.	Staphylococc sp.

Molecular characterization of the isolated protease producing bacteria

Among the above 6 bacterial isolates, 2 bacterial isolates showing highest proteolytic activity i-e., HG-1 and HD-1 were sent to Macrogen, Korea for sequencing. HG-1 was identified as Acinetobacter baumannii and HD-1 was identified as Pseudomonas aeruginosa. HG-1 showed

highest similarity with 10 different Acinetobacter species , while HD-1 showed highest similarity with 10 distinct Pseudomonas species.

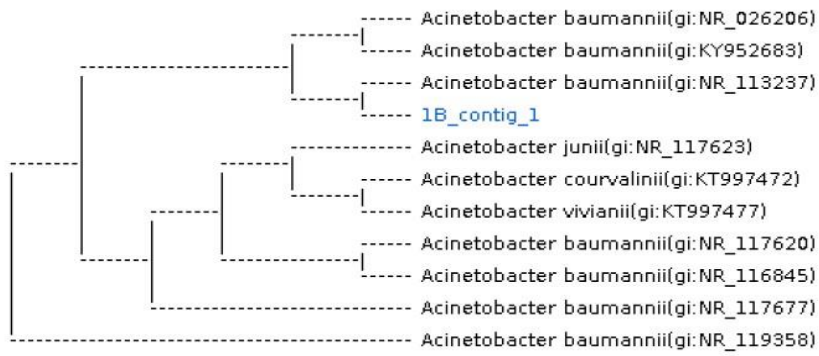

Figure 1. Phylogenetic tree showing the query sequences being identified as Acinetobacter baumannii.

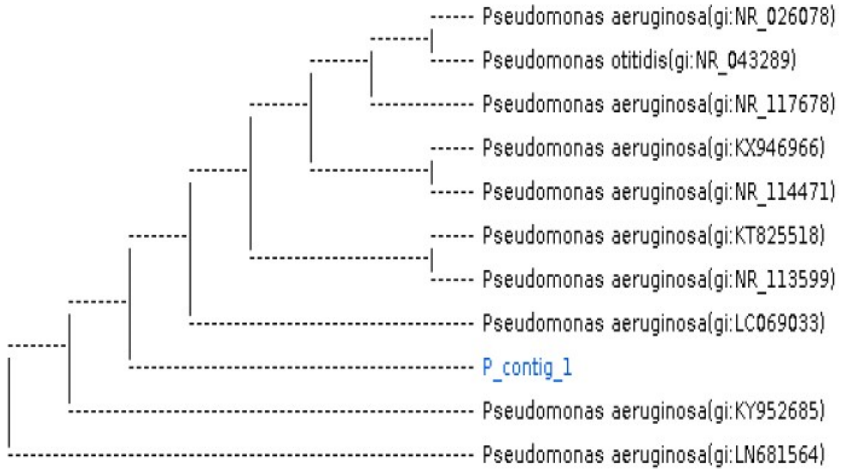

Figure 2. Phylogenetic tree showing the query sequences being identified as Pseudomonas aeruginosa.

Partial purification of proteases enzymes

The crude enzyme extract from HG-1 and HD-1 or P isolate were partially purified by adding 20 %, 50 % and 70 % of ammonium sulphate solution. Table 4.4 depicts the absorbance measured at OD 540 nm.

Table 4. Partial purification of proteases from HG-1 and HD-1/P isolate

Ammonium sulphate Precipitation	**Absorbance at 540 nm**	
	HG-1 (A. baumannii	**HD-1/P (P. aeruginosa)**
Crude enzyme	1.513	1.713
20 %	0.054	0.060

50 %	0.164	0.301
70 %	0.235	0.098

Optimization of several culture conditions for protease producing bacteria

The efficient protease producers i-e., Acinetobacter baumannii and Pseudomonas aeruginosa were selected based on secondary screening findings in order to optimize physiochemical conditions for maximum protease production.

Optimization of Incubation time

Protease producing A. baumannii and P. aeruginosa showed increased activity after 24 hours of incubation.

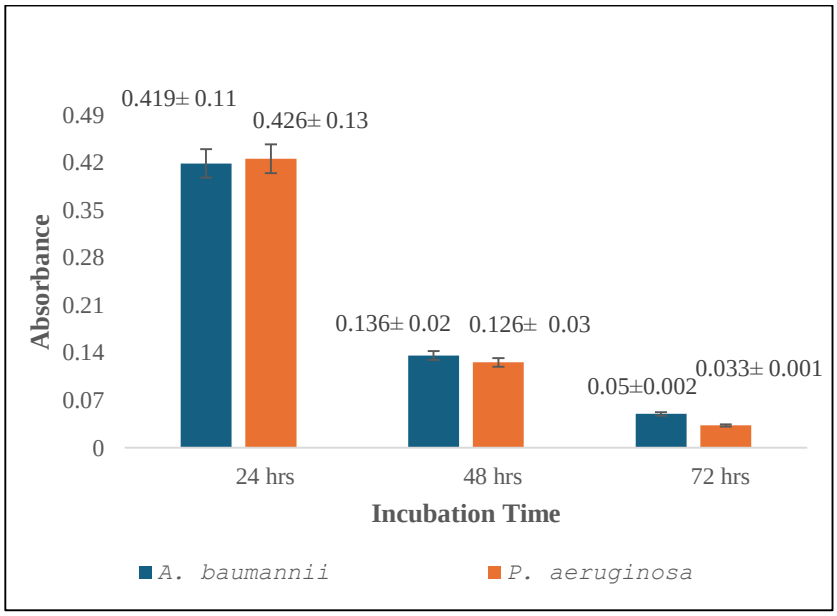

Fig 3. Optimization of incubation time for maximum production of proteases by A. baumannii (HG-1) and P. aeruginosa (HD-1/P)

Optimization of pH

The partially purified proteases of both the isolates was significantly active over a broad pH range from 6-9 having the maximum activity at pH 8. The enzyme activity began to diminish above this pH.

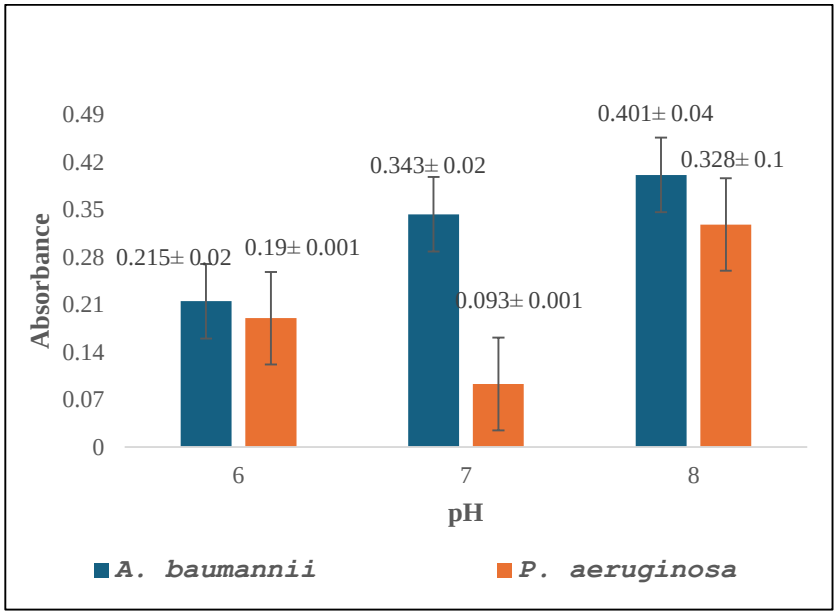

Fig 4 Optimization of pH for maximum production of proteases by A. baumannii (HG-1) and P. aeruginosa (HD-1/P)

Optimization of Temperature

The enzyme activity was evaluated at various temperatures. The optimum temperature for protease production by A. baumannii and P. aeruginosa was 37 °C. The protease production was decreased considerably when temperature increased above 40 °C, indicating that the enzyme was moderately thermophilic.

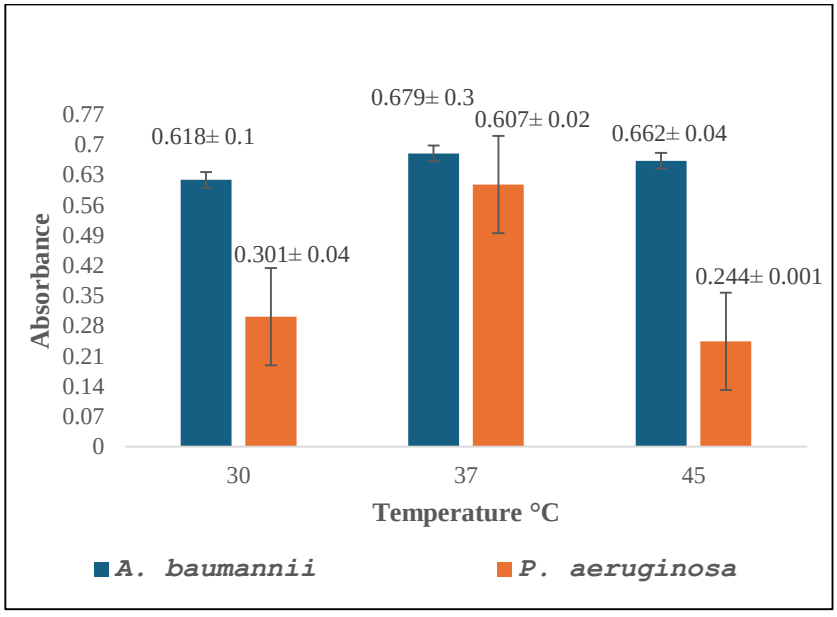

Fig 5. Optimization of temperature for maximum production of proteases by A. baumannii (HG-1) and P. aeruginosa (HD-1/P)

De-hairing activity of the isolated protease producing bacteria

Chicken feathers were treated with enzymes isolated from Pseudomonas aeruginosa and Acinetobacter baumannii. Chicken feathers treated with distilled water was used as a control. Complete dehairing of the feathers was achieved after 7 days of incubation. The feathers were easily removed as compared to the control when pulled with a forcep, which indicated positive

dehairing activity of the isolated proteases. In control, hair loosening was not observed, even by the mechanical action of a forceps.

Table 5. De-hairing activity of the isolated protease producing bacteria

S. No.	Isolates	Codes	Dehairing activity		
			After 2 days	After 4 days	After 7 days
1.	Acinetobacter baumannii	HG-1	Weak Dehairing	Moderate Dehairing	Complete Dehairing
2.	Pseudomonas aeruginosa	HD-1	Weak dehairing	Moderate dehairing	Complete Dehairing

Discussion

Microorganisms are major sources of industrial proteases due to their rapid growth, ease of cultivation, and high enzyme yield. Proteases account for nearly 60 percent of global enzyme consumption and are widely used in food, detergent, leather, and pharmaceutical industries (Mamo & Assefa, 2018; Toghueo & Boyom, 2021). Since soil is a rich reservoir of enzyme producing bacteria, this study aimed to isolate protease producers from dairy and garden soils. Six bacterial isolates exhibited clear proteolytic zones, with a maximum diameter of 16 mm. Similar findings have been reported from poultry farm and industrial soils (Seid, 2022; Sarwan, 2022). Both Gram positive and Gram negative protease producers were identified, consistent with earlier studies (Luang In et al., 2019; Lemenh et al., 2021). Molecular analysis identified the most active isolates as Acinetobacter baumannii (HG-1) and Pseudomonas aeruginosa (HD-1). Comparable reports have documented protease producing strains of Pseudomonas aeruginosa and Acinetobacter baumannii from diverse soil environments (Ali et al., 2022; Suresh et al., 2022). Optimization experiments demonstrated maximum enzyme production at pH 8 and 37 °C, aligning with previous studies highlighting alkaline conditions as optimal for protease synthesis (Rahman et al., 2018; Al-Dhabi et al., 2020). Temperature dependent enhancement of activity has also been reported for Pseudomonas species (Suberu et al., 2019). Given that feathers contain approximately 90 percent protein and are resistant to degradation, microbial proteases offer an eco-friendly solution for waste management. In this study, enzymes from Pseudomonas aeruginosa and Acinetobacter baumannii showed effective dehairing activity, supporting earlier findings on their potential for poultry waste bioconversion (Moonnee et al., 2021; Shaikh et al., 2023).

Conclusion

The current study revealed that among the six bacterial isolates studied, HG-1 identified as A. baumannii and HD-1 identified as P. aeruginosa exhibited the highest proteolytic activity. Partially purified proteases showed significant specific activities. The optimum temperature and pH for these proteases were 37 °C and 8, respectively. Furthermore, the isolated proteases exhibited promising dehairing activity when applied chicken feathers. After 7 days, complete dehairing was achieved, as evidenced by the ease of feather removal compared to the control. In

conclusion, the study highlights the potential of A. baumannii and P. aeruginosa as efficient protease producers with applications in dehairing industries.

References

Akhter, S., Iqbal, A., Khan, M. M., & Rashid, M. (2020). Assessment of dehairing activity of microbial proteases on poultry feathers. International Journal of Biological Macromolecules, 158, 213–221.

Ali, S., Khan, M. A., Rehman, I. U., & Akhtar, S. (2022). Molecular identification of protease-producing Pseudomonas aeruginosa from agricultural soil. BMC Microbiology, 22(1), 123–130.

Arora, S., Dutt, R., & Singh, S. (2020). Protease-producing bacteria from tannery waste soil and their enzymatic activity analysis. Environmental Science and Pollution Research International, 28(39), 54842–54851.

Benguenab, K., & Chibani, S. (2021). Isolation and screening of enzyme-producing bacteria from soil samples. Journal of Applied Biosciences, 153, 17115–17125.

Gurumallesh, P., Lakshmi, B. A., & Ramakrishnan, B. (2019). Systematic reconsideration of proteases: Classification and applications. International Journal of Biological Macromolecules, 128, 254–267.

Heckmann, T., & Paradisi, F. (2020). History and origin of the term “enzyme.” Catalysis Science & Technology, 10(7), 2782–2791.

Hossain, M. M., Rahman, M., & Hasan, M. M. (2021). Biochemical and molecular characterization of protease-producing soil bacteria. Journal of Microbiology and Biotechnology, 31(6), 847–856.

Kaur, P., Singh, J., & Kaur, A. (2019). Historical use of enzymes in food and beverage production. Food Science and Biotechnology, 28(3), 609–618.

Lemenh, Y. A., Biru, T. G., Chernet, A. Z., & Lema, F. B. (2023). Isolation and identification of protease-producing bacteria from sludge and sediment soil around Adama, Ethiopia. Indonesian Journal of Biotechnology, 14(2), 123–134.

Madzharova, E., Kastl, P., Sabino, F., & Auf dem Keller, U. (2019). Post-translational processing by proteases: Functional implications in cell biology. Molecular & Cellular Proteomics, 18(4), 663–676.

Mahato, K., Sharma, P., & Gupta, N. (2019). Shift from plant and animal to microbial enzyme production. Biotechnology Reports, 23, e00330.

Mamo, J., & Assefa, F. (2018). The role of microbial aspartic protease enzyme in food and beverage industries. Journal of Food Quality, 2018, Article 7957269.

Moonnee, P., Prasertsee, T., & Chaiyaso, T. (2021). Dehairing activity of bacterial proteases on poultry feathers. Journal of Applied Microbiology, 130(2), 420–430.

Rahman, M. H., Pranto, S. M., Das, M., Chakroborty, K., Marma, M., Alam, M. S., & Rafiquzzaman, S. M. (2025). Characterization of extracellular protease-producing bacteria isolated from the coastal environment of Bangladesh and optimization of parameters for enzyme production. Asia-Pacific Journal of Science and Technology, 30(06), APST-30.

Sarwan, S., Khan, M., & Malik, N. (2022). Isolation of protease producing bacteria from industrial soils. Journal of Industrial Microbiology & Biotechnology, 49(5), 789–798.

Seid, M. (2022). Protease producing bacterial isolates from poultry farm soil: Hydrolytic activity and enzyme characterization. International Journal of Biological Sciences, 18(4), 920–932.

Shekhawat, G., Thakur, P., & Dutt, S. (2019). Molecular characterization of protease producing soil bacteria by 16S rRNA sequencing. Archives of Microbiology, 201(8), 1105–1115.

Solanki, S., Gupta, N., & Kumar, R. (2021). Industrial applications of microbial proteases: A comprehensive review. Biocatalysis and Agricultural Biotechnology, 35, Article 102085.

Suberu, F., Adekunle, A., & Oladipo, O. (2019). Optimization of protease production from Pseudomonas aeruginosa and Bacillus subtilis. Journal of Enzyme Technology, 11(3), 154–165.

Toghueo, R. M. K., & Boyom, F. F. (2021). Microbial proteases: Production, applications, and bioengineering prospects. Journal of Industrial Microbiology & Biotechnology, 48(5–6), 1–13.

Wajahat, A., Ahmed, Z., & Raza, M. (2022). Partial purification and characterization of protease enzymes from soil bacteria. Journal of Enzyme Research, 27(2), 115–124.

Xu, J., Zhang, Y., & Wang, L. (2021). Structural and functional properties of enzymes. International Journal of Molecular Sciences, 22(4), 1955.