International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

Check for updates

Characterization of protease produced by *Lactobacillus plantarum* strain KCB4

Obiageli Comfort Makuachukwu * and Obichukwu Anselm Dike

Department of Applied Microbiology and Brewing, Enugu State University of Science and Technology, Agbani, P.M.B 01660, Enugu, Nigeria.

International Journal of Science and Research Archive, 2026, 20(01), 659–665

Publication history: Received on 10 June 2026; revised on 17 July 2026; accepted on 20 July 2026

Article DOI: https://doi.org/10.30574/ijsra.2026.20.1.1476

Abstract

Proteases are among the most important industrial enzymes owing to their extensive applications in the food, feed, pharmaceutical, detergent, leather, and biotechnological industries. The industrial value of these enzymes depends not only on high production yield but also on their biochemical properties and catalytic stability under different processing conditions. This study aimed to characterize the partially purified extracellular protease produced by *Lactiplantibacillus plantarum* KCB4 following optimization of fermentation conditions. The organism was cultivated under previously optimized conditions using banana peel and soybean waste as carbon and nitrogen sources, respectively. The crude enzyme was recovered, partially purified by 90% ammonium sulphate precipitation, desalted by dialysis, and assayed using casein as the substrate. The biochemical properties of the enzyme were evaluated by determining its optimum temperature and pH, thermal and pH stability, and hydrolytic activity on soybean meal. The protease exhibited maximum activity of 8.1 U/mL at 37 °C and retained appreciable activity up to 77 °C, indicating moderate thermostability. Optimum enzyme activity was recorded at pH 8 (6.5 U/mL), while maximum pH stability was observed at pH 7, with the enzyme retaining high residual activity between pH 7 and 9. The protease also demonstrated strong hydrolytic activity on soybean meal, recording 31.6 U/mL, indicating efficient degradation of soybean proteins. These findings demonstrate that the extracellular protease produced by *Lactiplantibacillus plantarum* KCB4 possesses desirable catalytic properties, broad operational stability, and considerable hydrolytic potential, making it a promising candidate for applications in food processing, feed improvement, and other industrial biotechnological processes.

Keywords: *Lactiplantibacillus plantarum* KCB4; Protease; Enzyme characterization; Thermostability; pH stability; Hydrolytic activity; Soybean meal

1. Introduction

Proteases (EC 3.4) are hydrolytic enzymes that catalyze the cleavage of peptide bonds in proteins and constitute the largest segment of the global industrial enzyme market because of their extensive applications in the food, dairy, detergent, leather, pharmaceutical, textile, feed, and environmental industries (Adetunji *et al*., 2023). Microbial proteases are preferred over plant- and animal-derived enzymes owing to their high catalytic efficiency, broad substrate specificity, ease of genetic manipulation, rapid microbial growth, and cost-effective large-scale production through fermentation. Their remarkable catalytic versatility, biochemical diversity, and adaptability to a wide range of industrial conditions have established microbial proteases as indispensable biocatalysts for sustainable industrial biotechnology, waste valorization, and the production of value-added biomolecules (Pessela *et al*., 2023).

Lactic acid bacteria (LAB), particularly *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), possess sophisticated proteolytic systems comprising cell-envelope proteinases, peptide transporters, and intracellular peptidases that hydrolyze proteins into peptides and amino acids required for growth and metabolism. Beyond supplying nitrogen for cellular growth, these proteolytic enzymes contribute significantly to flavor development,

* Corresponding author: Makuachukwu Comfort Obiageli

texture improvement, enhanced protein digestibility, and the production of bioactive peptides with antimicrobial, antioxidant, antihypertensive, and immunomodulatory properties in fermented foods (Demirci *et al*., 2023). Moreover, the proteolytic capacity of *L. plantarum* is highly strain dependent because of differences in proteinase genes, peptidase composition, regulatory pathways, and enzyme expression, making strain-specific characterization essential before industrial exploitation (Kieliszek *et al*., 2021).

Although optimization of fermentation conditions can substantially increase enzyme yield, production efficiency alone does not determine industrial applicability. The commercial value of a protease depends primarily on its biochemical characteristics, including optimum pH and temperature, catalytic stability, substrate specificity, kinetic properties, and tolerance to metal ions, inhibitors, surfactants, oxidizing agents, and organic solvents. These physicochemical properties govern enzyme performance under industrial processing conditions and provide the basis for selecting enzymes for applications in food processing, detergents, pharmaceuticals, leather processing, and environmental biotechnology (Kieliszek *et al*., 2021).

In our previous study, fermentation conditions for protease production by *Lactiplantibacillus plantarum* KCB4 were successfully optimized. However, the biochemical properties of the extracellular protease remain unknown. Since enzyme characteristics are critical determinants of industrial applicability, the present study aimed to characterize the partially purified protease by evaluating its physicochemical and catalytic properties to determine its potential for industrial and biotechnological applications (Song *et al*., 2023; Kieliszek *et al*., 2021).

2. Materials and methods

2.1. Source of test organisms

The proteolytic test organism was obtained from stock cultures previously isolated and characterized by Makuachukwu and George-Okafor. (2025).

2.2. Post Optimization of Protease Production

Post-optimization protease production by *Lactobacillus plantarum* KCB4 was carried out under the optimized fermentation conditions established in the preceding experiments. The production medium consisted of modified MRS broth supplemented with 2.5% (w/v) banana peel as the carbon source, 2.5% (w/v) soybean waste as the nitrogen source, 1% (w/v) skim milk, 0.5% (w/v) sodium acetate trihydrate, 0.2% (w/v) K_2HPO_4, 0.02% (w/v) magnesium citrate, and 0.0005% (w/v) $MgSO_4 \cdot 7H_2O$. The initial pH of the medium was adjusted to 4.5 before sterilization at 121°C for 15 min. Thereafter, each flask was inoculated with 5% (v/v) of a 24-h-old culture of *L. plantarum* KCB4 and incubated at 40°C on a rotary shaker (120 rpm) for the optimized fermentation period of 48 h. At the end of fermentation, the culture broth was centrifuged at 10,000 rpm for 20 min at 4°C, and the cell-free supernatant was collected as the crude protease for subsequent purification and characterization. This procedure was adapted from previously reported optimization studies on *Lactobacillus plantarum* protease production.

2.3. Ammonium Sulphate Precipitation of the Crude

The recovered crude enzyme was first precipitated with 90% saturated ammonium sulphate at 4°C for 15min. Thereafter, the precipitate was recovered by centrifugation at 10,000 rpm for 20 min at 4°C and reconstituted with 100 ml of 0.2M Phosphate buffer (pH 6.5). Then the crude enzyme solution was desalted against the same 5M of buffer via dialysis with dialysis tubing (3.5 k MWCO). The final concentrated volume of 40 ml was maintained at 4 °C.

2.4. Enzyme Activity Assay

A method by Cupp-Enyard, (2008) was adopted. The reaction mixture which consisted of 1 ml of 1% casein in 0.2M phosphate buffer (pH 8) and 1 ml of the enzyme solution was incubated at 30 °C for 30 min. The reaction mixture was stopped by the addition of 2 ml of 5% (w/v) of trichloroacetic acid (TCA). The reaction mixture was filtered with Whatman No. 1 filter paper and the filtrate collected. Thereafter, 5 ml of 0.5M Na_2CO_3 solution was added to 1 ml of the filtrate, followed by the addition of 0.5 ml of 3-fold diluted folin-ciocalteau reagent. The mixture was allowed to stand at room temperature for 30 min prior to absorbance reading at 660 nm with Spectronic-21 spectrophotometer. A blank tube which contained no enzyme served as the control. One unit (U) of the protease was defined as the amount of protease that released 1 mg of tyrosine from casein per minute under the applied condition. The two best isolates with highest protease production were selected for optimization study.

2.5. Characterization of Protease Enzyme from *Lactobacillus* spp.

The partially purified proteases were characterized as described below:

2.5.1. Effect of Temperature on Protease Activity and Stability

The effect of temperature on the produced protease activity was determined by incubating equal volume (1 ml) of the enzyme and 1% casein solution in phosphate buffer (pH 6.5). The enzyme mixture was incubated at varying range of temperature of 17 to 77 °C. After incubation for 30 min, the protease activity was assayed as earlier described.

Thermostability of the enzyme was determined by pre-incubating each enzyme for 30 min at different temperature range of 17 to 77 °C. The exposed enzyme was re-incubated with equal volume of the substrate at pre-determined optimal temperature for 30 min. Thereafter, activity of the enzyme was assayed as earlier described. Residual activity was then expressed as a percentage using the formula below:

$$\text{Residual Activity (\%)} = \frac{\text{Enzyme activity after treatment}}{\text{Enzyme activity of untreated control}} \times 100$$

2.5.2. Effect of pH on Enzyme Activity and Stability

The method of Sarker *et al*. (2013) was adopted. The effect of pH on enzyme activity was determined by incubating the enzyme with 1 ml of 1% casein of buffers at different pH (3-12). The casein was prepared with citrate buffer for pH 3-6, phosphate buffer for pH 7-8 and Glycine-NaOH for pH 9-12 for 30 min at 37 °C.

The pH stability of the enzyme was determined by pre-incubating the protease with each 0.2 M of citrate, phosphate, Glycine-NaOH buffers of pH of 3 to 12 and residual activity was evaluated as described earlier.

2.5.3. Protease Hydrolytic Activity Assay on Soybean

The hydrolytic potential of the produced enzyme on soybean substrate (one of the major components of the poultry feed) was assessed. A method by Cupp-Enyard (2008) was adopted. The reaction mixture which consisted of 1 ml of 1% Soya bean powder in 0.2M phosphate buffer (pH 8) and 1 ml of the enzyme solution was incubated at 30°C for 10 min. The hydrolytic activity was determined as earlier stated.

3. Results

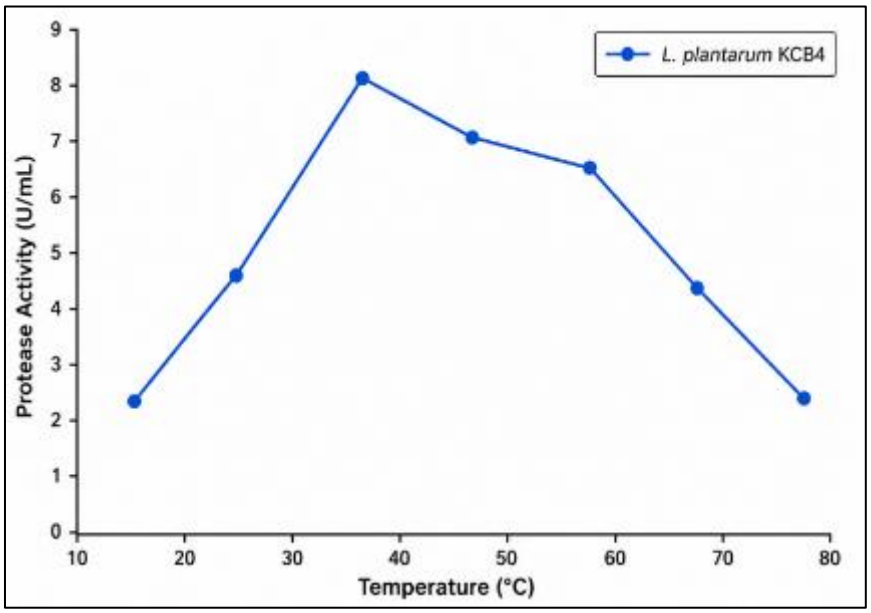

Figure 1 Temperature Activity Profile of the Protease from *L. plantarum* KCB4

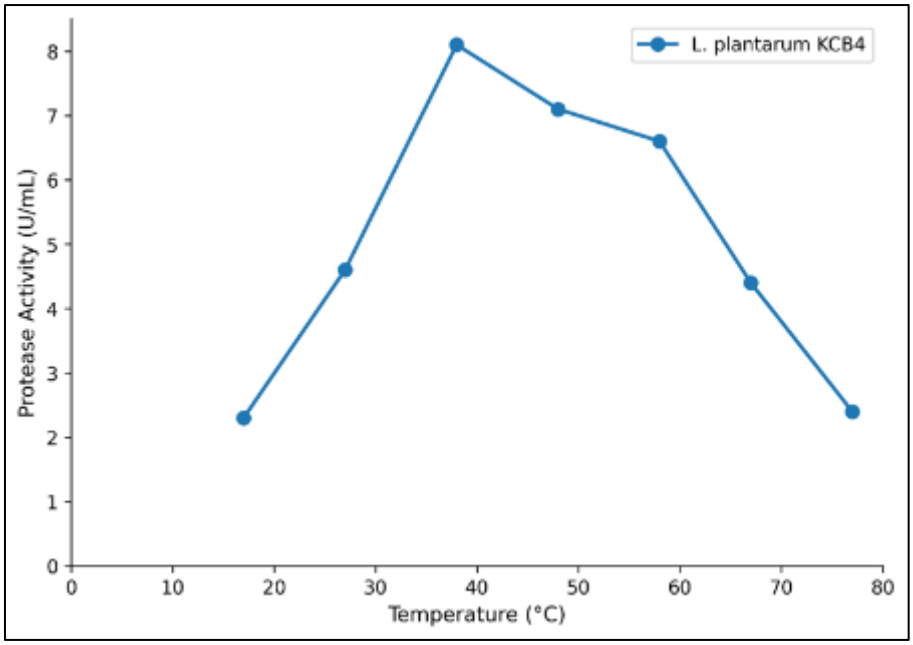

Figure 2 Thermostability Activity of Protease from *L. plantarum* KCB4

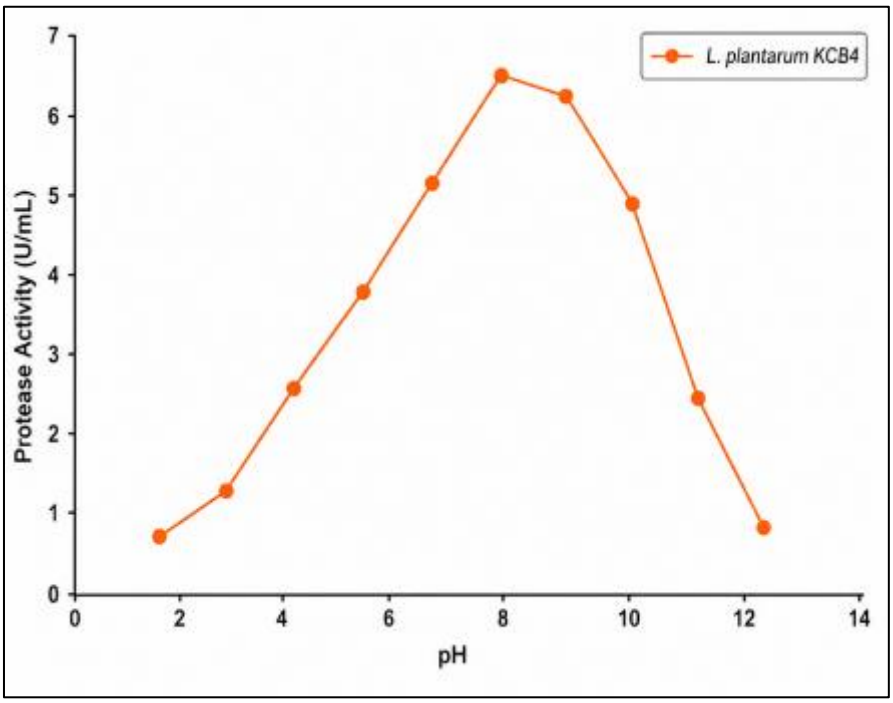

Figure 3 pH Profile of the Protease Activity of *L. plantarum* KCB4

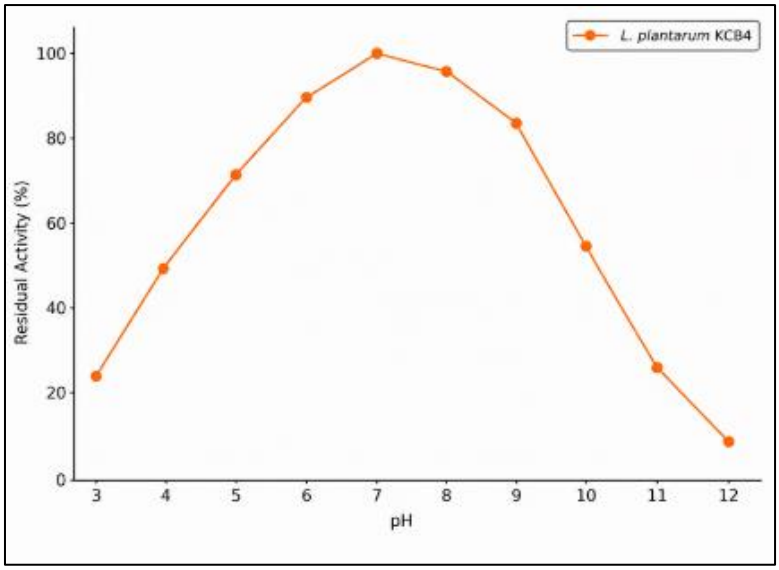

Figure 4 pH stability of Protease Activity of *L. plantarum* KCB4

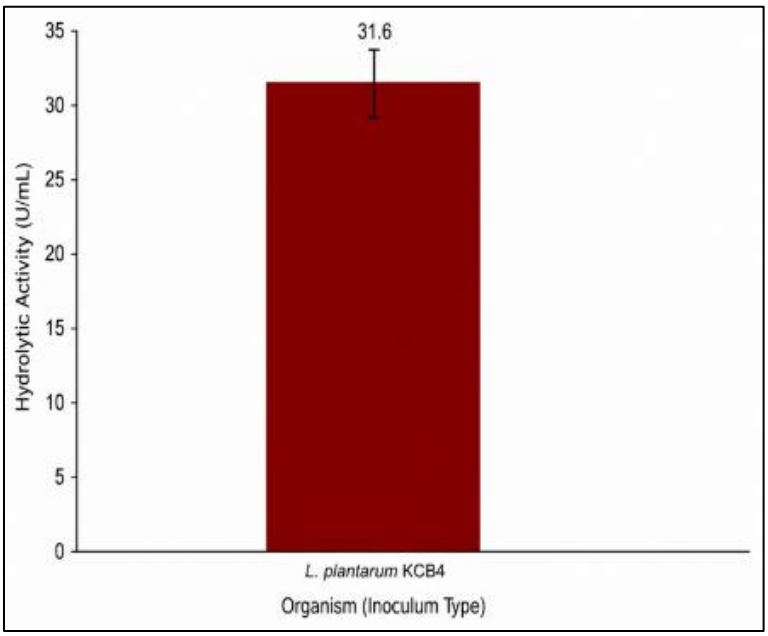

Figure 5 Hydrolytic Activity of Protease on Soyabean Meal

4. Discussion

Figure 1 shows the protease activity of *Lactiplantibacillus plantarum* KCB4 increased progressively with temperature, rising from 2.3 U/mL at 17 °C to a maximum of 8.1 U/mL at 37 °C, after which it declined gradually to 2.4 U/mL at 77 °C. The optimum activity recorded at 38 °C indicates that the enzyme exhibits maximum catalytic efficiency under mesophilic conditions, which is consistent with reports that proteases from lactic acid bacteria generally display optimal activity between 35 and 40 °C (Sun *et al.*, 2021; Solieri and Gullo, 2022). The increase in activity up to the optimum temperature reflects enhanced molecular collisions and favorable enzyme–substrate interactions, whereas the subsequent decline at higher temperatures is most likely due to progressive thermal denaturation and reduced conformational stability of the enzyme. Nevertheless, the enzyme retained appreciable activity up to 57 °C (6.6 U/mL), indicating moderate thermal stability that may be advantageous for industrial processes requiring elevated operating temperatures. Similar thermal behavior has been reported for proteases from *L. plantarum*, although the exact optimum

temperature varies among strains because of differences in enzyme structure and physicochemical properties (Papadimitriou *et al*., 2024).

The protease produced by *Lactiplantibacillus plantarum* KCB4 exhibited good thermostability within the mesophilic temperature range, retaining high catalytic activity up to 37–47 °C, with a maximum activity of 8.1 U/mL at 37 °C (Figure 2). Thereafter, enzyme activity declined gradually to 7.1 U/mL at 47 °C, 6.6 U/mL at 58 °C, 4.4 U/mL at 67 °C, and 2.4 U/mL at 77 °C, indicating progressive thermal inactivation at elevated temperatures. This pattern agrees with previous reports that proteases from lactic acid bacteria generally exhibit maximum thermal stability and catalytic efficiency between 35 and 50 °C, followed by reduced activity as enzyme structure becomes destabilized by heat (Kieliszek *et al*., 2021; Solieri and Gullo, 2022). Although activity decreased above 57 °C, the enzyme retained appreciable activity within the 50–60 °C range, suggesting moderate thermostability that is suitable for many food fermentation and biotechnological applications. Similar strain-dependent differences in thermal stability have been reported for *L. plantarum* proteases, with variations attributed to differences in enzyme conformation and structural resilience (Demirci *et al*., 2023).

Protease activity of *Lactiplantibacillus plantarum* KCB4 was strongly influenced by pH, increasing progressively from 0.7 U/mL at pH 2 to a maximum of 6.5 U/mL at pH 8, after which activity declined gradually to 0.8 U/mL at pH 13 (figure 3). High enzyme activity was maintained between pH 7 and 9, indicating that the protease functions optimally under neutral to mildly alkaline conditions. This observation is consistent with previous reports that proteases from *Lactiplantibacillus plantarum* and other lactic acid bacteria frequently exhibit maximum catalytic activity within the neutral to slightly alkaline pH range, depending on the strain and enzyme type (Demirci *et al*., 2023). The marked reduction in activity under highly alkaline conditions is likely due to disruption of the enzyme's tertiary structure and changes in the ionization state of catalytic amino acid residues, leading to reduced substrate binding and catalytic efficiency (Kieliszek *et al*., 2021; Solieri and Gullo, 2022). Although some *L. plantarum* proteases have been reported to exhibit acidic pH optima, particularly those associated with dairy fermentations (Sun *et al*., 2021), the present findings demonstrate that the extracellular protease produced by *Lactiplantibacillus plantarum* KCB4 is most active under mildly alkaline conditions, highlighting its potential suitability for industrial processes operating near neutral or alkaline pH.

The protease produced by *Lactiplantibacillus plantarum* KCB4 exhibited maximum stability at pH 7, retaining 100% residual activity, after which stability declined gradually with increasing alkalinity (figure 4). Residual activity increased from 24% at pH 3 to 90% at pH 6, remained high between pH 7 and 9 (84–100%), and then decreased markedly to 55% at pH 10, 26% at pH 11, and 9% at pH 12. This indicates that the enzyme is highly stable under neutral to slightly alkaline conditions but becomes progressively destabilized under strongly acidic and highly alkaline environments. Similar pH stability profiles have been reported for proteases from lactic acid bacteria, which generally retain maximum stability around neutral pH before losing activity due to structural alterations and changes in the ionization state of catalytic residues under extreme pH conditions (Kieliszek *et al*., 2021; Solieri and Gullo, 2022). Although some *Lactiplantibacillus plantarum* proteases have been reported to maintain good stability under mildly alkaline conditions (Shu *et al*., 2021), others exhibit greater stability in acidic environments depending on strain origin and enzyme characteristics (Sun *et al*., 2021). The present findings demonstrate that the extracellular protease from *Lactiplantibacillus plantarum* KCB4 possesses broad pH stability, particularly between pH 6 and 9, making it a promising candidate for industrial applications operating under neutral to moderately alkaline conditions.

Figure 5 shows that the protease produced by *Lactiplantibacillus plantarum* KCB4 exhibited a high hydrolytic activity of 31.6 U/mL on soybean meal, demonstrating its strong ability to degrade soybean proteins. This high proteolytic activity suggests that the enzyme can efficiently hydrolyze complex storage proteins into smaller peptides and amino acids, thereby improving protein availability and digestibility. Such enzymatic modification of soybean meal is particularly valuable in animal nutrition because it enhances nutrient utilization while reducing anti-nutritional protein components. Similar studies have shown that enzymatic or microbial hydrolysis of soybean meal improves its nutritional quality and feeding value in poultry and other livestock (Irawan *et al*., 2022; Zheng *et al*., 2023). The high hydrolytic activity observed in the present study therefore indicates that the extracellular protease from *Lactiplantibacillus plantarum* KCB4 possesses considerable potential for the bioprocessing and biological pretreatment of soybean meal for feed and other industrial applications.

5. Conclusion

The partially purified protease produced by *Lactiplantibacillus plantarum* KCB4 exhibited desirable biochemical characteristics, including optimum activity under mesophilic temperature and mildly alkaline pH conditions, with good thermal and pH stability over a broad operational range. The enzyme also demonstrated strong hydrolytic activity

against soybean meal, highlighting its efficiency in protein degradation and its potential to improve the nutritional quality of feed ingredients.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

References

[1] Adetunji, A. I., Olaitan, M. O., Erasmus, M., and Olaniran, A. O. (2023). Microbial proteases: A next generation green catalyst for industrial, environmental and biomedical sustainability. *Food Materials Research, 3*, Article 12. https://doi.org/10.48130/FMR-2023-0012

[2] Cupp-Enyard, C. (2008). Sigma's non-specific protease activity assay—Casein as a substrate. *Journal of Visualized Experiments, 19*, e899. https://doi.org/10.3791/899

[3] Demirci, T., Akpınar, O., and colleagues. (2023). Proteolytic activities and bioactive peptide production by Lactiplantibacillus plantarum strains during food fermentation. *Current Research in Food Science, 7*, 100620. https://doi.org/10.1016/j.crfs.2023.100620

[4] Irawan, A., Jayanegara, A., Yulianti, D., and Wiryawan, K. G. (2022). Protease-producing lactic acid bacteria with antibacterial properties and their potential use in soybean meal fermentation. *Chemical and Biological Technologies in Agriculture, 9*, Article 40. https://doi.org/10.1186/s40538-022-00303-5

[5] Kieliszek, M., Pobiega, K., Piwowarek, K., and Kot, A. M. (2021). Characteristics of the proteolytic enzymes produced by lactic acid bacteria. *Molecules, 26*(7), 1858. https://doi.org/10.3390/molecules26071858

[6] Makuachukwu, O. C., and George-Okafor, U. O. (2025). Isolation and characterization of proteolytic bacteria strains from poultry-waste contaminated soil. *World Journal of Advanced Research and Reviews, 26*(3), Article 2297. https://doi.org/10.30574/wjarr.2025.26.3.2297

[7] Pessela, B. C., Noma-Okamoto, D., and Cabral, H. (2023). Editorial: Microbial proteases: Biochemical studies, immobilization and biotechnological application. *Frontiers in Microbiology, 14*, 1126989. https://doi.org/10.3389/fmicb.2023.1126989

[8] Sarker, P. K., Talukdar, S. A., Deb, P., Sayem, S. M. A., and Mohsina, K. (2013). Optimization and partial characterization of culture conditions for the production of alkaline protease from *Bacillus licheniformis*. *Bangladesh Pharmaceutical Journal, 16*(2), 129–136.

[9] Solieri, L., Sola, L., Vaccalluzzo, A., Randazzo, C. L., Martini, S., and Tagliazucchi, D. (2022). Characterization of cell-envelope proteinases from two *Lacticaseibacillus casei* strains isolated from Parmigiano Reggiano cheese. *Biology, 11*(1), 139. https://doi.org/10.3390/biology11010139

[10] Shu, G., Huang, J., Chen, L., Lei, N. and Chen, H. (2018). *Characterization of Goat Milk Hydrolyzed by Cell Envelope Proteinases from Lactobacillus plantarum LP69: Proteolytic System Optimization, Bioactivity, and Storage Stability Evaluation.* Molecules, 23(6), 1317. https://doi.org/10.3390/molecules23061317

[11] Song, P., Zhang, X., Wang, S., Xu, W., Wang, F., Fu, R., and Wei, F. (2023). Microbial proteases and their applications. *Frontiers in Microbiology, 14*, 1236368. https://doi.org/10.3389/fmicb.2023.1236368

[12] Sun, F., Tao, R., Liu, Q., Wang, H., and Kong, B. (2021). Effects of temperature and pH on the structure of a metalloprotease from *Lactobacillus fermentum* R6 isolated from Harbin dry sausages and molecular docking between protease and meat protein. *Journal of the Science of Food and Agriculture, 101*(12), 5016–5027. https://doi.org/10.1002/jsfa.11146

[13] Zheng, H., Wang, L., Yu, H., Wang, W., Wu, G., and Qin, G. (2023). Protease-producing lactic acid bacteria with antibacterial properties and their potential use in soybean meal fermentation. *Chemical and Biological Technologies in Agriculture, 9*, Article 40. https://doi.org/10.1186/s40538-022-00303-