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(RESEARCH ARTICLE)

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Optimization of fermentation parameters for enhanced protease production by *Lactobacillus plantarum* Strain KCB4

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Abstract

Proteases are among the most commercially important industrial enzymes owing to their extensive applications in the food, pharmaceutical, detergent, leather, and biotechnological industries. The present study investigated the optimization of fermentation parameters for enhanced protease production by *Lactobacillus plantarum* KCB4, a proteolytic isolate previously recovered and characterized from poultry waste-contaminated soil. Preliminary protease production was carried out by submerged fermentation using modified MRS broth, after which the crude enzyme was partially purified by ammonium sulphate precipitation and dialysis. Protease activity was determined using the casein hydrolysis method, while a one-factor-at-a-time (OFAT) approach was employed to optimize the effects of initial pH, temperature, carbon source and concentration, nitrogen source and concentration, inoculum size, and fermentation period. Pre-optimization studies showed that *L. plantarum* KCB4 produced 4.754 U/mL of protease. The optimum conditions for enhanced protease production were pH 4.5, 40°C, 2.5% (w/v) banana peel as the carbon source, 2.5% (w/v) soybean waste as the nitrogen source, 5% inoculum concentration, and 48 h fermentation period. Under these optimized conditions, protease production increased from 4.80 U/mL to 5.80 U/mL, representing an approximately 21% improvement in enzyme yield. The results demonstrate that systematic optimization of fermentation variables significantly enhances protease production by *L. plantarum* KCB4 and highlight the potential of utilizing inexpensive agro-industrial residues such as banana peel and soybean waste as sustainable substrates for cost-effective enzyme production. These findings support the industrial application of *L. plantarum* KCB4 as a promising microbial source of protease for food and other biotechnological processes.

Keywords: *Lactobacillus plantarum* KCB4; Protease; Fermentation optimization; Agro-industrial residues; Submerged fermentation; Enzyme production

1. Introduction

Proteases (EC 3.4) are hydrolytic enzymes that catalyze the cleavage of peptide bonds in proteins and represent the largest segment of the global industrial enzyme market because of their broad applicability across numerous biotechnological sectors (Pessela *et al.,* 2023). Microbial proteases are preferred over plant- and animal-derived counterparts due to their rapid production, high catalytic efficiency, broad substrate specificity, ease of strain improvement, and suitability for economical large-scale fermentation. These enzymes are extensively utilized in food processing, dairy fermentation, detergent formulation, leather processing, pharmaceuticals, textile manufacturing, waste management, environmental remediation, and peptide synthesis, making them indispensable biocatalysts in modern industries (Song *et al*., 2023).

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Lactobacillus plantarum is a Gram-positive, facultatively heterofermentative lactic acid bacterium widely distributed in fermented foods and the gastrointestinal tract, where it contributes to food preservation, probiotic functionality, and nutritional enhancement. Owing to its Generally Recognized as Safe (GRAS) status and remarkable metabolic versatility, *L. plantarum* has become an important microorganism in food biotechnology (Song *et al*., 2023). The organism possesses an elaborate proteolytic system comprising cell-envelope proteinases, peptide transporters, and intracellular peptidases that facilitate protein degradation into peptides and amino acids essential for growth while simultaneously generating bioactive peptides that improve food quality and confer health-promoting properties. Consequently, *L. plantarum* has attracted increasing attention as a safe and efficient microbial cell factory for enzyme production and the development of functional fermented foods (Savijoki *et al*., 2006).

Protease biosynthesis by microorganisms is strongly influenced by nutritional and environmental conditions, including the composition of carbon and nitrogen sources, medium pH, incubation temperature, inoculum size, aeration, agitation rate, and fermentation duration. These factors regulate microbial growth, metabolic activity, gene expression, and enzyme secretion, ultimately determining enzyme productivity (Savijoki *et al*., 2006). Consequently, optimization of fermentation conditions is an essential strategy for maximizing protease yield, reducing production costs, and improving the feasibility of industrial-scale enzyme manufacture. Recent studies have demonstrated that systematic optimization of fermentation parameters, using either conventional one-factor-at-a-time approaches or statistical optimization techniques, can substantially enhance protease production by microbial strains. However, because optimal culture conditions are highly strain-dependent, each promising isolate requires individual optimization to realize its full biotechnological potential. Therefore, the present study was undertaken to optimize the fermentation parameters for enhanced protease production by *Lactobacillus plantarum* KCB4 (Sun *et al*., 2023).

2. Materials and methods

2.1. Source of test organisms

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

2.2. Pre-Optimization Production of Protease by *Lactobacillus* spp.

Production of protease from probiotic *Lactobacillus* spp. was carried out in MRS medium (w/v) containing glucose, (2%); beef extract (1%), skim milk (1%); sodium acetate trihydrate (0.5%); $MgSO_4 \cdot 7H_2O$, (0.0005%) and K_2HPO_4, (0.2%) and magnesium citrate (0.02%). The initial pH of the medium was adjusted to 5.0 with phosphate buffer, autoclaved at 121°C for 15 min and inoculated with 5% (v/v) of 24 h old *Lactobacillus* spp. isolates. Shake-flasks fermentation was at 37°C with agitation of 150 rpm for 72 h. After the complete fermentation, the broth was centrifuged at 10000 rpm 4°C and recovered clear supernatant was used as the crude protease.

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. Optimization of Culture Conditions for Maximum Productivity

One factor at a time procedure was adopted for optimization study. The condition parameters that were tested were pH, temperature, nitrogen sources and carbon sources, inoculum concentration, and fermentation period.

2.5.1. Effect of pH on Optimal Protease Prodution

The effect of pH on protease production by *Lactobacillus* spp. was determined using formulated MRS medium containing (w/v): glucose, (2%); beef extract (1%), skimmed milk (1%); sodium acetate trihydrate (0.5%); $MgSO_4 \cdot 7H_2O$, (0.0005%) and K_2HPO_4, (0.2%), magnesium citrate (0.02%). The pH of the medium was adjusted to different pH values 3.5, 4.5, 5.5, 6.5, with citrate buffer 7.5, and 8.5 with phosphate buffer prior to sterilization at 121°C for 15 min. Each 100 mL portion of the fermentation medium in Erlenmeyer flasks was inoculated with 5% (v/v) of 24 h *Lactobacillus* spp. strain. The inoculated flasks were incubated in a rotary shaker incubator at 37°C with agitation at 120 rpm for 72 h. Fermentation recovery and enzyme activity were carried out as earlier described.

2.5.2. Effect of Temperature on Optimal Protease Production

The inoculation, fermentation and recovery were carried out as earlier described but incubation was at different temperatures (30, 35, 40, 45, 50, and 55 °C). The temperature at which the optimal protease activity was achieved was considered as the optimum and utilized for subsequent optimization studies.

2.5.3. Carbon Source Profile

The influence of different carbon sources on protease production on the protease was determined as earlier described. The variance was that glucose (2%) in the fermentation medium was substituted respectively with local agrowastes (corn waste, banana peels, potato peels, rice bran, yam peels) and the fermentation medium with glucose served as control.

2.5.4. Carbon Concentration Effect

To evaluate the effect of different carbon concentration on protease. The best Carbon source was prepared at different concentrations (%) of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 (w/v). The inoculation, fermentation and recovery were carried out as earlier described. The concentration with the best activity was considered optimal.

2.5.5. Determination of Nitrogen Source for Optimal Protease Production

The effect of different nitrogen sources on protease production was studied by replacing the nitrogen component (beef extract) of the medium respectively with groundnut cake, soybean waste, bean waste, eggshell, Bambara waste and beef extract used as control. Each nitrogen source was added respectively to the production medium at a concentration of 1% (w/v) of the previously optimized carbon source. Nitrogen source that stimulated maximum protease production was subsequently utilized throughout the optimization study.

2.5.6. Nitrogen Concentrations Profile

The effect of nitrogen concentration on protease production was studied by supplementing the fermentation broth with the optimal nitrogen source at respective concentrations of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0% (w/v). The concentration with the optimal enzyme output was chosen.

2.5.7. Protease Production at different Fermentation period

Fermentation broth for each isolate was formulated using optimal temperature, pH, carbon source and nitrogen sources. Each isolate was inoculated into the formulated fermentation medium and incubated at varying temperature of 12, 24, 36, 48, 60, 72, 84 and 96h at 37°C, with agitation at 120 rpm. Fermentation and recovery were carried out as earlier described.

2.5.8. Inoculum concentration Profile on Protease Production

The formulated fermentation medium containing selected carbon and nitrogen substrates was inoculated with varying concentration of each isolate (1-10ml). The incubation was at the determined optimal pH, temperature and time. The concentration of the isolate that supported maximum protease production was selected.

2.5.9. Post Optimization of Protease Production

Post optimization production of protease by probiotic *Lactobacillus* spp. was carried out under the optimal conditions, 2.5% banana peels (Carbon source), 1% soya beans waste (Nitrogen source), pH 4.5, 5% inoculum size at 40^0C. The inoculation, fermentation and recovery were carried out as earlier described.

3. Results

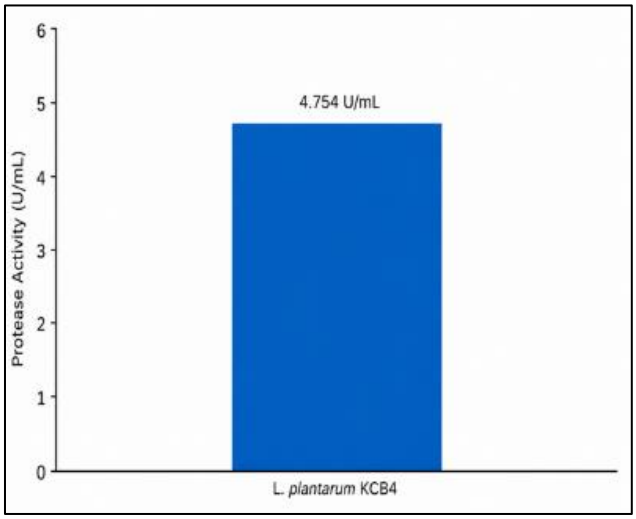

Figure 1 Pre-optimization Protease Production by *L. plantarum* KCB4

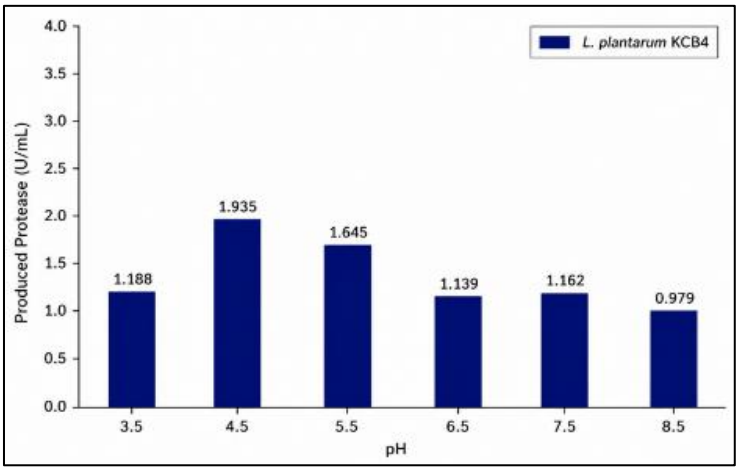

Figure 2 Optimal Protease Production at Different pH by *L. plantarum* KCB4

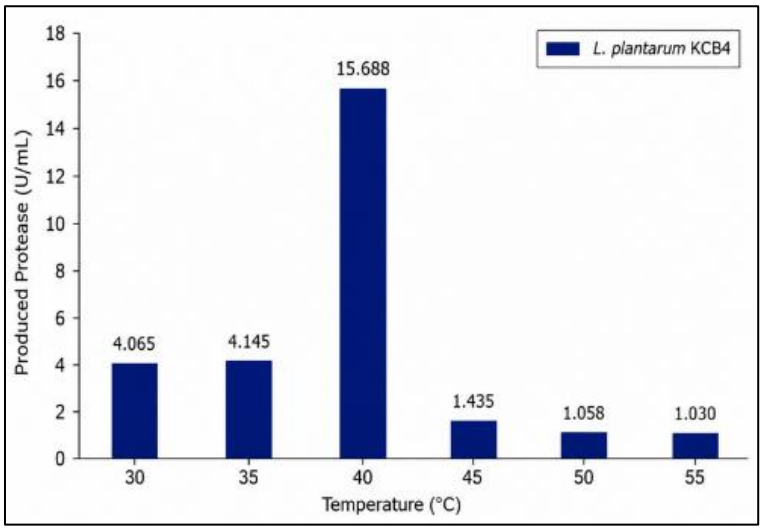

Figure 3 Optimal Protease Production at Different Temperature by *L. plantarum* strain KCB4

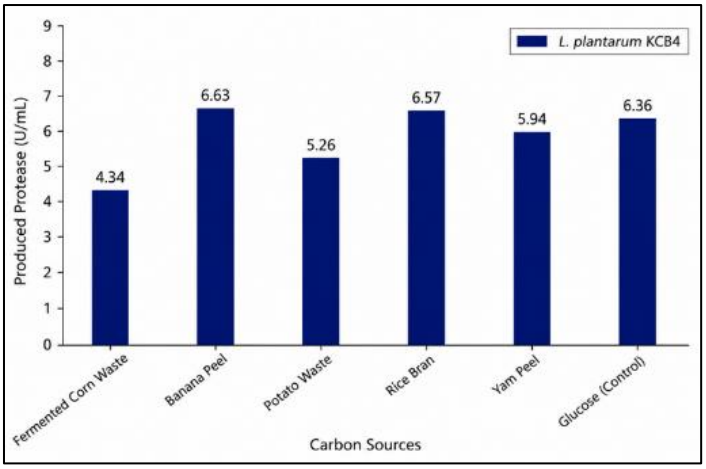

Figure 4 Effect of Carbon Sources at Optimal Protease Production by *L. plantarum* strain KCB4

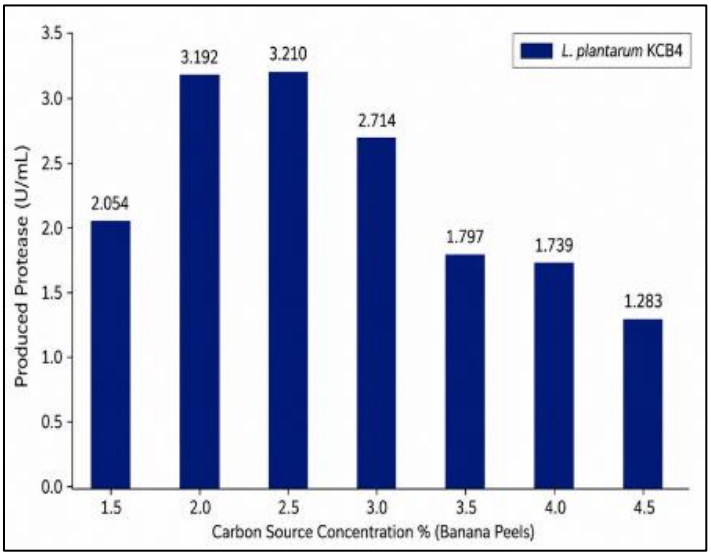

Figure 5 Carbon Concentration Profile on Protease Production

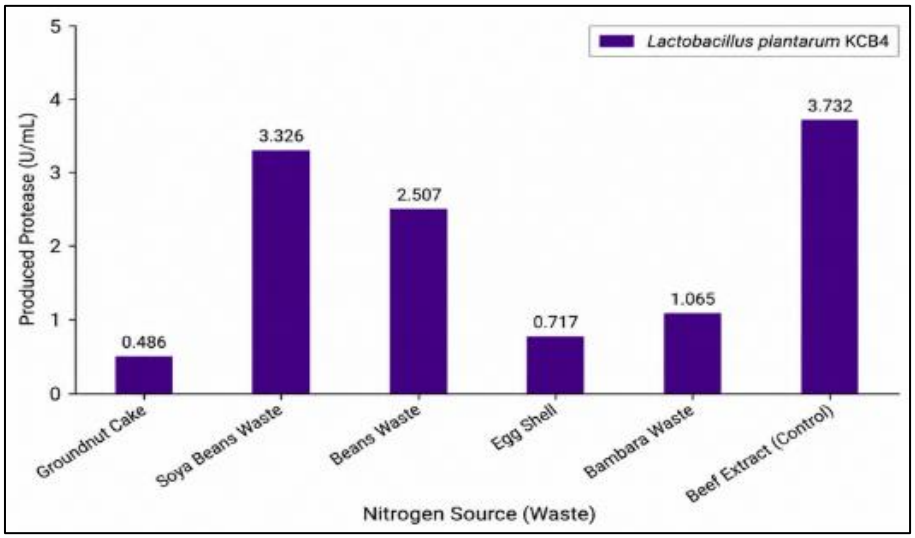

Figure 6 Nitrogen Profile on Optimal Protease Production by *L. plantarum* strain KCB4

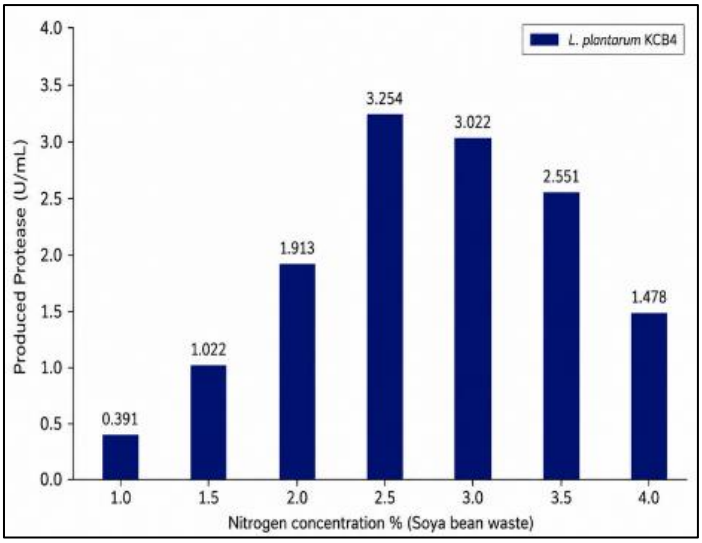

Figure 7 Nitrogen Concentrations profile on Optimal Protease Production by *L. plantarum* strain KCB4

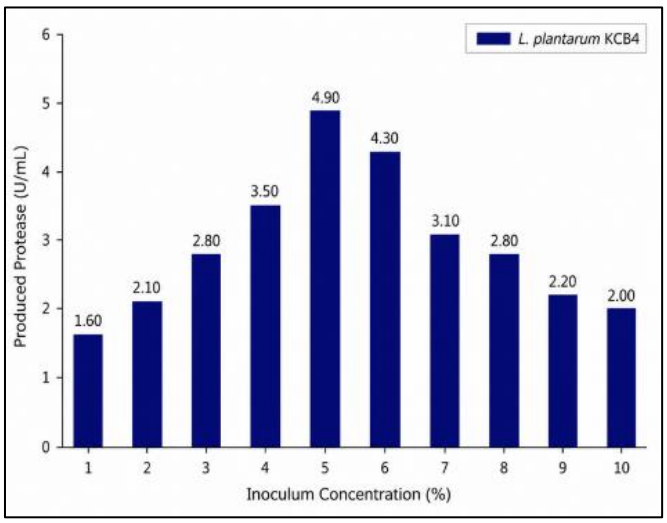

Figure 8 Inoculum Concentration Profile on Optimal Protease Production

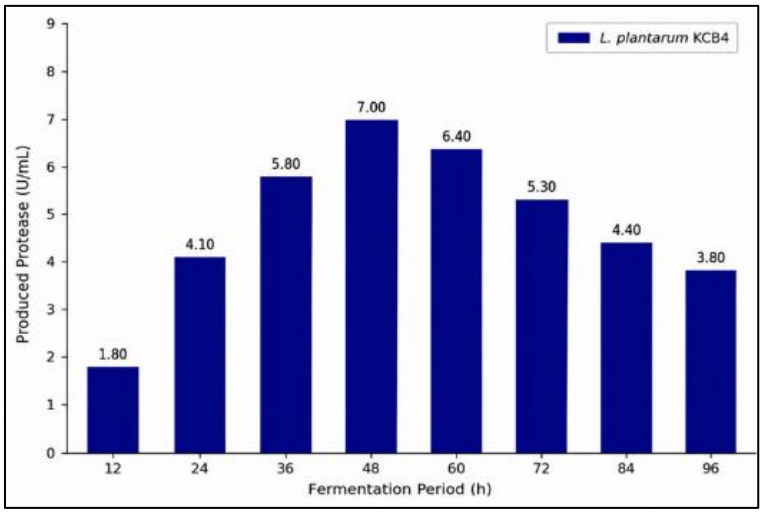

Figure 9 Effect of Fermentation period on Optimal Protease Production

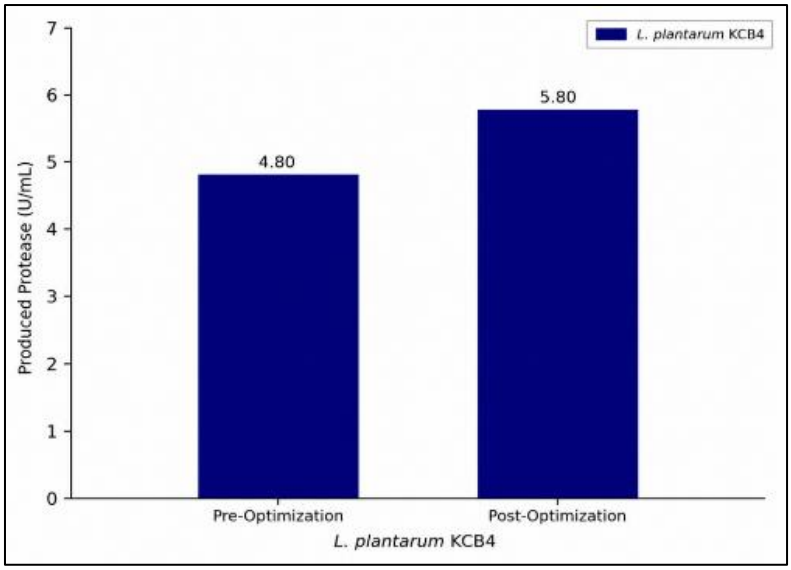

Figure 10 Pre and Post Optimized Protease Production

4. Discussion

The pre-optimization study revealed that *Lactobacillus plantarum* KCB4 produced 4.754 U/mL of protease (Figure 1), demonstrating strong proteolytic activity prior to optimization. This relatively high enzyme yield justified its selection for subsequent optimization studies aimed at enhancing protease production. Previous studies have reported that the proteolytic capacity of *L. plantarum* is strain-dependent, with high-producing strains exhibiting greater efficiency in protein hydrolysis and the generation of bioactive peptides during fermentation (Mahajan *et al*., 2023). Such enhanced protease production is associated with improved protein digestibility and increased potential for industrial and biotechnological applications (Demirci *et al*., 2023).

pH significantly influenced protease production by *Lactobacillus plantarum* KCB4 (Figure 2). Enzyme activity increased from 1.188 U/mL at pH 3.5 to a maximum of 1.935 U/mL at pH 4.5, after which it declined progressively to 0.979 U/mL at pH 8.5. This indicates that protease production by KCB4 is favored under mildly acidic conditions, with pH 4.5 identified as the optimum for enzyme synthesis. Similar observations have been reported for lactic acid bacteria, where acidic conditions promote protease production and enzyme stability (Razzaq *et al*., 2019). The reduction in protease activity at higher pH values may be due to decreased enzyme stability and altered metabolic activities associated with less favorable growth conditions (Kieliszek *et al*., 2021)

Temperature had a measurable effect on the protease production by *Lactobacillus plantarum* KCB4 (Figure 3). Enzyme activity increased slightly from 4.065 U/mL at 30°C to 4.145 U/mL at 35°C, reaching a maximum of 15.688 U/mL at 40°C, before declining sharply to 1.435, 1.058, and 1.030 U/mL at 45, 50, and 55°C, respectively. These results indicate that 40°C is the optimum temperature for protease production by KCB4, likely due to enhanced microbial metabolism and enzyme synthesis under mesophilic conditions. Similar optimum temperatures have been reported for protease-producing *L. plantarum* strains, while the decline at higher temperatures has been attributed to thermal inactivation of metabolic enzymes and reduced stability of the protease production system (López-Trujillo *et al*., 2023).

In Figure 4 Carbon source influenced protease production by *Lactobacillus plantarum* KCB4. Among the substrates evaluated, banana peel (6.63 U/mL) supported the highest enzyme production, followed by rice bran (6.57 U/mL) and glucose (6.36 U/mL), while fermented corn waste (4.34 U/mL) produced the lowest activity. The superior performance of banana peel may be attributed to its readily fermentable carbohydrates and essential nutrients that support microbial growth and enzyme biosynthesis. Similar findings have shown that agro-industrial wastes such as banana peel and rice bran are effective low-cost substrates for microbial protease production (Hao *et al*., 2024). The variation in enzyme yield across the different carbon sources further confirms that protease production in *L. plantarum* is highly dependent on the nature of the carbon substrate and the metabolic characteristics of the strain (Adetunji *et al*., 2023).

The concentration of banana peel was subsequently optimized after its selection as the most suitable carbon source (Figure 5). Protease production increased from 2.054 U/mL at 1.5% banana peel concentration to 3.192 U/mL and 3.210 U/mL at 2.0% and 2.5%, respectively, before declining progressively as the substrate concentration increased

further. The highest protease yield recorded at 2.5% suggests that moderate carbon availability is favorable for enzyme synthesis. Conversely, the reduction in protease production at higher concentrations may be associated with substrate inhibition and carbon catabolite repression, whereby excess carbon limits protease biosynthesis. Similar observations have been reported for *Lactobacillus plantarum* and other protease-producing microorganisms, where moderate carbon concentrations promoted enzyme production, whereas excessive substrate levels resulted in reduced protease yield (Gökmen *et al*. 2024); López-Trujillo *et al*., 2023). These results demonstrate that 2.5% (w/v) banana peel is the optimum carbon concentration for enhanced protease production by *Lactobacillus plantarum* KCB4.

The ability of *Lactobacillus plantarum* KCB4 to produce protease varied considerably with the type of nitrogen source supplied in the fermentation medium (Figure 6). Among the agro-waste nitrogen sources evaluated, soya bean waste (3.326 U/mL) supported the highest enzyme production, followed by beans waste (2.507 U/mL), whereas groundnut cake (0.486 U/mL) and eggshell (0.717 U/mL) resulted in the lowest activities. Although beef extract (control) produced the highest overall protease activity (3.732 U/mL), soya bean waste emerged as the most effective and economical alternative nitrogen source. The superior performance of soya bean waste is likely due to its high protein and amino acid content, which promotes microbial growth and protease biosynthesis. Similar findings have been reported for *Lactobacillus* species and other protease-producing bacteria cultivated on protein-rich agro-industrial residues (Pang *et al*., 2024).

Following the selection of soya bean waste as the optimum nitrogen source, its concentration was optimized (Figure 7). Protease production increased steadily from 0.391 U/mL at 1.0% to a maximum of 3.254 U/mL at 2.5%, after which enzyme production gradually declined to 1.478 U/mL at 4.0%. The reduction in protease yield beyond the optimum concentration may be attributed to excess nitrogen causing metabolic imbalance and repression of enzyme synthesis. Similar trends have been reported for *L. plantarum*, where moderate nitrogen concentrations favored protease production, whereas excessive nitrogen supplementation suppressed enzyme biosynthesis (Fan *et al*. (2024). These findings indicate that 2.5% (w/v) soya bean waste is the optimum nitrogen concentration for enhanced protease production by *Lactobacillus plantarum* KCB4 (Gökmen *et al*., 2024).

Protease production by *Lactobacillus plantarum* KCB4 was markedly affected by inoculum concentration (Figure 8). Enzyme activity increased progressively from 1.60 U/mL at 1% inoculum to a peak value of 4.90 U/mL at 5%, before declining gradually to 2.00 U/mL at 10% inoculum. The enhanced protease yield observed at 5% suggests that this inoculum level provides optimal conditions for microbial growth and enzyme biosynthesis. In contrast, the reduction in enzyme production at higher inoculum concentrations may be associated with rapid nutrient depletion, accumulation of inhibitory metabolites, and increased competition for available resources among the growing cells (Gökmen *et al*., 2024). Comparable observations have been reported for protease-producing *Lactobacillus* species, where moderate inoculum sizes favored maximum enzyme production, whereas excessive inoculum levels adversely affected protease synthesis (Gowthaman *et al*., 2020).

In Figure 9 fermentation period showed measurable influence on protease production by *Lactobacillus plantarum* KCB4. Enzyme activity increased steadily from 1.80 U/mL at 12 h to a maximum of 7.00 U/mL at 48 h, after which it declined progressively to 3.80 U/mL at 96 h. The peak enzyme production at 48 h suggests that protease biosynthesis is greatest during the exponential growth phase when microbial metabolism is most active. The subsequent decline may be attributed to nutrient depletion, accumulation of inhibitory metabolites, increased acidity, and possible enzyme degradation during prolonged incubation. Similar fermentation patterns have been reported for protease-producing *Lactobacillus* species, although the optimum fermentation time varies with strain and culture conditions (Kieliszek *et al*., 2021; Pang *et al*., 2024). These findings indicate that 48 h is the optimum fermentation period for enhanced protease production by *Lactobacillus plantarum* KCB4.

Optimization of the fermentation conditions enhanced protease production by *Lactobacillus plantarum* KCB4 (Figure 10). Enzyme activity increased from 4.80 U/mL under pre-optimization conditions to 5.80 U/mL after optimization, representing an approximately 21% improvement in protease yield. The increase demonstrates the effectiveness of optimizing fermentation parameters, including pH, temperature, carbon source and concentration, nitrogen source and concentration, inoculum size, and fermentation period, in stimulating enzyme biosynthesis. Similar improvements have been reported for protease-producing *Lactobacillus* strains following systematic optimization of culture conditions, highlighting the importance of optimizing fermentation variables to maximize enzyme production (Gökmen *et al*., 2024)).

5. Conclusion

This study demonstrated that *Lactiplantibacillus plantarum* KCB4 is a promising producer of extracellular protease and that optimization of fermentation conditions significantly enhanced enzyme production. Optimum protease yield was achieved under mildly acidic pH, mesophilic temperature, banana peel as the carbon source, soya bean waste as the nitrogen source, appropriate substrate concentrations, moderate inoculum size, and an intermediate fermentation period. The findings further showed that low-cost agro-industrial wastes can effectively support protease production, offering a sustainable and economical alternative to conventional substrates.

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