Pertanika J. Trop. Agric. Sci. 49 (S2): 173 - 199 (2026)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# Production of Antioxidant-Rich Feather Hydrolysate by *Alcaligenes faecalis* MEK1 and Its Evaluation in Mung Bean Under Salinity Stress

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## ABSTRACT

Poultry feather waste is a protein-rich resource whose valorisation is constrained by the recalcitrant structure of keratin. This study evaluated feather bioconversion by *Alcaligenes faecalis* MEK1 and the antioxidant and seed-priming properties of the resulting hydrolysate under salinity stress. Temperature, initial pH, feather concentration, and incubation time were evaluated using reductase-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency. Antioxidant capacity was determined using DPPH, ABTS, nitric oxide scavenging, and ferric-reducing assays, followed by mung bean germination and salinity-stress experiments. Based on the combined responses, 37 °C, pH 8.0, and 1.5% (w/v) feather substrate were selected as working conditions. Reduction-associated activity reached 129.30 U/mL at 72 h, followed by maximum keratinase activity of 5.74 U/mL at 96 h, whereas soluble protein concentration and feather degradation reached 1.18 g/L and 74.90%, respectively, at 168 h. The hydrolysate exhibited concentration-dependent antioxidant activity across the four assays. Seed priming with 0.5% (v/v) hydrolysate increased germination from 87.87% to 98.24%, reduced mean germination time from 2.874 to 1.758 days, and increased root length from 5.918 to 7.060 cm. Under 100 mM NaCl, hydrolysate treatment reduced hydrogen peroxide from 18.9 to 10.7 µmol/g fresh weight and malondialdehyde from 28.6 to 15.4 nmol/g fresh weight while increasing superoxide dismutase, catalase, and peroxidase activities. These findings demonstrate the potential of MEK1 feather hydrolysate as a seed-priming material for improving early mung bean responses under controlled salinity stress.

*Keywords:* Antioxidant, feather hydrolysate, feather waste valorisation, mung bean, salinity stress, seedling vigour

ARTICLE INFO

*Article history:*
Received: 26 May 2026
Accepted: 27 August 2026
Published: 30 September 2026

DOI: https://doi.org/10.47836/pjtas.49.S2.09

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## INTRODUCTION

Feather waste is a valuable protein-rich byproduct of poultry processing, but its valorisation is limited by the recalcitrant keratin structure. Feather keratin contains high proportion of sulfur-rich proteins stabilised by disulfide bonds, hydrogen bonding, and hydrophobic interactions, making the substrate insoluble and resistant to ordinary proteolysis (Chaitanya Reddy et al., 2021; Das et al., 2024; Kumar et al., 2026). Conventionally, feather waste has been disposed of through chemical treatment, open-air burning, incineration, and landfilling, which help reduce waste accumulation but often require high energy input, generate undesirable secondary waste, and result in the loss of valuable peptides and amino acids (Chaitanya Reddy et al., 2021; Kumar et al., 2026). Therefore, recent studies suggest that microbial conversion offers a more sustainable alternative because keratinolytic microorganisms can hydrolyse the feather keratin under milder conditions through keratinases and associated biochemical reactions that enhance the protein solubilisation and preserve the resulting hydrolysate (Brandelli et al., 2010; Qiu et al., 2020). This approach is relevant for circular waste management because it helps in the conversion of feather waste from the poultry industry into valuable protein hydrolysates, enzymes and nutrient-rich products with high potential in agriculture and industrial biotechnology (Enciso-Tenorio et al., 2025; Nad et al., 2024; Park et al., 2023).

Microbial feather degradation should not be defined only as a keratinase-driven bioprocess because native feather keratin remains protected by disulfide-stabilised structural packing. Biological reduction weakens these disulfide constraints and increases the exposure of peptide regions for keratinase-mediated hydrolysis into soluble proteins, peptides and amino acids (Qiu et al., 2020; Q. Wang et al., 2025). This reductive capacity provides a biological substrate-controlled opening mechanism compared to severe thermal or chemical solubilisation, which may compromise the quality of functional properties of the resulting hydrolysate through process severity and amino acid loss (Karuppannan et al., 2021; Sharma & Kango, 2021). Combined reductase and keratinase systems have been shown to accelerate feather degradation and increase soluble and functional bioactive protein or amino acid recovery compared with keratinase-centric systems, supporting the need to evaluate the reductive and proteolytic activities together (Fan et al., 2026; Ji et al., 2024; Li et al., 2023). Therefore, *A. faecalis* MEK1 feather hydrolysate is positioned in this study as a biologically produced hydrolysate generated through coordinated reductase-associated and keratinase activities for functional antioxidant application.

The potential agricultural value of feather hydrolysate is associated with its soluble peptides and amino acids, which may support nutrient availability, osmotic adjustment, and stress-responsive metabolism during seed germination. Protein-derived hydrolysates have been reported to improve germination, root and shoot development, biomass accumulation, and crop productivity after application (Adelere & Lateef, 2023; Kaur et al., 2021;

Roy & Jílková, 2025). Under salinity, amino acid- and peptide-based biostimulants may also enhance antioxidant defense, including superoxide dismutase, catalase, and peroxidase activities, thereby limiting hydrogen peroxide accumulation, lipid peroxidation, and associated growth inhibition (Gezgincioğlu & Atici, 2023; Zuluaga et al., 2023). These reported responses provide a scientific basis for evaluating MEK1 feather hydrolysate in salt-stressed mung beans. However, osmotic adjustment and nutrient uptake were not measured directly in the present study; therefore, hydrolysate effects were interpreted using germination, seedling growth, antioxidant enzyme activities, and oxidative-damage markers. Recent studies have shown that feather hydrolysates can exhibit DPPH, ABTS, and ferric-reducing activities and may improve germination, growth, or yield in mung bean, spinach, French bean, and other crops (Cavello et al., 2021; Devi et al., 2024; Ismail et al., 2025; Kaur et al., 2021; Kshetri et al., 2020; Stanly & Umesh, 2024). Studies involving *Alcaligenes* species have mainly focused on keratinase production, feather degradation, amino acid recovery, or organic-fertiliser applications (Otuyelu et al., 2025; Yusuf et al., 2016). However, limited research has integrated reduction-associated and keratinolytic activities with hydrolysate antioxidant performance and plant responses under salinity within the same experimental design. The effective concentration of an *A. faecalis*-derived hydrolysate for mung bean seed priming and its association with salinity-related oxidative stress also remain insufficiently defined. Therefore, the novelty of this study lies in producing feather hydrolysate using *A. faecalis* MEK1, characterising its antioxidant activity, and evaluating its concentration-dependent effects on mung bean germination and oxidative-stress responses under controlled salinity.

This study hypothesised that feather hydrolysate produced by *A. faecalis* MEK1 through reduction-associated and keratinolytic activities would improve mung bean germination and seedling vigour under salinity, accompanied by enhanced antioxidant defense and reduced oxidative damage. Feather bioconversion was evaluated across temperature, pH, substrate concentration, and incubation time using reduction-associated activity, keratinolytic activity, soluble-protein release, and degradation efficiency as responses. The resulting hydrolysate was subsequently assessed for antioxidant activity, concentration-dependent seed-priming effects, seedling growth, and biochemical responses to 100 mM NaCl. Accordingly, this study aimed to determine whether *A. faecalis* MEK1 could convert recalcitrant feather waste into an antioxidant-active hydrolysate capable of improving mung bean performance under controlled salinity stress.

## MATERIALS AND METHODS

### Materials, Chemicals, and Reagents

All chemicals and reagents used in this study were of analytical grade unless otherwise stated. Nutrient agar and nutrient broth were obtained from Oxoid Ltd. (Basingstoke, UK).

Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulfate heptahydrate, sodium chloride, sodium hydroxide, hydrochloric acid, trichloroacetic acid, potassium persulfate, potassium ferricyanide, ferric chloride, methanol, phosphoric acid, thiobarbituric acid, sulfosalicylic acid, ninhydrin, hydrogen peroxide and sodium hypochlorite were purchased from Merck KGaA (Darmstadt, Germany). 5,5′-Dithiobis-2-nitrobenzoic acid, L-cysteine, L-tyrosine, DPPH, ABTS, Trolox, ascorbic acid, sodium nitroprusside, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, nitroblue tetrazolium, riboflavin, methionine and guaiacol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent and bovine serum albumin were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Fresh chicken feathers were collected from a poultry slaughterhouse in Putrajaya, Malaysia, while mung bean seeds were obtained from a local agricultural supplier in Selangor, Malaysia. Distilled water was used for all reagent preparation and experimental procedures.

## Microorganism Identity, Revival, and Inoculum

*A. faecalis* MEK1 was used as a potent bacterial strain. The strain was previously identified by full-length 16S rRNA gene sequencing using the universal primers 27F and 1492R. Sequence similarity was determined using BLASTn, and phylogenetic analysis was performed using MEGA11 based on the maximum likelihood method with 1000 bootstrap replicates. The 16S rRNA gene sequence was deposited in GenBank with accession number PX096348.1. For inoculum preparation, *A. faecalis* MEK1 was revived from glycerol stock on nutrient agar. The agar was incubated at 37 °C for 24 h. Single colony was transferred into 10 mL nutrient broth and incubated at 37 °C with agitation at 150 rpm for 18 h. The bacterial culture was adjusted to $OD_{600}$ 0.8 ± 0.05 before use.

## Preparation of Chicken Feather Keratin Substrate

White chicken feathers were collected from a poultry slaughterhouse in Putrajaya, Malaysia. The feathers were used as the keratin substrate for the study. The feathers were washed with tap water and mild alkaline detergent to remove blood, dirt, and organic matter, followed by rinsing with distilled water. The cleaned feathers were dried at 60 °C until dry and cut into 0.5 to 1.0 cm fragments. The prepared feather substrate was sterilised at 121 °C for 15 min before use in submerged feather keratin bioconversion.

## Submerged Feather Keratin Bioconversion

Keratin bioconversion was done in 250 mL Erlenmeyer flasks containing 100 mL minimal basal media supplemented with chicken feather keratin substrate. The minimal basal media contained 0.4 g potassium dihydrogen phosphate, 0.3 g dipotassium hydrogen phosphate,

0.1 g magnesium sulfate heptahydrate, and 0.5 g sodium chloride in 1 L. Each flask was inoculated with 1% v/v *A. faecalis* MEK1 inoculum and incubated at 35 °C, initial pH 7.0, and 150 rpm for 7 days. The initial chicken feather keratin substrate concentration was at 1.0% w/v unless otherwise stated in the optimisation experiment.

**Hydrolysate Harvesting and Clarification**

After submerged feather keratin bioconversion, the culture mixture was filtered by vacuum filtration to separate the liquid feather hydrolysate from residual feather material. The recovered hydrolysate was centrifuged at 10,000 × g for 10 min, and the supernatant was collected as crude feather hydrolysate for subsequent enzymatic, soluble protein, antioxidant, and plant bioassay analyses. Residual feather material retained after filtration was used for determination of feather degradation efficiency.

**Preparation of Chemical Feather Hydrolysate Comparators**

Chemical feather hydrolysate comparators were prepared using NaOH-treated and NaOH plus autoclave-treated chicken feather keratin substrate. For NaOH treatment, feather substrate was mixed with 1 M NaOH at 1.5% w/v chicken feather keratin and incubated at 35 °C for 120 h. For NaOH plus autoclave treatment, the feather substrate was mixed with 1 M NaOH and autoclaved at 121 °C for 15 min. After treatment, all the hydrolysates were cooled, neutralised to pH 7, centrifuged at 10,000×g for 10 min, and filtered to obtain the soluble fraction. The soluble protein concentration of each hydrolysate was determined at approximately 0.65 g/L before antioxidant analysis to allow comparison with *A. faecalis* MEK1 feather hydrolysate.

**Enzymatic Activities, Soluble Protein and Feather Degradation Assays**

Keratinase activity was determined using crude feather hydrolysate as the enzyme source. The hydrolysate was centrifuged at 10,000×g for 10 min, and the supernatant was mixed with 0.05 M phosphate buffer at pH 7.4 and 1% w/v sterilised chicken feather substrate. The reaction mixture was incubated at 37 °C for 60 min and terminated with 0.4 M trichloroacetic acid. After centrifugation at 5,000×g for 10 min, absorbance was measured at 280 nm. Keratinase activity was calculated from an L-tyrosine standard curve and expressed as U/mL using Equation 1. 1 unit represents the amount of enzyme required to release 1 µmol of L-tyrosine per min under assay conditions.

$$Keratinase\ Activity\ (U/mL) = \frac{\mu mol\ of\ L-tyrosine}{volume\ (mL) \times time\ (min)} \quad [1]$$

Reductase-associated activity was determined using Ellman's assay based on sulfhydryl group release from chicken feather substrate. The crude enzyme was mixed with a Tris-EDTA buffer containing 30 mM Tris-HCl and 3 mM EDTA at pH 8.2, along with 1% w/v chicken feather substrate. The reaction mixture was incubated at 37 °C for 60 min and terminated with 0.4 M trichloroacetic acid. After centrifugation at 5,000×g for 10 min, the supernatant was reacted with 3 mM DTNB for 10 min at 25 °C, and absorbance was measured at 412 nm. Reductase-associated activity was calculated from an L-cysteine standard curve and expressed as U/mL using Equation 2. 1 unit represents the release of 1 μmol of sulfhydryl groups per min under the assay conditions.

$$Reductase-associated\ Activity\ (U/mL) = \frac{\mu mol\ of\ sulfhydryl\ groups}{volume\ (mL) \times time\ (min)} \quad [2]$$

For both keratinolytic and reduction-associated activity assays, substrate, enzyme, and reagent blanks were prepared by omitting the enzyme preparation, substrate, or both components, respectively, while maintaining the same final reaction volume. A zero-time control was prepared by adding the reaction-stopping reagent immediately after enzyme-substrate mixing without incubation. The heat-inactivated enzyme control was prepared by heating the enzyme preparation at 95 °C for 5 min, followed by immediate cooling on ice for 5 min, before analysis under the corresponding assay conditions. Complete inactivation was assumed from the severity of the heat treatment because residual activity was not independently quantified. For the TCA-precipitated control, the enzyme preparation was treated with 15% (w/v) trichloroacetic acid before substrate addition, incubated briefly to allow protein precipitation, and centrifuged at 8000 rpm for 10 min at 4°C; the resulting protein-depleted supernatant was then subjected to the respective assay, with its pH adjusted to the assay conditions where required. The same blank definitions, heat-inactivation procedure, and TCA-precipitation approach were applied consistently to both enzyme assays to correct background signals and determine whether the measured responses were attributable to active soluble proteins.

Soluble protein concentration was determined using the Bradford assay with bovine serum albumin as the standard, using Equation 3, and absorbance was measured at 595 nm. Feather degradation efficiency was determined by the dry weight loss of residual feather material after filtration and drying at 60 °C until constant weight. Degradation efficiency was calculated as the dried unhydrolysed feather keratin substrate using Equation 4.

$$Soluble\ Protein\ Concentration\ (g/L) = \frac{Absorbance\ at\ 595\ nm}{Slope\ of\ standard\ curve} \quad [3]$$

$$Feather\ Degradation\ (\%) = \frac{(Wo - Wt)}{Wo} \times 100\% \quad [4]$$

$W_0$ represents the initial dry feather mass before treatment, while Wt represents the remaining dry feather mass after treatment or incubation

**One Factor at Time Optimisation**

Keratin bioconversion was optimised using one factor at a time. The tested parameters included incubation temperatures at 25 to 50 °C, initial pH at 5.0 to 11.0, feather keratin substrate concentrations at 0.5 to 2.0% w/v, and incubation time up to 7 days. The effects of each parameter were evaluated based on reduction-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency. The optimum condition was selected based on the combined response of enzyme activities, protein solubilisation, and feather degradation.

**Antioxidant Capacity of Feather Hydrolysate**

The antioxidant capacity of *A. faecalis* MEK1 feather hydrolysate and chemical feather hydrolysate comparators was determined using DPPH radical scavenging, ABTS radical scavenging, nitric oxide scavenging, and ferric reducing power assays. Hydrolysate samples were tested at 0.02 to 0.65 g/L soluble protein. For DPPH analysis, the sample was mixed with 0.1 mM DPPH solution and incubated in the dark at 25°C for 30 min before absorbance was measured at 517 nm. For ABTS analysis, ABTS radical solution was prepared using ABTS and potassium persulfate, incubated in the dark for at least 12 h, diluted to an absorbance of 0.70 at 734 nm, and reacted with the sample for 6 min before measurement at 734 nm. Nitric oxide scavenging activity was determined using sodium nitroprusside and Griess reagent, with absorbance measured at 546 nm. Ferric reducing power was determined using potassium ferricyanide and ferric chloride, with absorbance measured at 700 nm. Ascorbic acid was used as the positive control for DPPH, nitric oxide scavenging, and ferric reducing power assays, while Trolox was used as the standard for ABTS analysis. Radical scavenging activity was calculated relative to the blank using Equation 5, and IC50 values for DPPH, ABTS, nitric oxide scavenging, and ferric reducing power were estimated from dose-response curves using nonlinear regression.

$$Radical\ Scavenging\ (\%) = \frac{A_c - A_s}{A_c} \times 100\% \quad [5]$$

Where $A_c$ is the absorbance of the control or blank reaction and $A_s$ is the absorbance of the sample.

**Determination of Hydrolysate Working Concentration for Mung Bean Seed Priming**

The seed-priming and germination procedure was adapted from previous studies evaluating feather-derived or animal-protein hydrolysates as plant biostimulants (W. Wang et al., 2022), with modifications to the hydrolysate concentration and priming duration. Mung bean seeds were sterilised with 1% w/v sodium hypochlorite for 15 min and rinsed with distilled water. The sterilised seeds were primed with feather hydrolysate at 0, 0.25, 0.5, 1.0, and 2.0% v/v for 6 h. After priming, 5 seeds were transferred onto sterile filter paper in each Petri dish. The seeds were incubated at 25 °C for 7 days. Germination and early seedling growth were evaluated based on germination percentage (Equation 6), mean germination time (Equation 7), germination index (Equation 8), root length, shoot length, total seedling length, fresh weight, and dry weight. The hydrolysate concentration that induces growth without inhibiting it was selected for salinity stress validation.

$$Germination\ (\%) = \frac{N_g}{N_t} \times 100\% \quad [6]$$

Where $N_g$ is the number of germinated seeds and $N_t$ is the total number of seeds.

$$Mean\ germination\ time = \frac{\sum n_i t_i}{\sum n_i} \quad [7]$$

Where $n_i$ is the number of seeds germinated on day $i$, and $t_i$ is the incubation time in days.

$$Germination\ index = \frac{\sum G_i}{\sum T_i} \quad [8]$$

Where $G_i$ is the number of seeds germinated each day and $T_i$ is the corresponding day of counting.

**Salinity Stress Validation and Plant Biochemical Assays**

Mung bean seedlings were evaluated for the stress-mitigating effect of MEK1 feather hydrolysate under salinity stress, with slight modifications to previously reported procedures (Shafique et al., 2019; Singh & Choudhary, 2025). Surface-sterilised seeds were primed for 6 h with either distilled water or 0.5% (v/v) feather hydrolysate and then transferred to sterile filter paper in Petri dishes. Salinity stress was imposed immediately after seed placement by moistening the filter paper with 100 mM NaCl solution. Four treatments were established: water-primed seeds supplied with distilled water as the untreated control, hydrolysate-primed seeds supplied with distilled water, water-primed seeds exposed to 100 mM NaCl, and hydrolysate-primed seeds exposed to 100 mM NaCl. The non-

saline treatments received an equal volume of distilled water, and the filter papers were maintained with the corresponding solution once throughout the experiment. All treatments were conducted in triplicate and incubated at 25 °C for 7 days. Thus, the NaCl-treated seedlings were exposed continuously to 100 mM NaCl from the beginning of germination until harvest. Fresh seedlings were collected on day 7 for the determination of hydrogen peroxide, proline content, malondialdehyde, superoxide dismutase, and catalase responses.

Hydrogen peroxide content was measured using the potassium iodide method and expressed as µmol/g fresh weight. Lipid peroxidation was determined as malondialdehyde content using the thiobarbituric acid reactive substances assay (Aguilar Diaz De Leon & Borges, 2020). Absorbance was recorded at 532 nm and corrected at 600 nm to remove nonspecific turbidity.

Proline content was measured using the acid ninhydrin method described by (Forlani & Funck, 2020). Fresh tissue was extracted in sulfosalicylic acid, reacted with acid ninhydrin, and the absorbance was measured at 520 nm. Proline concentration was expressed as µmol/g fresh weight. Crude enzyme extract was prepared from fresh seedling tissue by buffer homogenisation and centrifugation. Soluble protein concentration in the extract was measured by the Bradford assay using bovine serum albumin as the standard.

Superoxide dismutase activity was determined using the nitroblue tetrazolium inhibition method of Durak et al. (1993), where one unit represented 50% inhibition of NBT reduction. Catalase activity was measured by following hydrogen peroxide decomposition at 240 nm according to Hadwan et al. (2024). Peroxidase activity was measured from hydrogen peroxide-dependent substrate oxidation with adjustment from Nelson and Parsonage (2011). All antioxidant enzyme activities were normalised to soluble protein concentration and expressed as U/mg protein.

**Statistical Analysis**

All experiments were performed in triplicate, and results were expressed as average ± standard deviation. Raw data were organised using Microsoft Excel 365, while statistical analysis and graph preparation were performed using GraphPad Prism version 10. Data from one factor at a time of optimisation, antioxidant assays, and hydrolysate working concentration experiments were analysed using one-way analysis of variance followed by Tukey's multiple comparison test. For salinity stress validation, one-way analysis of variance was used to determine the effects of NaCl and feather hydrolysate. IC50 values for DPPH, ABTS, and nitric oxide scavenging activities were estimated from dose-response curves using nonlinear regression. Statistical significance was accepted at $p < 0.05$. Phylogenetic analysis of *A. faecalis* MEK1 was performed separately using MEGA11.

## RESULTS AND DISCUSSION

### Effect of Temperature, Initial pH and Feather Keratin Substrate Concentration

Temperature, initial pH, and feather substrate concentration affected feather bioconversion by *A. faecalis* MEK1, with different response patterns observed across reductase-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency (Figures 1 to 3). Temperature showed the clearest optimum at 37 °C, where reductase-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency reached 88.76 U/mL, 4.00 U/mL, 0.98 g/L, and 61.60%, respectively. These values were higher than those at 25 °C by 43.80%, 115.40%, 56.70%, and 54.70%, while all responses declined at 50 °C. For pH, reductase-associated activity was highest at pH 8.00 with 88.24 U/mL, while keratinase activity was highest at pH 7.00 with 4.19 U/mL and remained close between pH 8.00 and 9.00. However, soluble protein concentration and feather degradation efficiency increased further at pH 11.00, reaching 1.17 g/L and 77.10%, suggesting that higher alkalinity favored solubilisation and mass loss more than balanced enzymatic activity. For feather substrate concentration, reductase-associated activity peaked at 1.25% w/v with 90.23 U/mL, whereas keratinase activity, soluble protein concentration, and degradation efficiency were highest at 1.50% w/v with 4.06 U/mL, 0.99 g/L, and 63.23%, respectively. All responses declined at 2.00% w/v. Based on the combined response rather than a single maximum value, 37 °C, pH 8.00, and 1.50% w/v feather substrate were selected as the working conditions for MEK1 feather bioconversion.

The selected 37 °C, pH 8.00, and 1.50% w/v feather substrate agree with previous optimisation of feather-based keratinase production by *A. faecalis* IHB B 6507 and with other bacterial keratinolysis studies that commonly report mesophilic temperature, neutral to mildly alkaline pH, and moderate feather loading as suitable conditions for keratinase production and feather conversion (Akram et al., 2021; Otuyelu et al., 2025). However, the MEK1 result shows why reductase-associated activity should be included in process optimisation. Keratinase activity alone would favor pH 7.00, while soluble protein release and feather degradation would favor pH 11.00. In contrast, pH 8.00 retained keratinase activity and gave the highest reduction-associated activity, suggesting a more balanced condition for biological feather conversion. This agrees with studies showing that keratin degradation depends on proteolysis and weakening of the disulfide rigid structure either through sulfur metabolism, disulfide reduction, or combined reductase and protease systems (Fan et al., 2026; Ji et al., 2024; Kumari et al., 2024; Luo et al., 2026; Qiu et al., 2020).

Evidence from wool keratin studies also supports the same principle, where disulfide bond reduction improves protease accessibility, although these studies should be used only as structural support for feather keratin interpretation (Khumalo et al., 2020; Li et al., 2024; Q. Wang et al., 2025). Accordingly, pH 8.0 was selected as a balanced working condition because it maintained both reductase-associated and keratinolytic activities

without selecting the process solely based on alkaline solubilisation or feather mass loss. This supports a broader definition of keratinolytic performance in potency of keratinolytic microorganisms, reduction of disulfide bonds, keratinase activity, soluble protein release, and feather degradation are considered together.

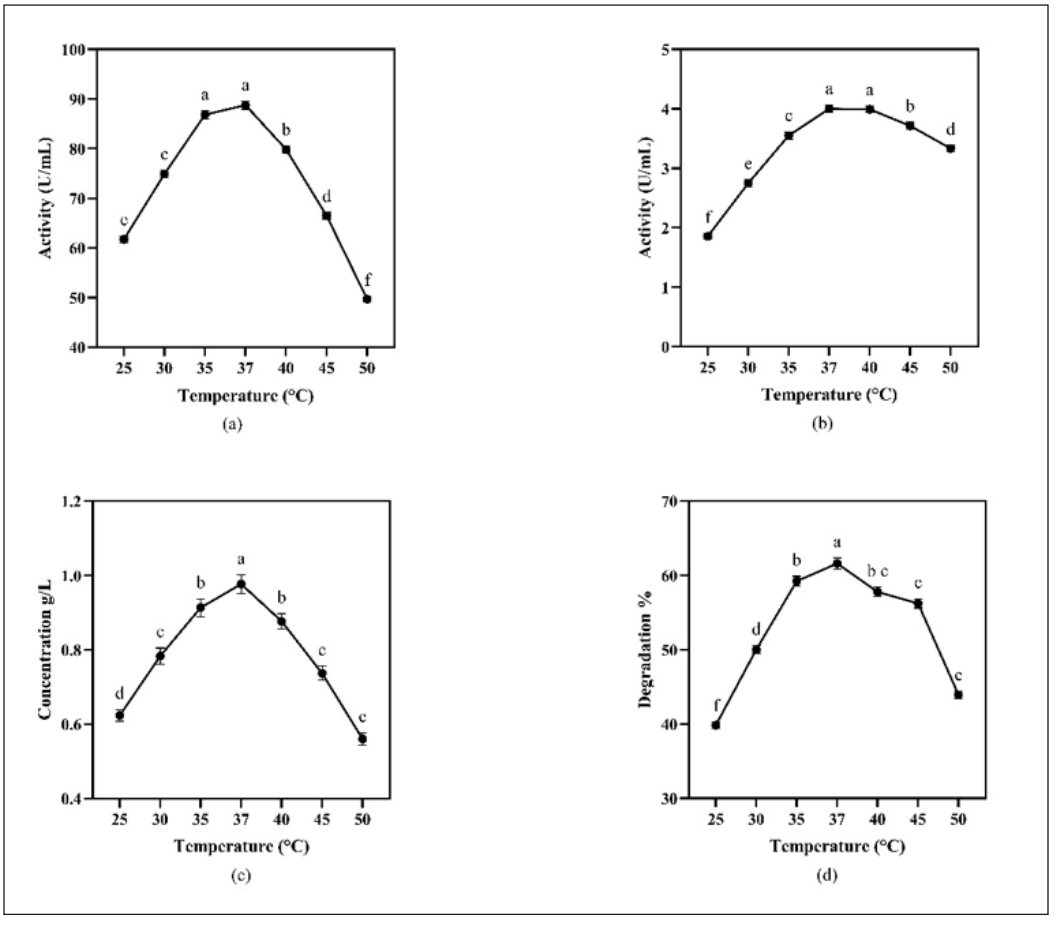


*Figure 1.* Effect of incubation temperature on feather bioconversion by *A. faecalis* MEK1. (a) reductase-associated activity, (b) keratinase activity, (c) soluble protein concentration, and (d) feather degradation efficiency

*Note.* Values are presented as mean ± standard deviation of three independent experiments (n = 3). For each panel, differences among incubation temperatures were evaluated separately using one-way analysis of variance followed by Tukey's multiple-comparison test. Values marked with different lowercase letters are significantly different at $p < 0.05$, whereas values sharing at least one letter are not significantly different.

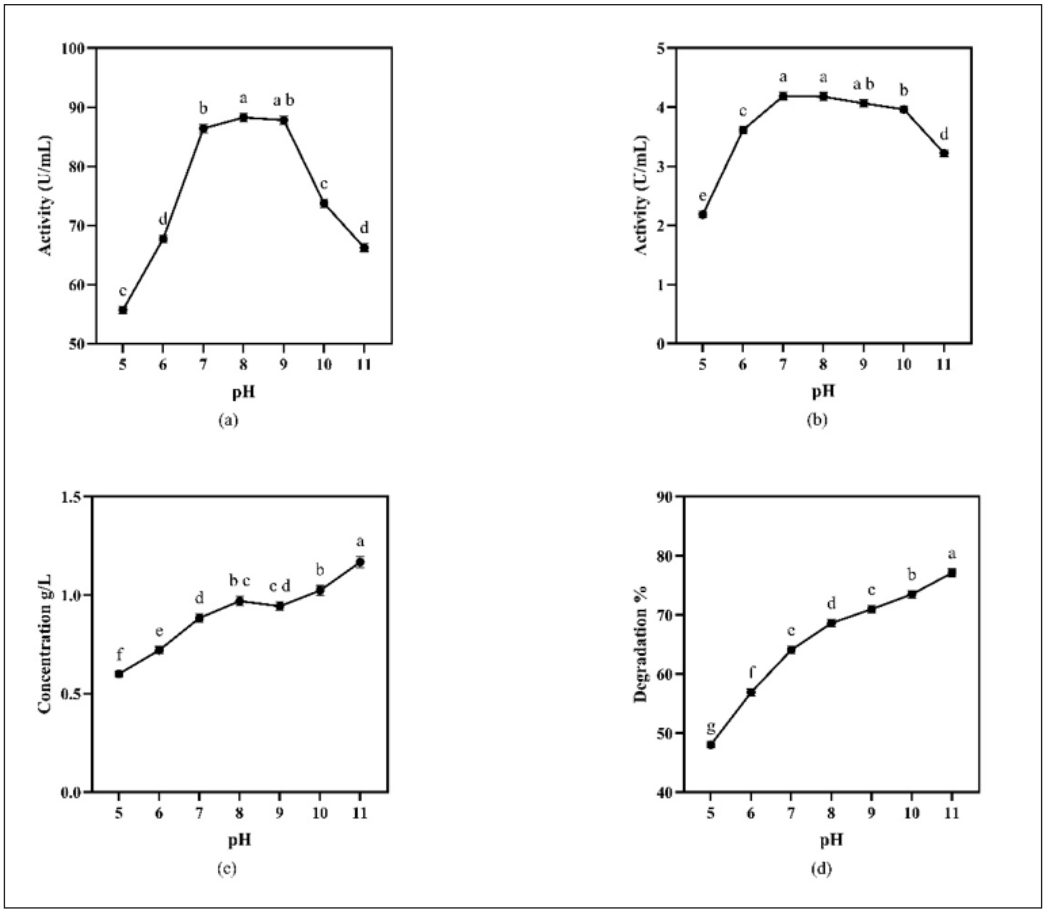


*Figure 2.* Effect of initial medium pH on feather bioconversion by *A. faecalis* MEK1. (a) reductase-associated activity, (b) keratinase activity, (c) soluble protein concentration, and (d) feather degradation efficiency

*Note.* Values are presented as mean ± standard deviation of three independent experiments (n = 3). For each panel, differences among initial pH levels were evaluated separately using one-way analysis of variance followed by Tukey's multiple-comparison test. Values marked with different lowercase letters are significantly different at $p < 0.05$, whereas values sharing at least one letter are not significantly different.

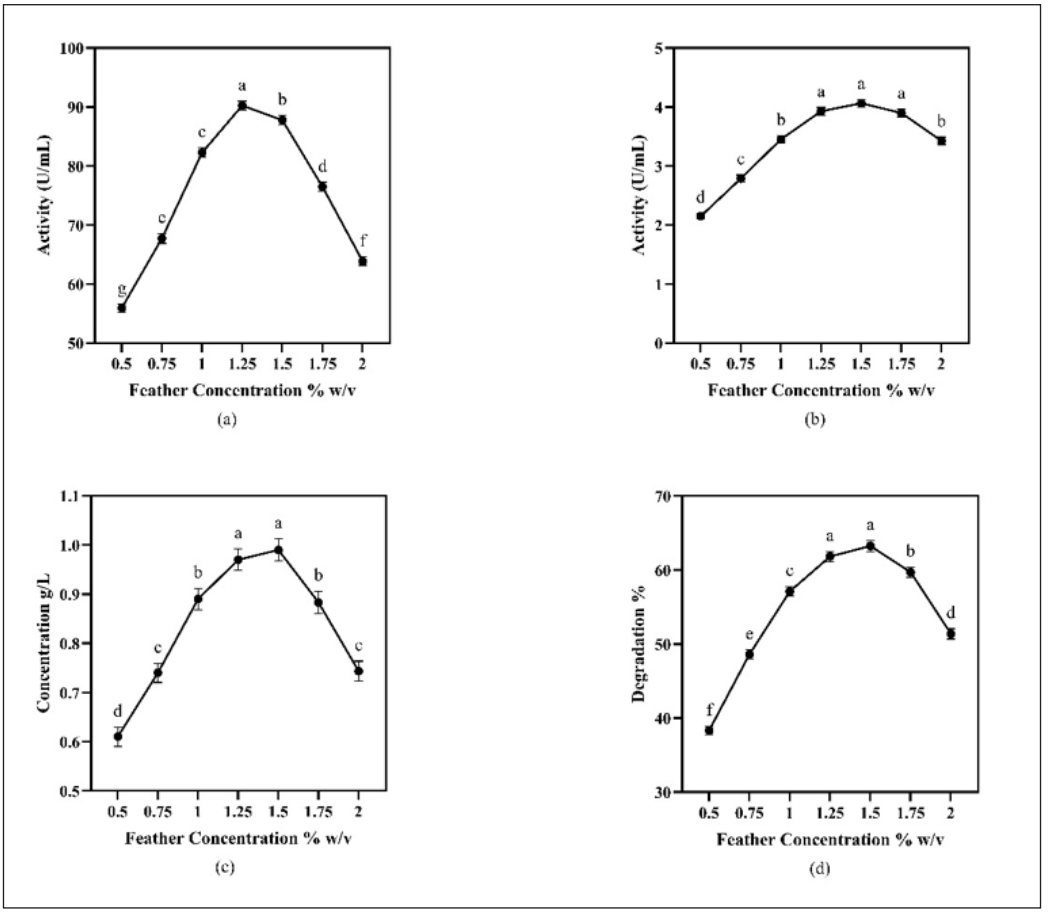


*Figure 3.* Effect of chicken feather keratin substrate concentration on feather bioconversion by *A. faecalis* MEK1. (a) reductase-associated activity, (b) keratinase activity, (c) soluble protein concentration, and (d) feather degradation efficiency

*Note.* Values are presented as mean ± standard deviation of three independent experiments (n = 3). For each panel, differences among feather substrate concentrations were evaluated separately using one-way analysis of variance followed by Tukey's multiple-comparison test. Values marked with different lowercase letters are significantly different at $p < 0.05$, whereas values sharing at least one letter are not significantly different.

## Effect of Incubation Time on Feather Bioconversion

Incubation time affected reductase-associated activity, keratinase activity, soluble protein concentration, and feather degradation efficiency during *A. faecalis* MEK1 feather bioconversion (Figure 4). Reductase-associated activity peaked earlier at 72 h with 129.30 U/mL, then declined to 73.50 U/mL at 168 h. Keratinase activity reached its highest later at 96 h with 5.74 U/mL, while soluble protein concentration and feather degradation efficiency continued to increase until 168 h, reaching 1.18 g/L and 74.90%, respectively. Compared with 24 h, the maximum keratinase activity, soluble protein concentration, and feather

degradation efficiency increased by 373.29%, 408.62%, and 477.49%, respectively. This result suggests that sulfhydryl group release was more evident during the earlier stage of feather bioconversion, while keratinase activity, protein release, and feather degradation became more noticeable during the later stage.

Reductase-associated activity peaked at 72 h, keratinase activity peaked at 96 h, and soluble protein concentration, together with feather degradation efficiency, reached their highest values at 168 h. The temporal sequence is consistent with a reduction-assisted model of keratinolysis in which weakening of disulfide constraints may increase substrate accessibility to subsequent proteolytic attacks.

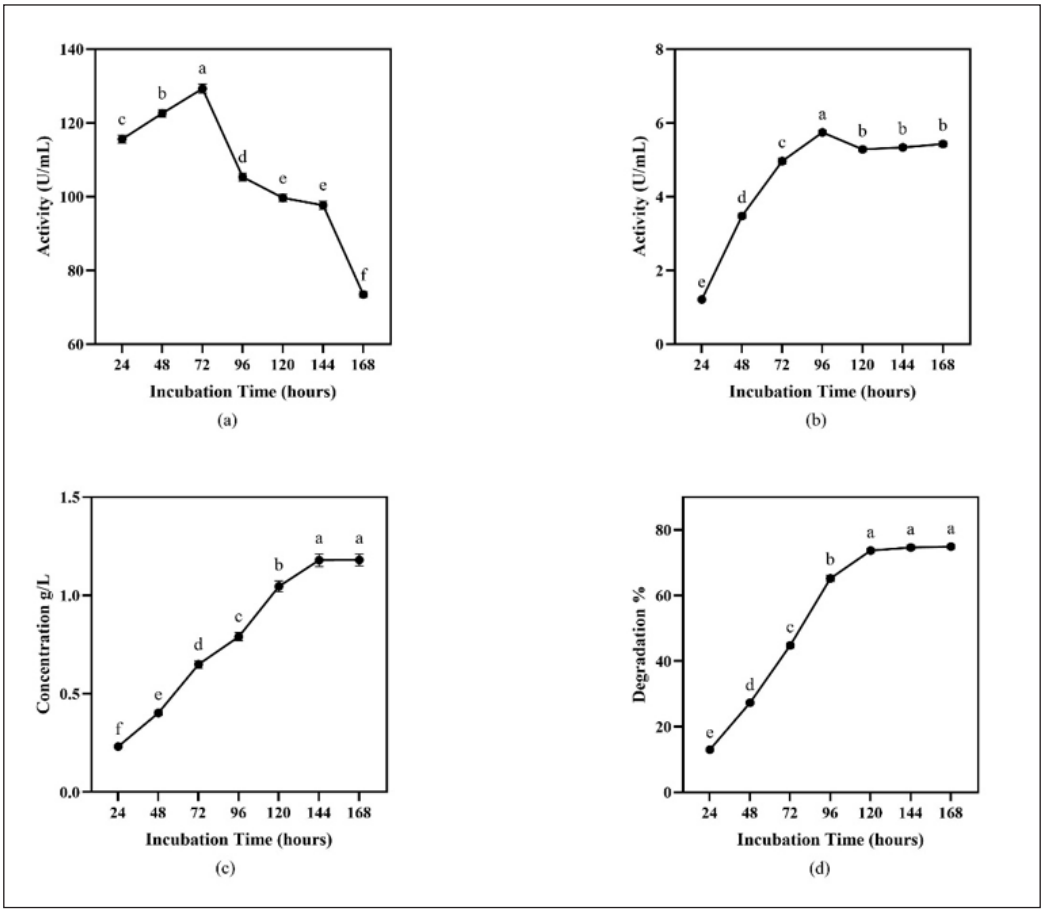


*Figure 4.* Effect of incubation time on feather bioconversion by *A. faecalis* MEK1. (a) reductase-associated activity, (b) keratinase activity, (c) soluble protein concentration, and (d) feather degradation efficiency

*Note.* Values are presented as mean ± standard deviation of three independent experiments (n = 3). For each panel, differences among incubation times were evaluated separately using one-way analysis of variance followed by Tukey's multiple-comparison test. Values marked with different lowercase letters are significantly different at $p < 0.05$, whereas values sharing at least one letter are not significantly different.

However, the present time-course experiment does not independently establish that the earlier reductase-associated response caused the subsequent increase in keratinase activity or feather degradation. Similar time-dependent patterns have been reported in *Bacillus* sp. P45, *Bacillus* sp. MBRL 575, *Stenotrophomonas maltophilia* R13, and *Chryseobacterium* sp. RBT, where sulfhydryl group release increased during early fermentation, followed by keratinase activity, soluble protein release, or feather degradation (Daroit et al., 2010; Gurav et al., 2020; Jeong et al., 2010; Kshetri et al., 2020).

Mechanistic-related studies also support this pattern by showing that sulfur metabolism, cysteine and cystine cycling, disulfide bond reduction, and coupled reductase and protease activity can contribute to keratin degradation, which we suggest that *A. faecalis* MEK1 may follow a similar reduction-assisted keratinolysis pattern (Lai et al., 2023; Peng et al., 2019; Qiu et al., 2020; B. Wang et al., 2024; Zhou et al., 2023). In *Alcaligenes* sp., previous studies have shown feather degradation and keratinase production by *Alcaligenes* sp. AQ05 001 and *A. faecalis* IHB B 6507, but the temporal relationship between reductase-associated activity, keratinase activity, protein release, and feather degradation has not been clearly validated (Otuyelu et al., 2025; Yusuf et al., 2016). The MEK1, therefore, adds a process-level insight by showing that in *Alcaligenes faecalis* sp., the reductase-associated activity appeared earlier than the keratinase activity, while soluble protein release and feather degradation increase over time.

**Antioxidant Capacity of Feather Hydrolysates**

The antioxidant capacity of *A. faecalis* MEK1 feather hydrolysate differed among assays, with clearer activity observed in DPPH radical scavenging activity, nitric oxide scavenging activity, and ferric reducing power than in ABTS radical scavenging activity (Figure 5). In the DPPH assay, MEK1 feather hydrolysate showed the lowest IC50 at 0.1933 g/L, compared with 0.2519 g/L for NaOH-treated hydrolysate and 0.4585 g/L for NaOH plus autoclave-treated hydrolysate. A similar pattern was observed for nitric oxide scavenging activity, where MEK1 feather hydrolysate recorded the lowest IC50 at 0.1674 g/L, followed by NaOH-treated hydrolysate at 0.2040 g/L and NaOH plus autoclave-treated hydrolysate at 0.2210 g/L. In ferric reducing power, MEK1 feather hydrolysate showed stronger reducing activity at lower concentration, although NaOH-treated hydrolysate showed a higher fitted maximum response. In contrast, ABTS radical scavenging activity showed that NaOH-treated hydrolysate had a slightly lower IC50 than MEK1 feather hydrolysate, while MEK1 feather hydrolysate showed the highest fitted maximum response.

The antioxidant capacity of MEK1 feather hydrolysate supports the earlier decision to avoid selecting bioconversion conditions based only on maximum solubilisation or feather mass loss. MEK1 feather hydrolysate showed lower IC50 values than the chemical hydrolysates in DPPH radical scavenging activity and nitric oxide scavenging activity, and

it also showed stronger ferric reducing power at lower concentration. The results indicate that microbial feather bioconversion produced a hydrolysate with comparatively strong antioxidant activity in several assay systems, although it did not produce the highest response in every assay. Differences in peptide size, amino-acid composition, and redox-active residues may have contributed to the assay-dependent responses. However, these compositional characteristics were not determined.

In contrast, NaOH plus autoclave-treated hydrolysate showed weaker activity in several assays, suggesting that severe chemical and thermal treatment may increase keratin solubilisation but compromise the resulting hydrolysate. This agrees with studies showing that antioxidant capacity in feather hydrolysates is influenced not only by soluble protein concentration, but also by peptide size, composition of amino acids, and redox-active residues such as tyrosine, cysteine, methionine, tryptophan, and histidine (Alahyaribeik et al., 2021; Bezus et al., 2021; Fakhfakh et al., 2011; Jana et al., 2022; Pei et al., 2023). Together, these findings show that MEK1 can convert feather keratin into an antioxidant active product under milder processing conditions. This supports its potential use as a value-added feather hydrolysate, while reducing dependence on chemical treatment during feather valorisation.

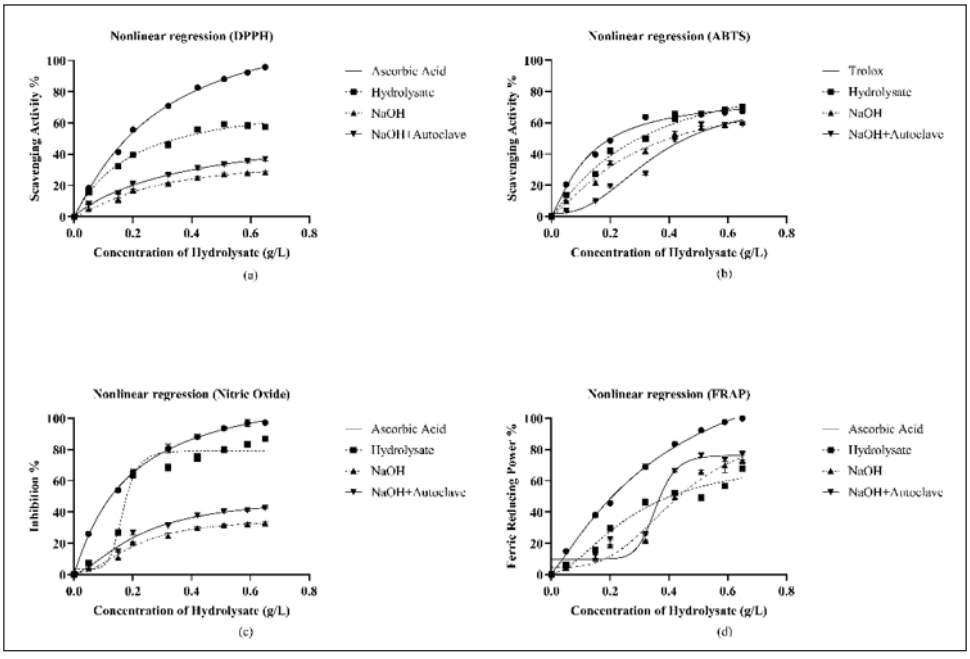


*Figure 5.* Antioxidant capacity of feather hydrolysates. (a) DPPH radical scavenging activity, (b) ABTS radical scavenging activity, (c) nitric oxide scavenging activity, and (d) ferric reducing power

*Note.* Ascorbic acid was used as the positive control for DPPH radical scavenging activity, nitric oxide scavenging activity, and ferric reducing power, while Trolox was used as the standard for ABTS radical scavenging activity.

## Determination of Effective Hydrolysate Concentration for Seed Priming

Hydrolysate concentration influenced mung bean germination and early seedling growth, with 0.5% v/v giving the best overall response (Figure 6). At this concentration, root length increased from 5.918 to 7.060 cm, shoot length from 0.988 to 1.676 cm, and total seedling length from 6.906 to 8.736 cm compared with the control. Fresh weight and dry weight also increased from 0.818 to 1.188 g and from 0.188 to 0.284 g, respectively. Germination percentage improved from 87.87% to 98.24%, while mean germination time decreased from 2.874 to 1.758 days. In contrast, higher hydrolysate concentrations at 1.0% and 2.0% v/v reduced most growth responses relative to 0.5% v/v. These results suggest that MEK1 feather hydrolysate improved germination and seedling growth within an effective concentration range, rather than showing a dose-dependent increase. Therefore, 0.5% v/v was selected as the working concentration for subsequent salinity stress validation because it produced the strongest non-inhibitory response across germination, seedling growth, and biomass accumulation.

The MEK1 feather hydrolysate was effective only within a suitable dose range, rather than increasing seedling growth. This pattern agrees with previous reports showing that feather hydrolysates can improve seed germination, root growth, shoot growth, biomass,

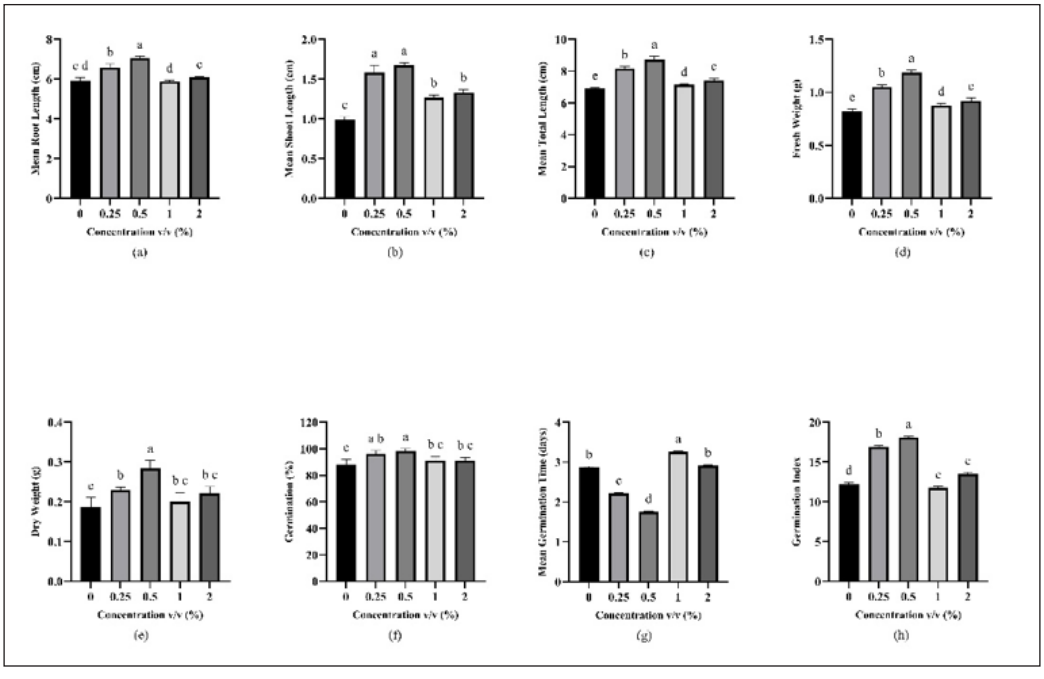


*Figure 6.* Effect of feather hydrolysate concentration on mung bean germination and early seedling growth. (a) mean root length, (b) mean shoot length, (c) mean total seedling length, (d) fresh weight, (e) dry weight, (f) germination percentage, (g) mean germination time, and (h) germination index

and early seedling establishment, with the best response commonly observed at low or intermediate concentrations (Adelere & Lateef, 2023; Bhange et al., 2016; Devi et al., 2024; Kaur et al., 2021; Paul et al., 2013; Stanly & Umesh, 2024). Similar findings have also shown that higher hydrolysate concentrations may reduce plant growth responses, possibly due to excess soluble peptides, amino acids, salts, or nitrogen compounds that can affect early seedling growth (Broutin et al., 2025; Jain et al., 2016; Możejko & Bohacz, 2023). In this study, 0.5% v/v MEK1 feather hydrolysate improved germination speed, seedling length, and biomass, suggesting that this concentration supported early growth without causing inhibition due to high concentration. This result provides a practical working concentration for seed priming and supports further testing of MEK1 feather hydrolysate under salinity stress, where the objective was to determine whether the hydrolysate could protect seedlings under stress rather than only improve growth under normal conditions.

**Salinity Stress Validation of Hydrolysate-Treated Mung Bean Seedlings**

Salinity stress increased oxidative stress markers and reduced antioxidant enzyme activity in mung bean seedlings, while 0.5% v/v MEK1 feather hydrolysate reduced these stress effects (Figure 7). Hydrogen peroxide increased from 8.4 µmol/g FW in the control to 18.9 µmol/g FW under 100 mM NaCl, while malondialdehyde increased from 11.2 to 28.6 nmol/g FW. In the salt plus hydrolysate treatment, hydrogen peroxide decreased to 10.7 µmol/g FW and malondialdehyde decreased to 15.4 nmol/g FW, corresponding to reductions of 43.4% and 46.2% compared with salt stress alone. Proline increased from 9.6 µmol/g FW in the control to 17.3 µmol/g FW under salt stress, but decreased to 11.2 µmol/g FW when hydrolysate was supplied with salt. Salt stress also reduced superoxide dismutase, catalase, and peroxidase activities to 48.7, 15.6, and 10.3 U/mg protein, respectively, while salt plus hydrolysate increased them to 61.8, 29.5, and 18.7 U/mg protein. The hydrolysate-only treatment remained close to the control, suggesting that 0.5% v/v MEK1 feather hydrolysate did not impose detectable oxidative stress under non-saline conditions.

Treatments were controlled, 0.5% v/v feather hydrolysate, 100 mM NaCl, and 100 mM NaCl plus 0.5% v/v feather hydrolysate. The stress validation result shows that MEK1 feather hydrolysate was associated with reduced oxidative damage and stronger antioxidant enzyme responses. The decrease in hydrogen peroxide and malondialdehyde suggests lower reactive oxygen accumulation and lower lipid peroxidation, while the recovery of superoxide dismutase, catalase, and peroxidase activities suggests stronger enzymatic antioxidant defense under salt exposure. The lower proline level in the salt plus hydrolysate treatment should be interpreted as a reduced stress burden rather than loss of osmotic adjustment, because it occurred together with lower oxidative damage

and higher antioxidant enzyme activity. This trend agrees with studies showing that protein hydrolysates and feather-derived hydrolysates can improve plant stress tolerance by supporting antioxidant defence and reducing oxidative injury during abiotic stress (Devi et al., 2024; Gezgincioğlu & Atici, 2023; Kaur et al., 2021; Stanly & Umesh, 2024; Zuluaga et al., 2023).

In relation to the earlier antioxidant assays, the salinity result provides biological validation that the hydrolysate was not only active in chemical antioxidant models but also associated with lower oxidative damage in seedlings. The observed response may involve combined contributions from soluble peptides, amino acids, antioxidant-active components, and modulation of endogenous antioxidant defense. However, their relative contributions were not determined.

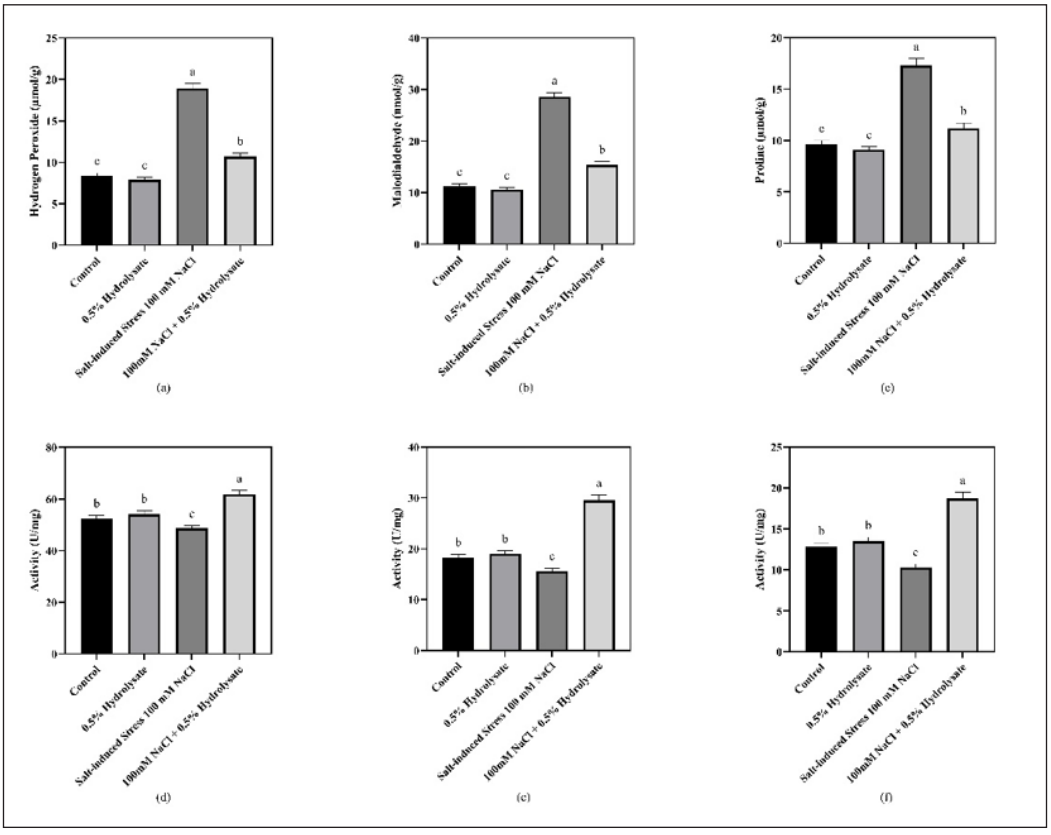


*Figure 7.* Effect of MEK1 feather hydrolysate on oxidative stress markers and antioxidant enzyme activity in mung bean seedlings under salinity stress. Panels show (a) hydrogen peroxide content, (b) malondialdehyde content, (c) proline content, (d) superoxide dismutase activity, (e) catalase activity, and (f) peroxidase activity

## CONCLUSION

*A. faecalis* MEK1 converted chicken feather keratin into an antioxidant feather hydrolysate through submerged fermentation. The optimum conditions were observed at 37 °C, pH 8.00, and 1.50% w/v feather substrate supported reductase-associated activity, keratinase activity, soluble protein release, and feather degradation. The time course pattern suggests that reductase-associated activity occurred earlier than peak keratinase activity, followed by soluble protein accumulation and feather degradation. This finding suggests that MEK1 feather bioconversion is better explained by combining sulfhydryl release and proteolysis, rather than keratinase activity alone. The hydrolysate also showed consistent antioxidant activity in DPPH radical scavenging, nitric oxide scavenging, and ferric reducing power assays, while chemical treatment did not consistently improve antioxidant performance. At 0.5% v/v, MEK1 feather hydrolysate improved mung bean germination, reduced germination time, and enhanced root growth, shoot growth, and biomass. Under salinity stress, hydrolysate reduced hydrogen peroxide, malondialdehyde, and proline accumulation while enhancing superoxide dismutase, catalase, and peroxidase activities. Further work should validate its performance under soil, greenhouse, and field conditions and identify the active peptide or amino acid fractions responsible for the observed plant response.

## IMPLICATION OF THE STUDY

The main implication of this study is that chicken feather waste should be viewed as a recoverable source of functional hydrolysate. *A. faecalis* MEK1 provides a mild biological route for converting feather keratin into an antioxidant-active hydrolysate with measurable effects on mung bean germination and salinity response. The study also suggests that MEK1 is a potent keratin degrader due to its ability to reduce disulfide bonds and proteolysis. This also prevents the process from being evaluated only by mass loss or chemical solubilisation, which may not reflect hydrolysate quality. The antioxidant assays and seedling results show that MEK1 hydrolysate has functional value at a practical working concentration of 0.5% v/v. At this concentration, it improved germination, seedling growth, and biomass, while under salinity stress, it reduced oxidative damage and supported antioxidant enzyme activity. These findings position MEK1 feather hydrolysate as a potential natural seed priming material for early stress protection. More broadly, the study strengthens the link between poultry waste valorisation, microbial bioconversion, and crop stress management through a product that can be evaluated by both biochemical function and plant response.

## LIMITATION AND RECOMMENDATION FOR FUTURE RESEARCH

Advanced optimisation of microbial feather conversion has increasingly shifted from sequential one-factor testing toward multivariable statistical designs that can identify significant process variables, quantify factor interactions, and generate predictive operating

models. Plackett-Burman screening has been used to identify influential parameters such as feather concentration, pH, temperature, inoculum level, and nutrient composition, followed by response surface methodology using central composite or Box–Behnken designs to refine the selected factors and maximise keratinase production or feather hydrolysis (Derhab et al., 2024; Ismail et al., 2025). These approaches have produced approximately 1.5- to 13.3-fold improvements over unoptimised conditions and have generally shown close agreement between predicted and experimentally validated responses (Ismail et al., 2025; Sharma et al., 2022). Statistical optimisation has also supported scale-up in solid-state and semi-pilot fermentation systems, although performance remains sensitive to substrate heterogeneity, oxygen and moisture transfer, pH control, enzyme stability, and reactor configuration (El Salamony et al., 2024). More computational approaches, including artificial neural networks, genetic algorithms, Bayesian optimisation, kinetic modelling, and model-based bioreactor control, may offer additional advantages for highly nonlinear or multi-response systems, but their application to feather-hydrolysate production remains comparatively limited. These methods were not used in the present study and are relevant mainly as future strategies for simultaneous optimisation of enzyme activity, soluble-protein release, feather degradation, product bioactivity, and scale-up efficiency.

Although microbial feather hydrolysates and related protein hydrolysate biostimulants have produced promising responses under controlled conditions, their agronomic effectiveness remains dependent on crop species, dose, soil properties, environmental conditions, and application method. Feather hydrolysate increased barley biomass in pot experiments but did not significantly improve barley yield under field conditions, demonstrating that laboratory or greenhouse responses may not translate directly into agronomic performance. Concentration-dependent effects have also been reported, with effective application levels ranging from 0.2-0.4% (v/v) for maize seedling elongation to approximately 5% (v/v) for broader improvements in plant growth, soil fertility, and microbial activity (Jagadeesan et al., 2023). Formulation and storage conditions, therefore, require systematic evaluation because peptide composition, soluble protein concentration, antioxidant activity, and microbial quality may change during storage, although short-term stability at 4 °C has been reported for chicken-feather protein hydrolysate (Jagadeesan et al., 2023). Microbial feather conversion can reduce the accumulation of keratin-rich waste and recover amino acids and bioactive peptides, but residual feather solids, microbial biomass, process water, energy consumption, and downstream treatment requirements must be considered when evaluating its overall environmental benefit (Jagadeesan et al., 2023; Jain et al., 2016). Accordingly, claims of circularity, reduced fertiliser dependence, or commercial feasibility remain provisional in the absence of comparative life cycle and techno-economic assessments. The present study did not include field validation, formulation development, long-term storage testing, waste-stream characterisation, or

formal techno-economic analysis. These aspects should therefore be evaluated before the laboratory-observed improvements in mung bean germination, seedling vigour, and oxidative-stress responses are interpreted as evidence of field performance or commercial applicability.

## ACKNOWLEDGEMENT

This work was supported by the Ministry of Higher Education Malaysia under the Fundamental Research Grant Scheme (FRGS), FRGS/1/2024/WAS04/UPM/02/25, awarded to Universiti Putra Malaysia. The authors gratefully acknowledge the financial support provided through this grant, which enabled the successful completion of this research.

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