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Research Article

Isolation, Identification and Optimization of Potential Keratinase Enzyme-Producing Bacteria from Poultry Soil: Synthesis of Zinc Oxide Keratinase-based Nanoparticles and Their Application in Plant Growth Promotion

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ABSTRACT

Poultry farming produces large amounts of feather waste, which is hard to manage due to its tough keratin content and limited eco-friendly disposal methods. Keratin-degrading microorganisms offer a cost-effective and viable alternative for the management of feather waste compared to chemical treatment methods. The present study aimed to focus on the identification and optimisation of potential keratinolytic enzyme-producing bacteria from the poultry soil. Through a molecular identification method (16S rRNA), the potent bacterium was identified as belonging to the species *Mesobacillus thioparans*. The one variable at a time approach (OVAT) investigation revealed that a high level of keratinase activity was detected at pH 7.0, 40°C, with glucose as a better carbon source and yeast as the preferred nitrogen source. Along with optimisation, we also demonstrated the beneficial use of the partially purified keratinase enzymes via synthesising zinc oxide-based nanoparticles (ZnO NPs) and evaluating their potential as plant growth promoters. According to this study, keratinase-ZnO NPs considerably improved the physiological and biochemical properties of the plants compared to the control group. There were no significant variations of chlorophyll producing plants' however, significant variations were observed in the protein and carbohydrate concentrations in enzyme treated plants, recording protein content of 3.02±0.43 µg/g and carbohydrate of -9.8±1.01 µg/g), compared to the control plants (protein is 1.24±0.16 µg/g and carbohydrate -5.3±0.76 µg/g). This study sheds new light on the application of microbially derived keratinase enzyme-based NPs for plant growth promotion potential.

1. Introduction

The growing demand for food, driven by population expansion, has significantly increased the scale of poultry farming, animal husbandry, and slaughterhouse operations, resulting in the generation of large quantities of keratinous waste, including feathers, wool, hooves, horns, and hair (Shalaby *et al.* 2021). The persistence of these non-degradable keratin-rich materials poses a serious threat to ecological balance (Nnolim and Nwodo 2020). Among these wastes, avian feathers are the most abundantly produced keratinous by-product globally (Sharma *et al.* 2022), comprising approximately 5–7% of a chicken's total body weight (Nnolim and Nwodo 2020). Feathers contain around 90% pure keratin (β-keratin), a rich supply of amino acids, proteins, and nitrogen (Sun *et al.* 2021). Keratins are a type of protein that is very fibrous and insoluble. They are made up of a-helix and b-sheet configurations and are classified into two types: soft and hard. The hard keratin present in feathers is particularly rich in sulphur-containing amino

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acids like cystine, forming extensive disulfide bonds that contribute to its structural complexity and resistance to degradation (Peng *et al.* 2019; Akram *et al.* 2020). Recent studies have highlighted microbial keratinases as promising tools to address this waste challenge (Hassan *et al.* 2020). Microbial keratinases have garnered considerable attention in the field of biotechnology due to their effectiveness in degrading resilient keratin-rich substrates and their contribution to environmentally sustainable waste management practices (Nnolim *et al.* 2020b). Although keratinases are secreted by various microorganisms, bacterial keratinases, particularly those produced by *Bacillus* species, are of special interest owing to their high activity levels and variability (Akram *et al.* 2020). Beyond their role in environmental remediation, the enzymatic degradation of keratinous waste results in the production of valuable by-products with diverse industrial applications (Nnolim and Nwodo 2020). These metabolites can be employed in the formulation of safe, non-toxic personal care products (Rai *et al.* 2020), the development of nitrogen-based fertilizers, protein-enriched animal feed, cosmetics, controlled drug delivery systems, and therapeutic products for dermatological conditions (Akram *et al.* 2020).

Keratinase enzymes have demonstrated significant potential in both agricultural and therapeutic applications. Recent research has increasingly focused on the synthesis of nanoparticles derived from keratinase enzymes, given their wide-ranging utility in biotechnology, particularly in targeted drug delivery systems. Nanoparticles are employed across numerous domains, including nanomedicine, agriculture, cell biology, food technology, and as antioxidants and antimicrobial agents. Their unique optical and electrical characteristics, stemming from their crystalline structure, along with excellent stability and dispersion, make them highly effective carriers. The biosynthesis of nanoparticles using biological entities such as enzymes, protein hydrolysates, plant extracts, and microorganisms is gaining momentum due to its eco-friendly nature (Diyanat *et al.* 2018).

Nanoparticles exhibit unique optical and electrical properties attributable to their crystalline structures, along with high stability and excellent dispersion capacity. Increasing attention is being given to the synthesis of biologically functional nanoparticles through the conjugation of proteins, pharmacological agents, ligands, and other biomolecules. Particularly, the eco-friendly synthesis of nanoparticles using enzymes, protein-rich hydrolysates, plant extracts, and microorganisms has gained significant momentum due to its sustainability and minimal environmental impact (Diyanat *et al.* 2018). Among various metallic nanoparticles explored for plant growth-promoting applications, zinc-based nanoparticles have attracted the most interest. Zinc is a vital micronutrient required for several physiological and biochemical functions in plants, such as enhancing water use efficiency, photosynthesis, protein biosynthesis, reactive oxygen species regulation, antioxidant defense, membrane stability, growth modulation, and gene expression (Noohpisheh *et al.* 2021). The application of zinc oxide nanoparticles (ZnO NPs) has been shown to enhance plant growth and development in a wide range of crops, including maize, onion, tomato, olive, capsicum, cucumber, wheat, and zucchini (Zhao *et al.* 2013; Munir *et al.* 2018; Neto *et al.* 2020; Awan *et al.* 2021; Regni *et al.* 2022).

The current study focuses on the isolation and optimization of a highly potent keratinolytic bacterial strain as well as the preliminary characterization of isolated keratinase enzyme. This study also discussing the application of keratinase enzyme, such as nanoparticle-based enzyme conjugation (keratinase-Zn oxide nanoparticle) and their application in plant growth.

2. Materials and Methods

2.1. Samples Collection

The soil samples were collected from the local poultry farm of Kozhikkode, Kerala, India (Lat 11.244737 Long. 75.861522) (Figure 1). Approximately, 50 g of soils were randomly collected from the different location of the poultry farm in a depth of 2-5 cm at areas designated for the disposal of poultry wastes, packed in a sterilized, air tight polythene bags, and immediately transferred to the microbiology laboratory.

2.2. Isolation and Screening of the Keratinolytic Bacteria

The keratinolytic bacteria were isolated through the implementation of the serial dilution and plating technique on keratinase isolation agar plates (composed of; 10 g keratin powder, 1.4 g K_2HPO_4, 0.7 g KH_2PO_4, 5 g NaCl, 0.1 g $MgSO_4$, and 20 g agar per litre dist. water, using the pour plate assay. A quantity of 1 g of each soil sample was individually mixed in 10 ml of sterile dist. water and mixed extensively. Serial dilution was performed up to a dilution factor of 10^{-5}. Afterward, 100 µl of the diluted sample was inoculated onto the surface of keratinase agar plates, and the plates were incubated at a 37°C for 24 h.

Bacillus agar and skim milk agar plate methods were employed for screening of the keratinolytic bacteria. The

A

B

Figure 1. Soil sample collection study sites (A) and (B)

composition of bacillus agar plate included 49.22 g of bacillus agar powder (Himedia, SKU M1651) mixed in 1,000 ml of dist. water, sterilized at 121°C for 15 min., and then poured aseptically in sterile petri plates for preparation the bacillus agar plates. In the case of skim milk agar plate, the composition involved 60 g casein powder, 1.4 g K_2HPO_4, 0.7 g KH_2PO_4, 5 g NaCl, 0.1 g $MgSO_4$, and 20 g agar per litre dist. water. To the agar plates, one loop of each bacterial culture was streaked on the plates to detect the keratinolytic bacteria. The seeded plates were incubated at 37°C for 24-72 h After incubation and. based upon the production of zones of

inhibition, the bacteria were selected and used for further studies.

2.3. Molecular Identification of the Potent Bacterium

The bacterium with the greatest hydrolysis zone or keratinolytic activity was subsequently selected for further investigations. For the DNA isolation, a pure culture was inoculated into Luria Bertani broth and incubated for 24 h at 37°C. The isolation of bacterial DNA was carried out according to Prashanthi *et al.* (2021). The universal primers used for the amplification of 16S rRNA fragments were a forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and a reverse primer 5′-GGTTACCTTGTTACGACT-3′. The PCR amplification parameter involved initial denaturation at 95°C for 5 min., followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1.30 min., and final extension at 72°C for 7 min (Sambrook and Russell 2001). After quality check of the sequence, this sequence was analysed in BLAST to identify the bacterial species and then deposited in NCBI GenBank (NCBI accession no. OQ410466.1).

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). The bootstrap consensus tree inferred from 100 replicates (Felsenstein 1985) was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates that were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) were shown next to the branches (Felsenstein 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with a superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among the sites of 5 categories (+G, parameter = 0.0500). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 39.02 % sites). This analysis involved 9 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. There were a total of 1520 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.* 2021).

2.4. Production of Potent Bacterium Keratinase Enzyme

For production of the keratinase enzyme, the selected bacterium was cultured in basal feather meal medium according to Rajesh *et al.* (2014) with some slight modifications. The medium composed of 0.5 g/L of NaCl; 0.4 g/L of K_2HPO_4; 0.3 g/L of KH_2PO_4; $MgSO_4$ 0.005 g/L; peptone 0.2 g/L, and feather 10 g/L. After sterilization, 1 mL of the bacterial inoculum was inoculated onto 50 mL of a production medium in a conical flask and incubated on a rotatory shaker at 37°C for 14 d. The biomass estimation was carried out for the keratinase production for up to 14 d, which was conducted in reference to Kumawat *et al.* (2017). Biomass estimation was carried out by centrifuging 1 mL of the culture medium at 5,000 rpm for 5 min. after every 48 h of incubation. After removal of the supernatant, the air died pellet was weighed.

Enzyme assay for keratinase production was carried at every 24 h of incubation. Enzyme assay was performed according to the method of Cai *et al.* (2008). Where, 1 % keratin in 0.05 M Tris HCl buffer of pH 7.0 served as the substrate, while the culture supernatant served as the crude enzyme. The assay began by mixing 1.75 mL of substrate solution and 0.25 mL of crude enzyme that was incubated at 40°C in a water bath for 10 mins. The reaction mixture without the addition of crude enzyme served as the control. After incubation, the reaction was terminated by adding 2 mL of TCA solution. Then the solution was centrifuged and the absorbance was measured at 280 nm using a UV spectrophotometer. The enzyme activity was calculated by the given equation (1):

$$U = 4 \times n \times A280 / (0.01 \times 10) \quad (1)$$

Where:
n is the dilution factor
4 is the final reaction volume (mL)
10 is the incubation time (min.)

2.5. Optimization of Keratinase Production

There are different kinds of optimisation methods employed for understanding the production of keratinase enzyme. The different optimisation factors used in the experiment analysis included different substrates (10 g/L of casein, peptone, and skim milk powder), nitrogen sources (0.02 % of tryptone, peptone, and beef extract),

carbon sources (10 g/L of glucose, sucrose, and maltose), incubation temperatures (30°C, 40°C, and 50°C), and initial pH values (5-9 using 1.0 N HCL and 1.0 N NaOH). The inocubated flasks were incubated for 4 d on a shaker at 200 rpm. All the assays were performed using one variable at a time approach (OVAT) (Revankar *et al.* 2023).

2.6. Partial Purification of Keratinase Enzyme

The bacterial culture was centrifuged at 5,000 rpm for 5 min. and the supernatant was collected. Afterward, ammonium sulphate was added slowly by continuous stirring using a magnetic stirrer for precipitating the protein until 80% saturation. Then it was allowed to stand overnight at 4°C and the pellet was collected by centrifugation. The obtained the pellet was dissolved in phosphate buffer (pH 8.0) and applied to a DEAE (diethylaminoethyl)-Sepharose Fast Flow (FF) column (1.9 cm × 20.0 cm), which was processed at a flow rate of 3 mL/ h with 20 mmol/L Tris-HCl buffer (pH 8.0), and every obtained 3 mL fraction were collected. After precipitation and purification, the amount of protein liberated was calculated by protein estimation using BSA as a standard referring to Lowry *et al.* (1951) method.

2.7. Preparation and Characterisation of Keratinase-Based Zinc Oxide Nanoparticles

Synthesis of zinc oxide nanoparticles (ZnONPs) (0.1 M zinc acetate dehydrate) using keratinase was conducted according to Jeyabharathi *et al.* (2022) with slight modifications. Stirring of zinc acetate dehydrate (0.7 mL) and partial column purified keratinase (0.3 mL) reaction solution was applied for 2 h, and 2 M of NaOH solution was incorporated (drop by drop until colour change). The precipitated suspension was incubated overnight. After the appearance of white precipitate, it was centrifuged to get the pellet, and then washed with dist. water to remove the impurities. The obtained sample was dried and used for further study.

The optical properties of the synthesised NPs were assessed with UV-Visible spectrophotometer (Labtronics LT291 spectrophotometer with a scanning range of 300-600 nm) and Fourier Transform Infrared (FTIR) spectroscopy (scanning range of 400-4,000 cm^{-1}), according to Bagewadi *et al.* (2016). FE-SEM EDAX was used for the morphological ultrastructural characterisation and determination of elemental composition of the synthesised ZnO NPs.

2.8. Plant growth promoting activity of synthesised keratinase-ZnONPs

During experimental analysis in the greenhouse, seeds of green gram (*Vigna radiata* L.) were preliminarily washed in running tap water for 5 min. in order to remove adhering soil particles or any debris. Then, they were washed in 70% of ethanol for 1 min., in 2% sodium hypochlorite for 1 min., and in sterilised disti. water 15 times (Anand and Ramani 2021). Fifty surface sterilized seeds were soaked in a keratinase-ZnONPs and keratinase solutions overnight at room temperature along with the control seeds. In this study, each pot (15 cm × 14 cm width) was filled with 250 g of steam sterilized (autoclaved) soil. Moisture content of the soil was maintained at 70% (v/v) of field capacity with continuous watering of the plants. After 15 days, the shoot length, root length, and leaf count, biochemical parameters, including chlorophyll, carbohydrate (Anthrone method), and protein contents were also documented in reference to Lowry *et al.* (1951); Hedge and Hofretter (1962); Ekanayake and Adeleke (1996).

2.9. Statistical Analysis

All the experiments were conducted in three independent assays with three replicate. The data were represented as mean ± SD. The variation among treatment groups was estimated using one-way ANOVA at significance level of 5% for all analyses and the sample size of the experiments was 10 (i.e., n = 10). All the experiments were conducted independently and the statistical significance was maintained as $p<0.05$. All the statistical analyses were performed in Origin 2025 statistical tool.

3. Results

3.1. Isolation of the Keratinolytic Bacterium

The study aimed at isolation of potential keratinase producing bacteria from the poultry soil. After initial screening of the soil samples, we selected a pure highly potent keratinolytic bacterial strain from the poultry farm soil. *Bacillus* agar was used for screening the keratinolytic *Bacillus* sp. bacteria. The isolated strain was subjected to keratin agar and skim milk agar aasays and the hydrolytic zone was observed. Compared to keratin agar, the isolated bacterium showed high level of hydrolytic zone in the skim milk agar (Figure 2). In the keratinase and biomass production of the selected

isolate, highest keratinase production was observed on the 8th day recording 0.066±0.007, and the biomass production was observed on the 10th day (0.681±0.009) (Figure 3).

The NCBI BLAST analysis of 16S rRNA fragment confirmed that the isolated bacteria show 100% similarity with *Mesobacillus thioparans*. This keratinolytic novel strain named as *Mesobacillus thioparans* strain JAS with NCBI accession No. OQ410466. The maximum likelihood phylogenetic analysis of the strain indicated a high degree of similarity with *B. jeotagli* and *B. cohnii* (Figure 4).

3.2. Optimization of Keratinase Production

In the optimum condition for keratinase production was observed in the pH of 7 (0.884±0.07 U/mL) and the temperature was 40°C (0.844±0.06 U/mL). In the case of effect of substrate analysis, highest amount of enzyme activity was observed in the casein (0.745±0.01 U/mL) and followed by skim milk (0.734±0.01 U/mL) and peptone (0.593±0.04 U/mL). Yeast extract was the good nitrogen source which showing high keratinase activity (0.863±0.08), and lowest by tryptone (0.699±0.06). Glucose act as good carbon source for isolated strain which was showing high degree of

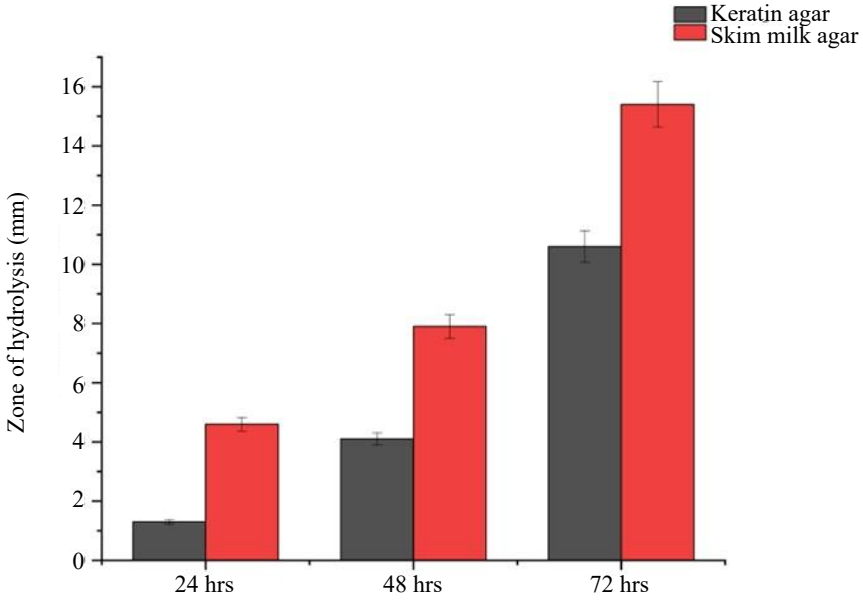

Figure 2. Hydrolytic activity of highly potent isolated strain, *Mesobacillus thioparans* strain JAS in keratin and skim milk agar

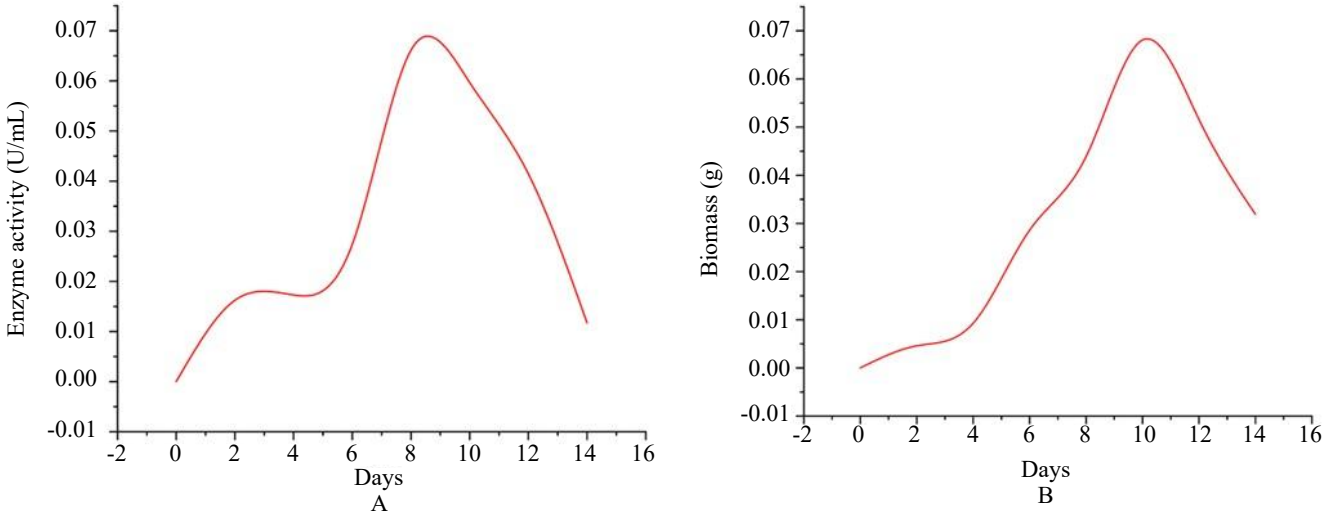

Figure 3. (A) Keratinase enzyme activity (B) biomass production of *Mesobacillus thioparans* strain JAS

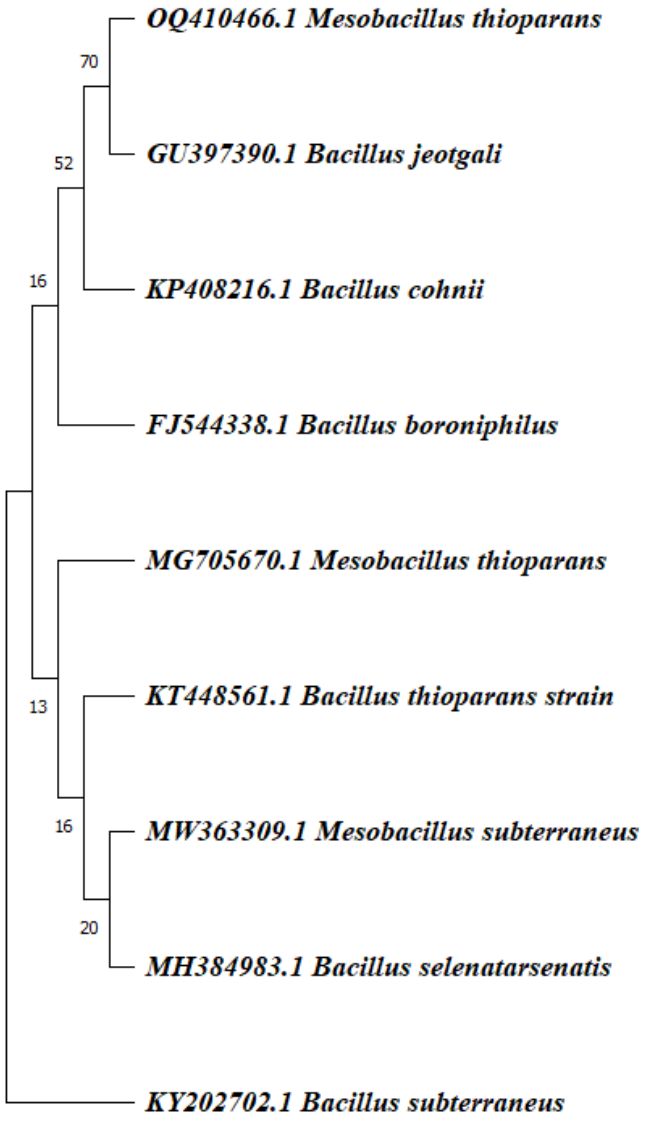

Figure 4. Maximum likelihood MUSCLE aligned, 1,000 bootstrap based phylogeny analysis of potent keratinolytic bacterial strain with closely related species

keratinolytic activity (0.898±0.08) and followed by beef extract (0.631±0.04) and sucrose (0.502±0.09). The enzyme activity and biomass production are exclusively correlated with each other (Figure 5).

3.3. Partial Purification of Keratinase Enzyme

The crude sample, ammonium sulphate precipitated and column purified samples were subjected to protein estimation. The highest amount of protein was documented in the crude sample (0.631 mg/mL) and followed by column purified (0.603 mg/mL) and ammonium sulphate participated (0.446 mg/mL).

3.4. Characterization of Keratinase-ZnO Nanoparticle

The ZnONP synthesis was initially confirmed by the transformation in the colour of reaction mixture from colourless to milky white precipitate. The UV-Visible spectrum (Figure 6A) absorption peak at 372.4 nm indicated as the presence of zinc oxide nanoparticle. In the FTIR analysis (Figure 6B), the peak at 470.63 cm^{-1} could be due to zinc and oxygen bonding vibration. The presence of peaks at 424.34 cm^{-1} for ZnONPs.

The FE-SEM analysis revealed the morphology of synthesised nanoparticle and their size range in between 24.50 nm to 52.70 nm at 1 µm magnification, and the shape was round and spherical (Figure 6C). The concentration of zinc oxide was measured in EDAX analysis, the synthesised nanoparticle contains 47.55% of zinc oxide nanoparticle (Figure 6D).

3.5. Effect of Keratinase-ZnO NP on Growth of Green Gram

The keratinase-ZnONP significantly ($p<0.05$) improve the growth of plant (Figure 7). In the case of shoot length, after 15 days, the control plant showed 7.0±0.98 cm whereas the synthesised NPs showed 13.5±1.01 cm. Same kind of impact was also documented in leaf count, high number of leaves was observed in keratinase-ZnONPs treated groups and followed by keratinase and control group (Figure 7 and 8A).

The plant physiological activity was documented through estimating by chlorophyll a, b, total, carbohydrate and protein. There is no significant variations was observed in the keratinase-ZnONPs (chlorophyll a – 9.367±0.98 µg/g; chlorophyll b – 17.772±1.23 µg/g; total chlorophyll – 27.220±2.56 µg/g) treated plant chlorophyll content as compared to control (chlorophyll a – 8.699±0.74 µg/g; chlorophyll b – 16.681±1.03 µg/g; total chlorophyll – 25.455±1.98 µg/g) and keratinase (chlorophyll a – 9.068±0.76 µg/g; chlorophyll b – 17.510±1.46 µg/g; total chlorophyll – 26.655±2.16 µg/g) treated group (Figure 8B). Significant variations ($p<0.05$) were observed in the case of protein and carbohydrate estimation in the treated and control groups. In the case of protein, control plant showed 1.24±0.16 µg/g protein whereas the keratinase treated sample is 3.02±0.43 µg/g and keratinase-ZnONP treated sample is 4.0±0.21 µg/g (Figure 8C). Highest concentration of carbohydrate

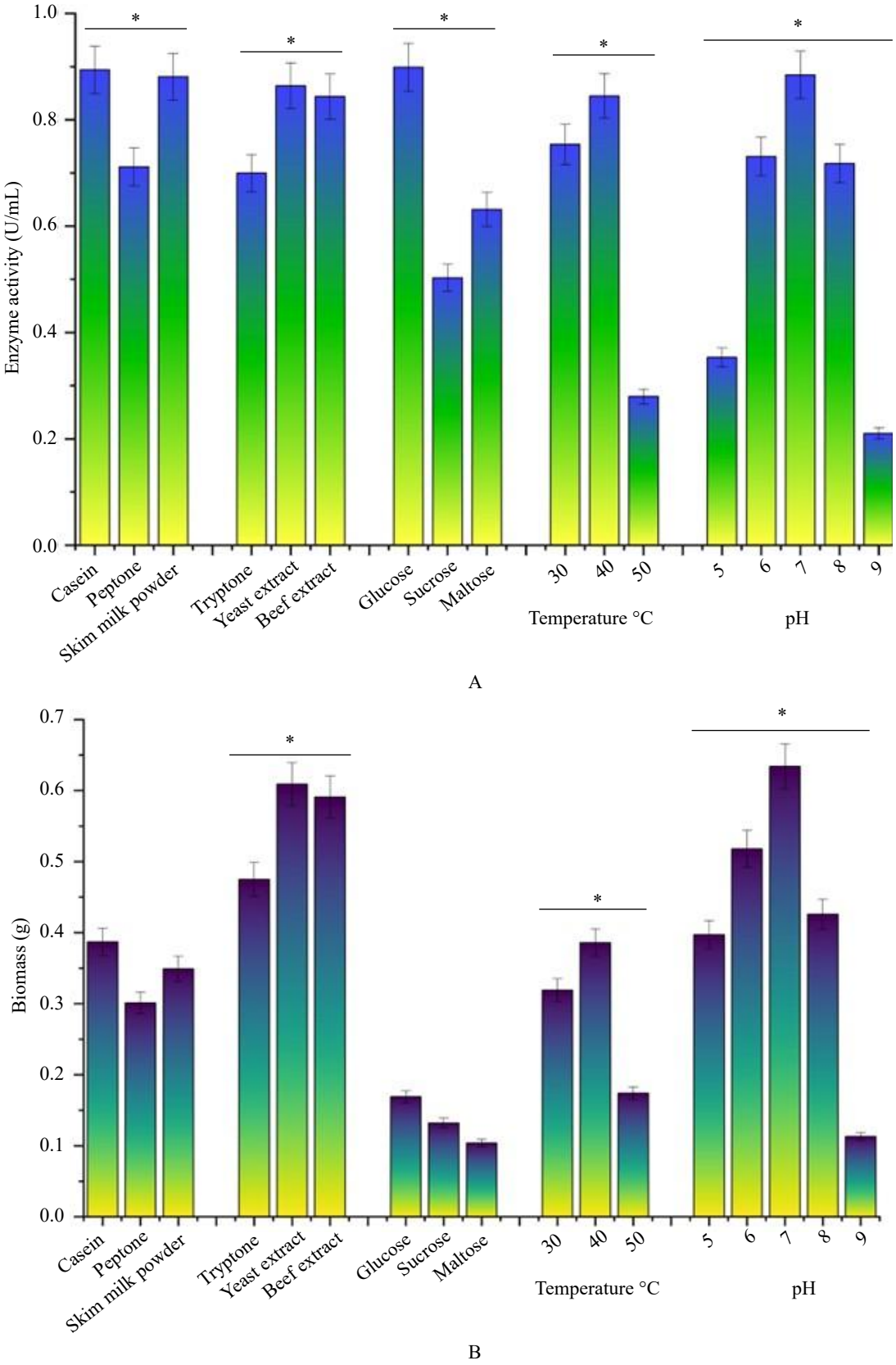

Figure 5. Optimization of Mesobacillus thioparans strain JAS (A) enzyme activity, and (B) biomass production

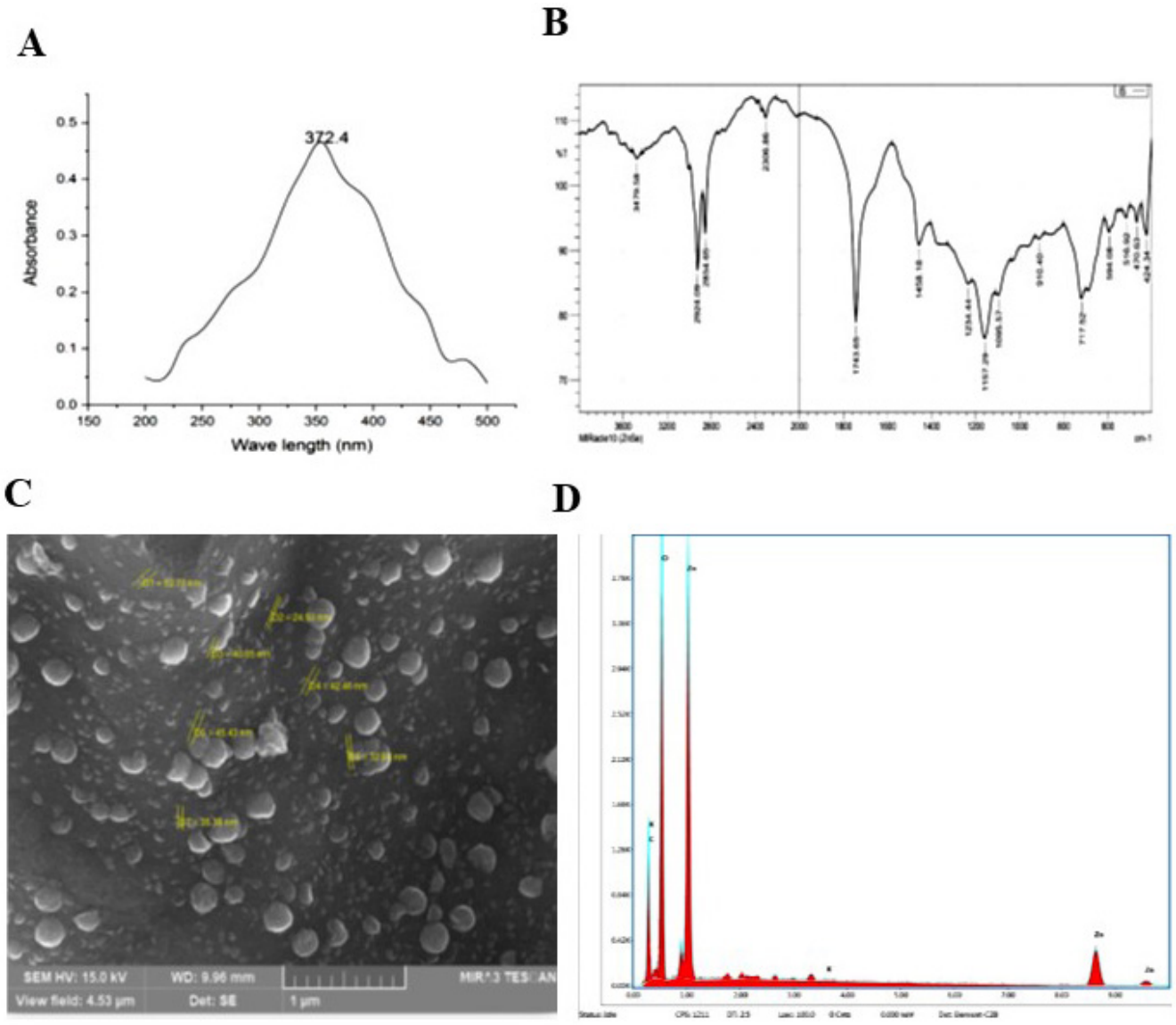

Figure 6. Characterization of Zinc oxide nanoparticles (A) UV-Visible spectrophotometer (B) FTIR (C) FE-SEM image and (D) EDAX analysis

was observed in the keratinase-ZnONP treated group (9.8±1.01 µg/g) and followed by keratinase (7.5±0.93 µg/g) and control group (5.3±0.76 µg/g) (Figure 8D).

4. Discussion

Keratinolytic bacteria are capable of colonizing a wide array of keratin-rich substrates, enzymatically degrading them to utilize keratin as a source of carbon and nitrogen. These microorganisms are identified based on their ability to break down keratinous materials such as feathers and hair, often accompanied by the production of keratinase enzymes. The present study aimed to isolate efficient keratinolytic bacterial strains from poultry farm environments, facilitate the biosynthesis of nanoparticles using the isolated strains, and evaluate their application in promoting plant growth.

In the present study, various keratinolytic bacterial strains were screened for their enzymatic activity. Among them, one strain exhibited the highest keratinase activity. Molecular identification using 16S rRNA sequencing confirmed the strain as *Mesobacillus thioparans*. Numerous keratin-degrading bacteria have been reported from diverse environments. For instance, Bacillus sp. NDS-10 was isolated from soil

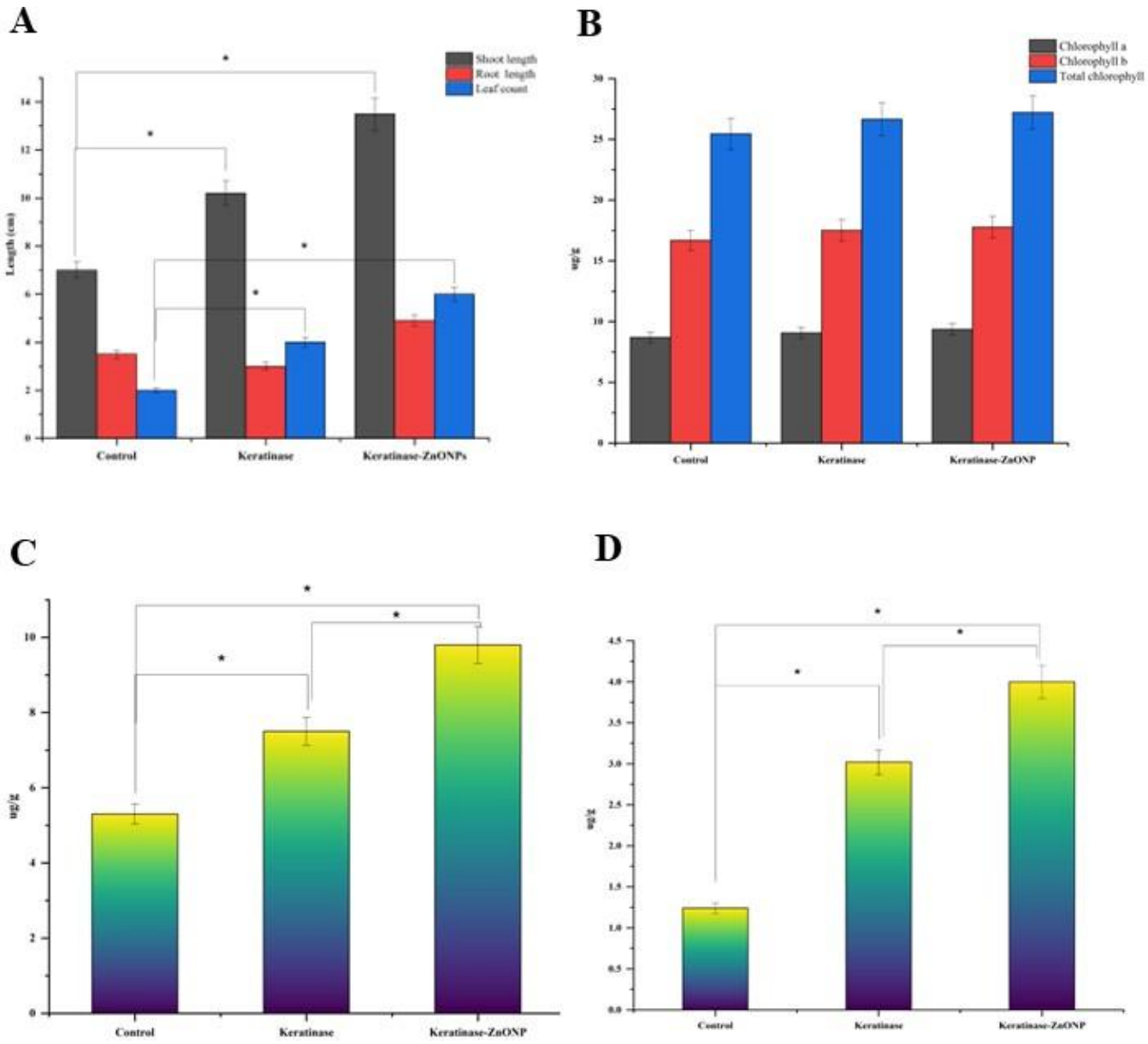

Figure 7. Plant growth promoting activity (A) Growth, (B) Chlorophyll content, (C) protein, and (D) carbohydrate. N = 10, *p<0.05

(Akram *et al.* 2020), *Pseudomonas aeruginosa* 4-3 from slaughterhouse waste (Pei *et al.* 2023), *Bacillus cereus* from poultry dump soil (Yadav *et al.* 2022), *Bacillus thuringiensis* strain MT1 from cattle yards (Hassan *et al.* 2020), and *Bacillus pumilus* AR57 from slaughterhouse soil (Jagadeesan *et al.* 2020). Among these, *Bacillus* species have been consistently recognized for their efficiency in bioremediating keratin-rich proteinaceous waste, which is generally considered recalcitrant (Revankar *et al.* 2023).

The isolated bacterial strain exhibited notable hydrolytic activity on both keratin and skim milk agar. Peak enzyme production was observed between days 8 and 10, after which activity declined, likely due to biomass accumulation. Saibabu *et al.* (2013) suggested that keratinase production is influenced by the concentration and availability of keratin, where excessive feather meal can inhibit enzyme synthesis through catabolic repression. Optimization of the culture conditions revealed that casein supported greater enzyme activity than peptone, and among nitrogen sources, yeast extract proved superior to tryptone and beef extract. These results align with those of Sivakumar *et al.* (2013), who reported that yeast extract significantly enhances keratinase activity, while peptone-based media yield comparatively lower

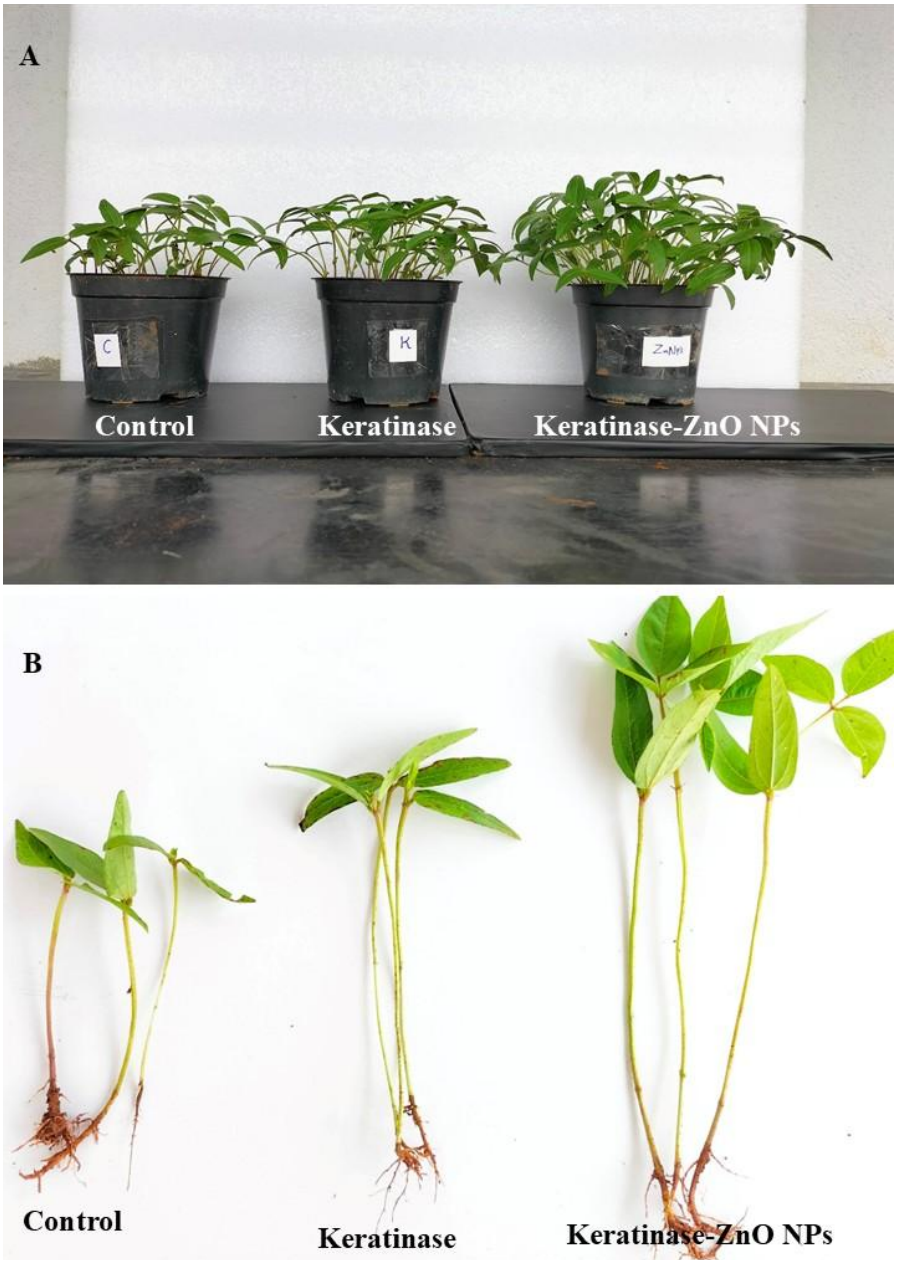

Figure 8. Plant growth promoting activity (A) Experiment set-up, (B) Growth activity of plant

levels. Additionally, enzyme synthesis was found to be highly sensitive to culture parameters such as carbon and nitrogen sources, pH, and temperature. Similar observations were made by Suh *et al.* (2001), who demonstrated that *Bacillus subtilis* and *Bacillus pumilus* produced maximum keratinase activity at 40°C.

In this investigation, keratinase-based zinc oxide (ZnO) nanoparticles were successfully synthesised using partially purified keratinase. The nanoparticles ranged in size from 24.50 to 52.70 nm and exhibited a high zinc content. While most previous studies have focused on the use of crude keratinase for nanoparticle synthesis, the present work highlights the synthesis using partially purified enzyme. The formation of nanoparticles was confirmed by UV–Visible spectrophotometry and FTIR analysis, and their morphological characteristics were further validated using FE-SEM.

Evaluating the effect of nanoparticles on plant growth is essential for assessing both their potential toxicity and suitability for agricultural application. Parameters such as plant height, leaf area, and

biomass serve as key phytotoxicity biomarkers (Ali *et al.* 2015). In our study, plants treated with keratinase-ZnO nanoparticles exhibited significantly enhanced shoot length and leaf numbers compared to both control and keratinase-only treated groups. Moreover, the application of keratinase-ZnO NPs improved photosynthetic efficiency and increased carbohydrate concentrations in the treated plants. These results are consistent with findings by Mukherjee *et al.* (2016), who proposed that such enhancements may be attributed to the nutritional behaviour of the nanoparticles or their dissociated ions, along with zinc's known regulatory role in plant physiology. Zinc contributes to enzyme activation, protein synthesis, cell elongation, membrane integrity, and overall metabolic efficiency (Cakmak 2008; Boonchuay *et al.* 2013; Singh *et al.* 2013).

Our findings suggest that keratinase can effectively degrade keratinous waste, enriching the soil, while ZnO NPs enhance plant metabolic function. The integration of keratinase with ZnO nanoparticles presents a promising and novel approach for agricultural enhancement. However, further research is required to fully characterise and purify the keratinase enzyme, sequence its gene, and explore its potential industrial applications via genetic engineering.

4.1. Limitations of the Study

While this study demonstrates the potential of keratinase-ZnO nanoparticles in enhancing plant growth, several limitations should be acknowledged. First, the enzymatic activity and stability of the keratinase-ZnO complex were not assessed under variable environmental conditions, such as fluctuating pH, temperature, or field-relevant stressors. Second, the study focused on short-term pot experiments without evaluating long-term effects or environmental fate of the nanoparticles, including potential soil accumulation or impacts on microbial communities. Third, nutrient release (e.g., zinc ions or keratin hydrolysates) was not quantitatively analyzed, limiting mechanistic understanding. Additionally, comparisons with conventional zinc fertilizers or commercially available nanoformulations were not included, which would help assess relative efficiency. Finally, the scalability and formulation aspects for field application were not addressed. Future studies should include detailed nutrient profiling, soil ecotoxicity assessments, and field-scale trials to validate the broader agricultural applicability of this nano-biofertilizer approach.

In conclusion, this study successfully isolated and optimised Mesobacillus thioparans strain JAS, a strong keratinase-producing bacteria from poultry soil. Under optimal conditions, the enzyme showed considerable keratinolytic activity (pH 7.0, 40°C, glucose as a carbon source, and yeast extract as a nitrogen source). Furthermore, the study used a novel technique to synthesise keratinase-based zinc oxide nanoparticles (ZnO NPs) and assess their impact in boosting plant growth. ZnO NPs' nanoscale size and stability were validated through characterisation, and plant growth assays demonstrated significant improvements in shoot length, leaf count, chlorophyll content, protein, and carbohydrate levels. These findings demonstrate the potential of keratinase-ZnO NPs as an environmentally friendly biotechnological tool for agricultural applications. Future research should prioritise large-scale production, field trials, and genetic alterations to improve keratinase activity for broader industrial and environmental uses.

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