Keratinase-Mediated Zinc Oxide Nanoparticles from Poultry Feather Waste Valorization: Characterization, Stability and Reusability

K.P. Jhansi Rani[1], Suvarnalatha Devi Potireddy[2*], V.V. Lakshmi[3]

[1,2,3] Department of Applied Microbiology, Sri Padmavati Mahila Visvavidyalayam, Tirupati -517502, Andhra Pradesh, India.
*Corresponding author: drsuvarnaspmvv@gmail.com,

ABSTRACT
The principles of green technology emphasize sustainable, energy-efficient, and environmentally benign processes that minimize chemical waste while maximizing resource recovery. Within this paradigm, nanoparticles-based formulations have emerged as synergistic strategies to enhance industrial biocatalysts. Nanoparticles approaches improve enzyme utilization by confining enzymes or microbial cells within a distinct phase, while still permitting free diffusion of substrates and products. Among biocatalysts, keratinase has become pivotal for waste valorization and circular bioeconomy applications. Enabling repeated catalytic cycles can reduce costs and improve efficiency and reusability. The present study shows the results of keratinase zinc nanoparticles synthesis, characterization, and reusability capacity. Characterization studies included UV-visible spectroscopy, Fourier- transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), Dynamic light scattering (DLS), and scanning electron microscopy (SEM) analysis. The keratinase-assisted KZnNPs synthesis follows similar green-friendly routes, producing nanoparticles with more stability and industrial applicability. Enzyme activity remained maximum up to 12 consecutive batches of feather degradation**.** The keratinase zinc nanoparticles were reused up to six cycles with 67.11% residual activity, while 53.6% activity was retained till the 12th cycle. This is crucial for industrial use as it decreases expenses and boosts efficiency. This biogenic pathway avoids hazardous reducing agents, ensuring safer, sustainable production while maintaining catalytic activity. These innovations enhance enzyme stability, reusability, and catalytic efficiency, while valorizing keratin-rich waste into valuable products, Such as amino acids, peptides, biofertilizers, and bioplastics. Overall, keratinase-mediated ZnO nanoparticles demonstrate a promising role in feather waste valorization, enabling eco-friendly, cost-effective applications across environmental and industrial domains.

Keywords: Keratinase, Green technology, Nanoparticles, valorization, Eco-friendly, cost effective.

Introduction

Recent advances focus on nano-immobilization, where nanocarriers provide a high surface-area-to-volume ratio, protecting enzymes from denaturation and enabling controlled release or recovery. The term nano-keratinase encompasses two related concepts: keratinase immobilized on or within nanoscale supports and systems integrated with nonmaterial to enhance the catalytic function of keratinase. In both cases, nanoscale architecture modifies enzyme behavior, yielding enhanced catalytic efficiency, improved stability, higher storage resilience, and easier recovery. Decorating nanoparticles with keratinase creates dense reaction zones, accelerating keratin waste degradation compared to free enzymes. Nanocarriers such as carbon nano-tubes, magnetic nanoparticles (MNPs), and metal-organic frameworks (MOFs) have proven effective, combining biocompatibility, biodegradability, and eco-friendly characteristics (Brandelli et al.,2010; Hassan et al.,2020).Nanotechnology has gotten a lot of attention because it can help solve problems in biotechnology, keep the environment clean, and drive progress. Among all the nonmaterial that have been studied, zinc oxide (ZnO) nanoparticles have stood out because of their physical and chemical features. Fighting germs makes them good choices for uses like coatings on surfaces, sensors for biology, ways to deliver medicine, packaging for food, cleaning water, and tools for health care (Sirelkhat et al*.,* 2015).Keratinase-based nanostructures, metal nanoparticles-notably zinc (ZnNPs)-are particularly significant. Keratinase can act as both a reducing and stabilizing agent, enabling biogenic synthesis that eliminates toxic chemicals and aligns with green chemistry principles. Recent studies of (Sharma et al*.,* 2023; Saba et al*.,* 2025). Demonstrate that keratinase from *Pseudomonas aeruginosa* or *Bacillus* species reduces silver ions to metallic nanoparticles, yielding stable, catalytically active AgNPs, suitable for feather degradation, antimicrobial coatings and biocatalysis. Keratinase-assisted ZnNPs synthesis follows similar eco-friendly routes, producing nanoparticles with high stability and industrial applicability. This biogenic pathway avoids hazardous reducing agents such as sodium borohydride, ensuring safer, sustainable production while maintaining catalytic activity. Keratinase-assisted ZnNPs synthesis follows similar biogenic principles (Gopinath et al., 2015; Srivastava et al., 2021). Recent studies highlight the potential of green-synthesized silver nanoparticles as carriers for keratinase, enabling efficient valorization of poultry feather waste while avoiding toxic reagents.

However, some limitations remain, such as scale-up challenges, high nanoparticles costs in low-margin waste applications, and environmental safety concerns. Thus, nano- Keratinases should be positioned not as universal replacements but as specialized strategies for selected applications where operational gains justify added complexity. By integrating immobilization and nanotechnology, keratinase systems exemplify the transition from conventional chemical hydrolysis to sustainable biocatalytic processes. These innovations enhance enzyme stability, reusability, and catalytic efficiency, while valorizing keratin-rich waste into valuable products such as amino acids, peptides, biofertilizers, and bioplastics. Together, they reinforce the principles of Green Chemistry and advance the circular bioeconomy (Hassan et al*.,* 2020; Martinez et al., 2020; Saba et al*.,* 2025). By integrating enzyme-based approaches with nanotechnology, this work aims to contribute to the development of environmentally conscious strategies for poultry-waste valorization and the potential utilization of biologically synthesized nonmaterial. However, compared to plant- or microbe-based methods, enzyme-assisted synthesis of ZnO nanoparticles has received relatively less attention (Yugay Y., and Shkryl, Y., 2026). Keratinase, an extracellular protease capable of breaking down highly recalcitrant keratin-rich materials such as feathers, hair, and wool with unique potential in several areas. Keratinase combines biodegradation capacity with a proteinaceous structure that contains amino, carboxyl, hydroxyl, and sulfhydryl groups. These functional groups can interact with metal ions, regulate nanoparticles nucleation and growth, and prevent aggregation by acting as natural stabilizers (Hanna, D. H., et al*.,* 2025 and Mapaka, L., et al*.,* 2025). Due to excellent substrate specificity and biodegradation property, keratinase has gained a significant interest in waste valorization, environmental biotechnology and industrial bioprocessing. Thus, keratinase not only serves as a catalyst for nanoparticles synthesis but also as a capping agent that enhances stability and reusability (Gupta and Ramnani, et al*.,*2006; Vikash, V., et al*.,* 2025).In this study, we present an eco-friendly approach for the biogenic synthesis of ZnO nanoparticles using keratinase and utilize it for degradation of poultry feather waste batches. This study explores the integration of waste valorization, enzyme biotechnology, and nanotechnology for the development of functional nonmaterials. Keratinase-assisted ZnO nanoparticles offer potential advantages in terms of stability, reusability, and biocompatibility. These properties suggest opportunities for their further investigation in biomedical, environmental, and industrial applications, while highlighting the importance of sustainable approaches to nanoparticles development.

MATERIALS AND METHOD

Source of keratin substrate and Production of Keratinase enzyme

Feather samples are used as source of keratin were collected from a native poultry farm at Tirupati, Andhra Pradesh, India. The source of keratin for the enzyme production was washed, dried and grounded into fine powder. The production process was carried out following the method described by Jeevana Lakshmi et al., (2013) and Jhansi Rani et al., (2026), with slight modifications. After fermentation, broth was centrifuged at 5000 rpm for 20 min at 4℃ to discard the remnant substrate and microbial biomass. The clear solution collected after centrifugation was taken as the crude keratinase.

Keratinase Enzyme Purification

The culture filtrate obtained was partially purified by Ammonium sulphate precipitation by following the method of Bernfeld (1955) with continuous addition of solid 60% ammonium sulphate at 4°C under continuous stirring. The proteins precipitated by this salting out method were recovered by centrifugation in a cooling centrifuge at 12,000 rpm for 15 minutes target protein was collected as a pellet and suspended in 50 mM Tris HCL buffer (pH 8.0). The sample was dialyzed at cooling temperature against a 23 kD cut-off to remove residual salt ions. The dialyzed sample collected was used for further purification and characterization studies (Riffel, A*.,* et al*.,* 2003; Brandelli, A*.,* et al., 2010).

Fast Protein Liquid Chromatography and Molecular Mass Determination

The dialysate obtained from the above step was concentrated by Sephadex-G-25 GE- Healthcare, AKTA purifier Column, NaCl with different concentrations (pH 8.0.) Column equilibrated with 100mM Tris HCL and 20mM Sodium chloride, Sample elution was carried with 250Mm Tris HCL, pH 8.0, 20mM Sodium chloride. The pure protein fractions eluted were characterized with the same buffer at a retention time of 1ml / min of the fraction collected. After column purification, the collected fractions were checked for quality of the expressed protein on 12% SDS-PAGE gels stained with Coomassie Brilliant Blue stain and destained with acetic acid and methanol. Polyacrylamide gel electrophoresis PAGE (12%w/w) in the presence of sodium dodecyl sulphate (SDS) was carried out by the method of Laemmli (1970). The electrophoresed protein gels were stained with Coomassie brilliant blue R250 Sigma, (USA).

Protein Quantification and Keratinase Activity Assay

The crude and purified keratinase enzyme samples were tested for their protein content and keratinase activity to determine the efficiency of the purification process. The total protein content was measured using Lowry's method (Lowry et al*.,* 1951), with BSA (bovine serum albumin) used as the standard. The absorbance was measured at 660 nm. Keratinase activity was measured using feather powder as the substrate, following the method described by Yu et al., (1968), with slight modifications. Distilled water was used as the control. One unit (U) of keratinase activity was defined as the increase in absorbance at 280 nm per minute under the assay conditions (Yu et al., 1968 and Koutb et al., 2023).

Biosynthesis of Keratinase-Conjugated Zinc Nanoparticles (KZnNPs)

In this study, Zinc nanoparticles associated with Purified keratinase (KZnNPs) were synthesized using keratinase obtained from *Bacillus* sp. MBF20. The enzyme preparation obtained under the optimized conditions established by Response Surface Methodology (RSM) and purified enzyme as mentioned above was used for nanoparticles synthesis. For the biosynthesis of KZnNPs, 45 ml of 50 mM Zinc acetate solution was prepared and mixed with 5 ml of the respective keratinase enzyme preparation. The reaction mixture was continuously stirred and incubated at 37°C for 2 hours**.** Following this incubation, the reaction mixture was further maintained at room temperature for 16 hours to facilitate nanoparticles formation. (Alahdal et al., and Jeyabharathi et al., 2003).The formation of KZnNPs was initially monitored visually by observing the change in colour of the reaction mixture. The appearance of a Brownish-yellow colour was considered a preliminary indication of nanoparticles formation. A corresponding zinc acetate solution without keratinase was maintained as a control, where applicable. Following completion of the reaction, the nanoparticles suspension was centrifuged at 10,000 rpm for 20 min to recover the synthesized KZnNPs. The resulting pellet was collected and washed three times with distilled water to remove unreacted zinc ions and other soluble components. The washed nanoparticles were subsequently dried at 50°C. The dried KZnNPs powder was stored appropriately and used for further physicochemical characterization and biological evaluation.

The synthesized KZnNPs were characterized using complementary analytical techniques to determine their optical properties, particle size, surface charge, functional groups, crystalline structure, and surface morphology. The characterization studies included UV–Visible spectroscopy, dynamic light scattering (DLS), zeta-potential analysis, Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (SEM–EDX).

The formation of KZnNPs was confirmed using UV–Visible spectroscopy. The nanoparticles were dispersed in 50 mM phosphate buffer (1 mg/mL), and the absorption spectrum was recorded from 190 to 600 nm using a Jasco V-630 UV–Vis spectrophotometer. The characteristic absorption peak confirmed nanoparticles formation (De Leersnyder et al., 2019). The average particle size, size distribution, and surface charge of KZnNPs were determined using Dynamic Light Scattering (DLS) with a Horiba SZ-100 analyzer. These results provided information about nanoparticles size, uniformity, and colloidal stability (Karmakar et al., 2019).

FTIR spectroscopy was used to identify the functional groups involved in the formation and stabilization of KZnNPs (Pasieczna-Patkowska et al., 2025). Approximately 10 mg of KZnNPs were mixed with potassium bromide (KBr) and made into pellets. The FTIR spectrum was recorded from 400 to 4000 cm^{-1} using a Bruker Alpha FTIR spectrometer. The crystal structure of KZnNPs was analyzed using an X-ray diffractometer (Panalytical Aeris XRD, Spectris Technologies). The obtained diffraction patterns were compared with standard patterns to confirm the crystalline nature of the nanoparticles (Cullity and Stock et al., 2014).

Scanning Electron Microscopy (SEM) was used to observe the surface morphology of KZnNPs. Energy Dispersive X-ray (EDX) analysis was used to determine their elemental composition. For SEM–EDX analysis, the samples were placed on aluminium stubs, coated with a thin layer of gold, and examined at different magnifications. EDX analysis was used to confirm the presence of zinc and other associated elements (Yacouba et al., 2023).

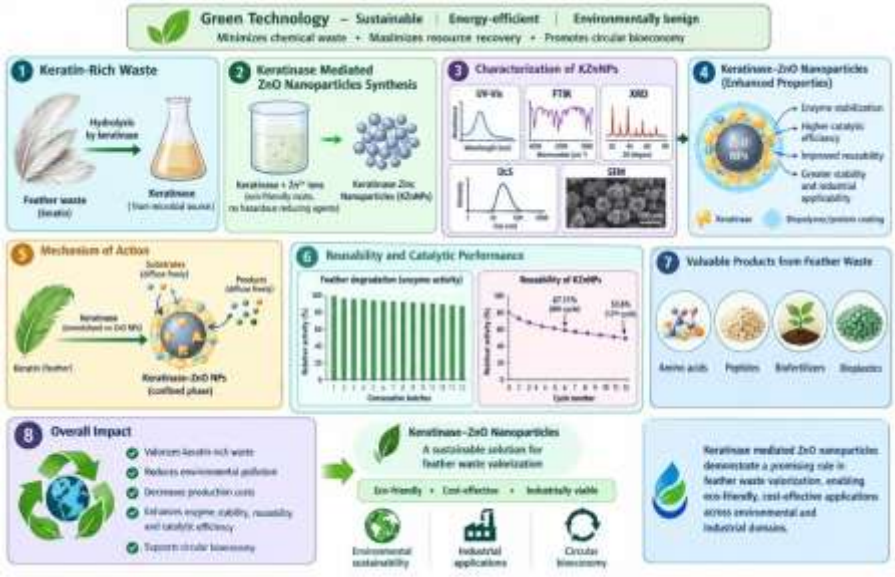


Fig 1: Schematic representation of Keratinase mediated Zinc Nanoparticles synthesis, characterization and its application in feather waste valorization

Feather Degradation, Recycling and Reusability of Synthesized KZnNPs

Feather degradation experiments were performed to evaluate the keratinolytic activity of the synthesized KZnNPs. The ability of KZnNPs to degrade chicken feathers and produce keratin Hydrolysates was evaluated based on keratinase activity. Keratinase activity was determined according to the method described by Riffel et al., (2003), with slight modifications, and the activity was expressed as an increase in absorbance at 280 nm (Lateef et al., 2015). This recovery and reuse procedure was repeated for successive degradation cycles. After each cycle, the soluble feather hydrolysate was collected for subsequent product analysis, while the recovered nanoparticles were employed in the next batch. The retained keratinolytic activity was determined using the keratinase assay described, and the relative residual activity was calculated by considering the activity observed during the first cycle as 100%. The reusability of the nanoparticles-bound enzyme was assessed based on its ability to retain keratinolytic activity over successive degradation cycles.

Storage Stability of KZnNPs

The storage stability of KZnNPs was assessed by storing the nanoparticles at 4°C for 12 months. UV–Visible spectra and keratinase activity were monitored at three-month intervals

Evaluation of Keratinase Activity

The amount of the enzyme keratinase was estimated by Jeevana Lakshmi (2013) and Riffel et al., (2003) spectrophotometric method.

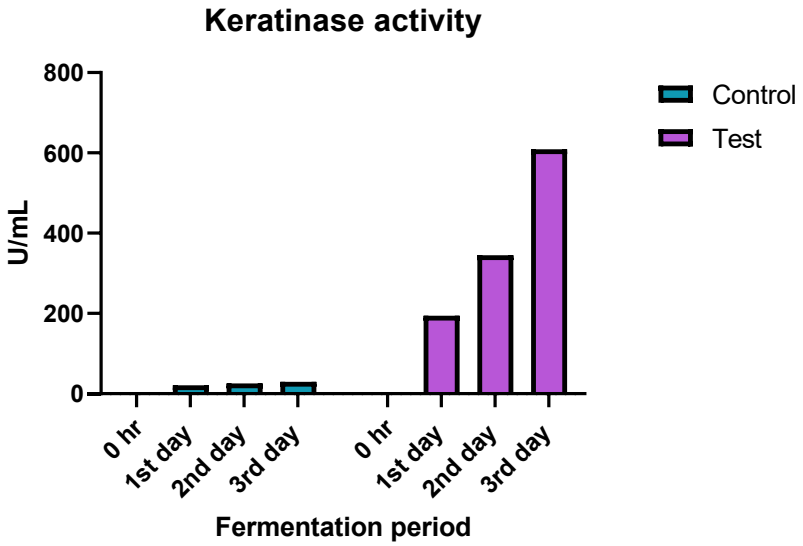


Fig-2: The activity of Keratinase during fermentation

Keratinase production increased progressively during the fermentation period. No enzyme activity was detected at 0 h in either the control or test samples. The test culture showed keratinase activity maximum on 3rd day with 609.0 KU/ml.In contrast, the control showed negligible activity throughout the incubation period, ranging from 0.00 to 2.29 KU/ml. Complete degradation of the keratin substrate was achieved within 3 days of incubation, indicating efficient keratinolytic activity of the bacterial isolate.

The gradual increase in keratinase production suggests active microbial growth and secretion of extracellular enzymes in response to keratin substrate utilization. The maximum enzyme activity (609 KU/ml) was observed on the third day, coinciding with complete feather degradation. Similar observations have been reported in keratinolytic bacteria, where maximum enzyme production generally occurs after 48–72 h of fermentation due to enhanced substrate induction and nutrient utilization.

Purification of Keratinase

Overall it was observed that Specific activity 221.34 KU/mg with fold increase of 5.14 (table 1) Total protein, enzyme activity, purification fold and specific activity of the enzyme were measured at different stages to evaluate the purification efficiency.

Samples	Protein content (mg/ml)	Enzyme Activity (KU/ml)	Specific Activity (KU/mg)	Purification Fold
Crude keratinase Extract	14.15	609	43.03	1.00
Precipitation 60%	11.90	729	61.2	1.42

Dialysis	9.50	900	94.73	2.20
Sephadex G-25 Gel filtration	5.81	1283	221.34	5.14

Table 1: The purification profile of keratinase enzyme

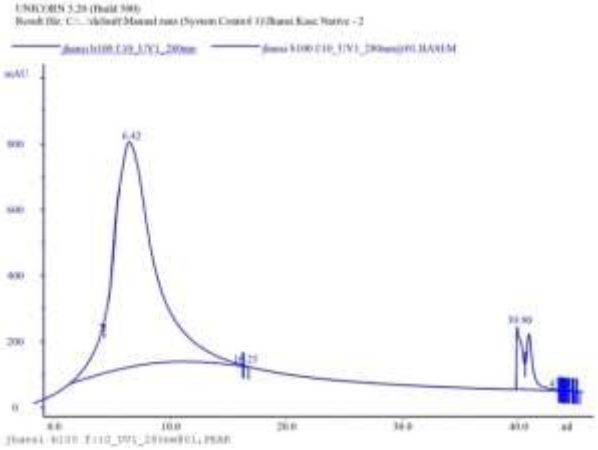


Fig -3 FPLC peak for Native Keratinase enzyme

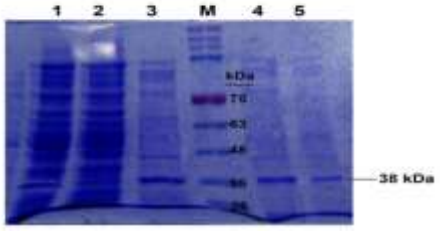


Fig -4 Well 1and 2 Crude, 3rd well Dialyzed, M marker, well 4and 5 eluted samples from FPLC

Synthesis of Keratinase-Mediated Zinc Nanoparticles

The biosynthesis of zinc nanoparticles (KZnNPs) was successfully achieved using purified keratinase as a reducing agent and stabilizing agent. In the reaction, a clear colour change was observed from pale yellow to dark yellow, which was a preliminary sign of the formation of nanoparticles.

The formation of dark yellow colour is due to the interaction of incident light with nanoparticles, and its optical properties were observed as shown in Figure 5.These visual changes indicated successful conversion of zinc ions to nanoparticle forms mediated by biomolecules from the keratinase.

This mechanism of formation of the nanoparticles is based on the bio-reduction of Zn^{2+} ions from the zinc acetate solution by amino acids and peptides produced during hydrolysis of keratin. The functional group that can donate electrons of amino acids like cysteine, tyrosine and tryptophan can help reduce the zinc ions to zinc nuclei. These nuclei then proceed to nucleate and grow into stable zinc nanoparticles.

UV –VISIBLE Spectroscopic Analysis of Keratinase-Mediated Zinc Nanoparticles (KZnNPs)

UV–Visible spectroscopy was used to check the formation of keratinase-mediated zinc nanoparticles (KZnNPs). The spectrum was obtained in the range of 200 to 800 nm. The synthesized nanoparticles had characteristic absorption in the UV region, showing the distinct maximum absorption peak at 370nm as shown in Figure 5, which is characteristic of zinc oxide nanoparticles and corresponds to the electronic transition. This absorption peak has been reported with biogenic production of ZnO nanoparticles.

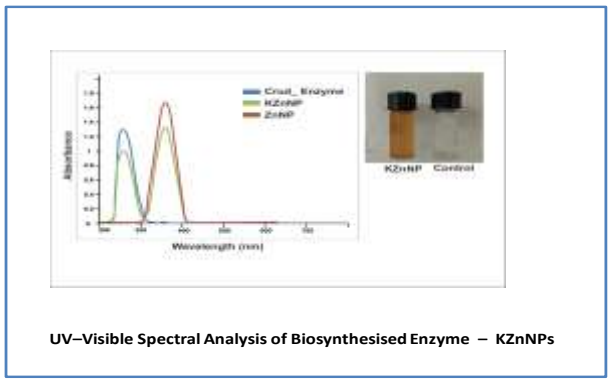


Fig-5: UV-Visible spectroscopic analysis of Keratinase Zinc nanoparticles

The sharp absorption peak suggests that the nanoparticles have been successfully synthesized and have a relatively narrow distribution of particle sizes. The presence of some broad peaks in visible region indicates that there was little aggregation of nanoparticles, probably because of the stabilizing effect of biomolecules produced by keratinase which acts as a natural capping agent.

The outcomes align with the successful synthesis of stable zinc nanoparticles with the aid of biogenic keratinase-mediated synthesis and its applications in biomedical and antibacterial field.

Particle size analysis of KZnNPs and Zeta potential analysis

The result shows that the keratinase mediated zinc nanoparticles (KZnNPs) have an average particle size of 53nm and a polydispersity index (PDI) of 0.2208 which shows that the particle distribution is moderately uniform. The single dominant peak was found; indicating that the designed nanoparticles were successfully synthesized and are dispersed. The results from the zeta potential analysis indicated that their surface charge was −49 mV which indicated good colloidal stability, and less nanoparticle aggregation as illustrated in figure-6. Therefore, these results suggest that the synthesis of zinc nanoparticles using keratinase would result in stable nanoparticles of the metal which should be useful to biomedical and environmental applications

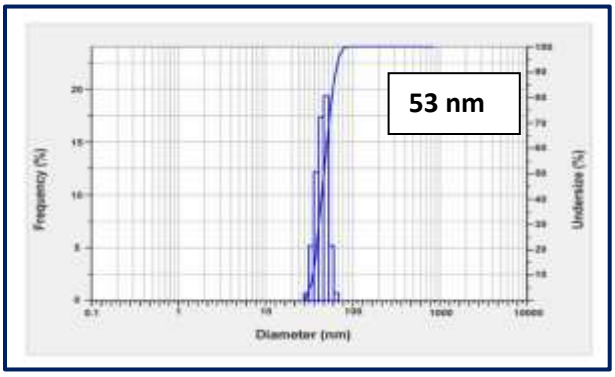


Fig-6 A: Particle size Analysis of KZnNPs

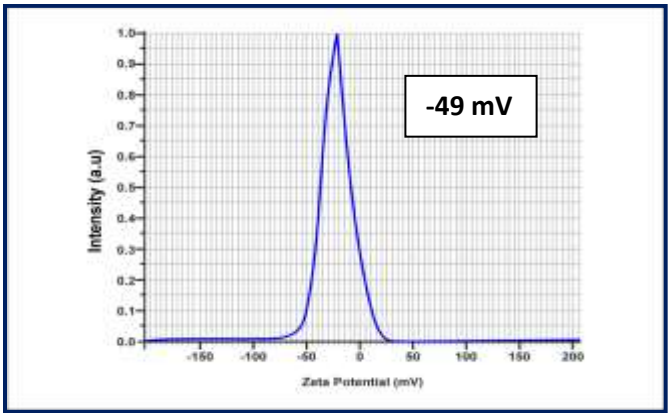


Fig-6 B: Zeta potential of KZnNPs

Fourier-transform infrared (FTIR) spectroscopic analysis of KZnNPs

FTIR analysis was conducted to identify the functional groups in the process of zinc nanoparticles (KZnNPs) reduction, capping and stabilization by keratinase. FTIR spectrum of KZnNPs appeared as shown in figure -7, which shows a very large band at 3367.99 cm^{-1} attributed to O–H stretching vibrations, suggesting interaction of biomolecules and water molecules with the surface of the nanoparticles. The N–H stretching band appeared at 2926.69 cm^{-1} and C=O had stretching vibrations in the peak at 1620.61 cm^{-1} which were attributed to amide groups. The absorption band at 1402.27 cm^{-1} is due to the O–H bending of carboxylic acids. These functional groups may be attributed to amino acid, peptides and proteins in the keratinase Hydrolysates which are likely to play a role in the synthesis and stabilization of the nanoparticles. Other peaks seen at 840.80 cm^{-1} and 554.83 cm^{-1} were tentatively identified as alkene and halo compound vibrations. Importantly, a prominent absorption peak at 426.78 cm-1 was attributed to Zn–O stretching vibrations, indicating successful Nanoparticles preparation. The overall results of FTIR show that the biomolecules derived from keratinase was used as natural reducing and capping agents in the green synthesis of KZnNPs.

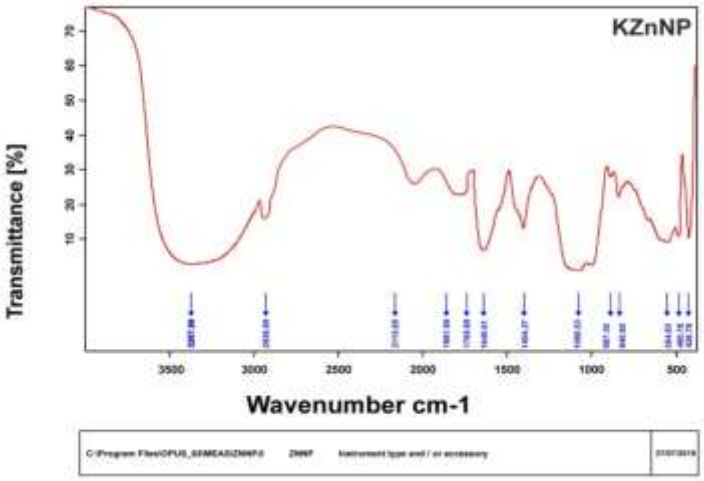


Fig-7: Fourier-transform infrared (FTIR) spectroscopic analysis of KZnNPs

X-ray Diffraction (XRD) Analysis of Keratinase-Mediated Zinc Nanoparticles (KZnNPs)

The crystalline nature of the derived keratinase-mediated zinc nanoparticles was analyzed with X-ray diffraction (XRD) analysis, and the diffraction patterns exhibited characteristic peaks at 2θ values of 32.8°, 36.0°, 37.7°, 38.3°, 41.1°, 45.7°, 52.9°, and 77.7°. Of these, the highest peak was found at 36.0°, which is the (101) crystal plane of the crystal of zinc oxide as depicted in figure-8, diffraction peaks obtained were in good agreement with those of the standard hexagonal wurtzite structure used for ZnO nanoparticles (JCPDS Card No. 36-1451), which indicates the successful crystalline formation of ZnO nanoparticles. The reflections of the (100), (002), (101), (102), and (110) planes further confirmed the existence of a hexagonal crystal lattice.

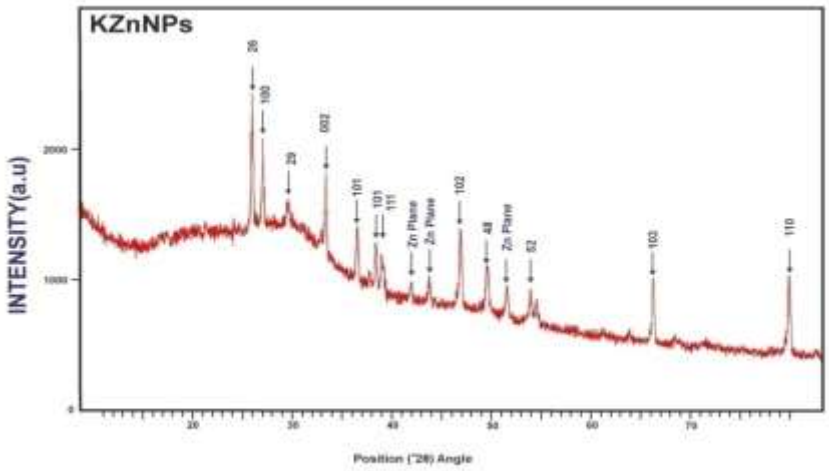


Fig-8: X-ray Diffraction (XRD) Analysis of KZnNPs

Good crystallinity of the synthesized nanoparticles, with some peak broadening as expected in nanometer-sized crystallites, is suggested by the sharp diffraction peaks. The crystallite size calculated with the Debye–Scherrer formula was around 42-50 nm, which is in accordance with the Nano size of the particles from the particle size analysis.

Moreover, the lack of peaks due to impurities indicated that the synthesized ZnO nanoparticles are of high purity. This indicates that the green synthesis is a promising method for the preparation of highly crystalline and stable ZnO nanoparticles using keratinase enzyme for application in biomedical and environmental fields.

SEM-EDX Analysis of Keratinase-Mediated Zinc Nanoparticles (KZnNPs)

The synthesized keratinase zinc oxide nanoparticles were found to have pseudo-spherical morphology with significant agglomeration by SEM analysis, as presented in Figure 9. The rough surface morphology confirmed successful synthesis and capping of nanoparticles with biomolecules using keratinase. The aggregation seen was traced to the high surface energy of the ZnO nanoparticles and intermolecular interactions between the protein-coated nanoparticles.

The SEM image showed that the individual nanoparticles have a size of 40 to 80 nm, but some degree of aggregation led to larger clusters.

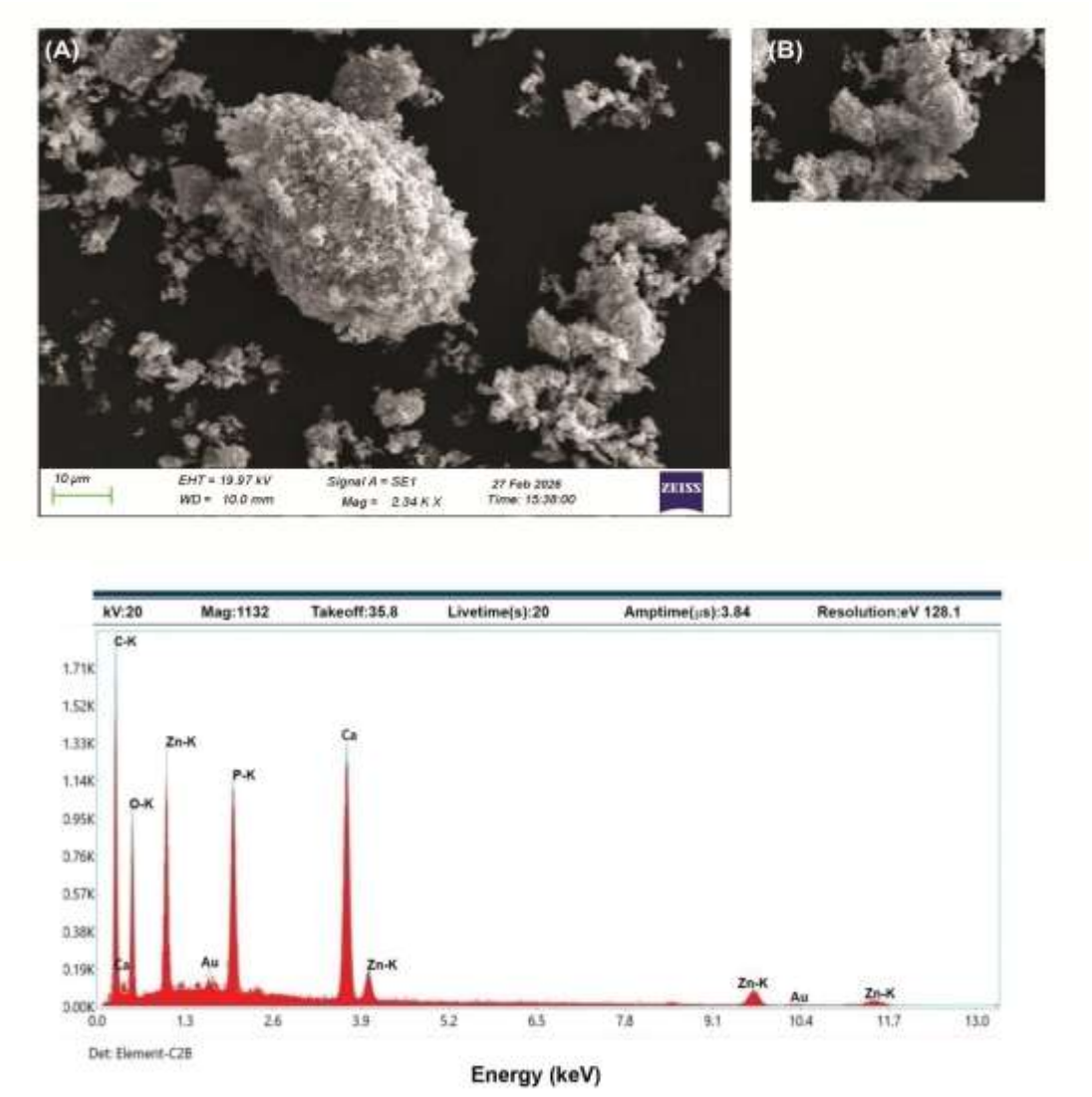


Fig 9: SEM-EDX Analysis of Keratinase-Mediated Zinc Nanoparticles (KZnNPs)

The EDX graph represents the characteristic peaks for zinc (Zn) and oxygen (O), indicating the successful synthesis of zinc oxide nanoparticles. Small peaks of carbon (C) were also observed, likely due to the presence of organic biomolecules such as keratinase and conductive carbon tape used in the sample preparation process. There were extra gold (Au) peaks from gold sputter coating prior to SEM analysis and a small niobium (Nb) peak from the

instrument/sample holder. The SEM–EDX analysis in total confirmed the efficient biosynthesis of ZnO nanoparticles of nanoscale size and high elemental purity using keratinase.

Recycling of Keratinase Zinc Nanoparticles

By contrast, Table 3 and Figure 10 illustrate the graph results for KZnNPs synthesized using the purified keratinase, which proved to be very good with respect to recyclability. Regarding the keratinase activity, the use of nanoparticles did not significantly reduce the activity of keratinase (more than 604 KU/mL) until the 9th batch. However, a starting drop was seen after the 10th cycle and in Batch 11 and 12 the activity was reduced by 568 KU/mL,368 and 123KU/mL respectively. A high percentage of enzymatic activity after several cycles of use indicates the stability of purified KZnNPs and their potential for reusability.Enzyme activity remained maximum up to 12consecutive batches of feather degradation**.** Figure 13.The keratinase zinc nanoparticles were recycled up to 6 cycles with 67.11% residual activity, while 53.6% activity was retained till the 12th cycle. These results indicate the effectiveness of the noncomplex up to 12 consecutive cycles. (Isha Sharma et al., 2013)

Sample Batch	Keratinase activity KU/ml
Blank	0
Control	41
1 Batch	1183
2 Batch	1097
3 Batch	1008
4 Batch	989
5 Batch	867
6 Batch	789
7 Batch	699
8 Batch	646
9 Batch	604
10 Batch	568
11 Batch	368
12 Batch	123

Table-3: Recycling of Keratinase Zinc Nanoparticles from purified enzyme

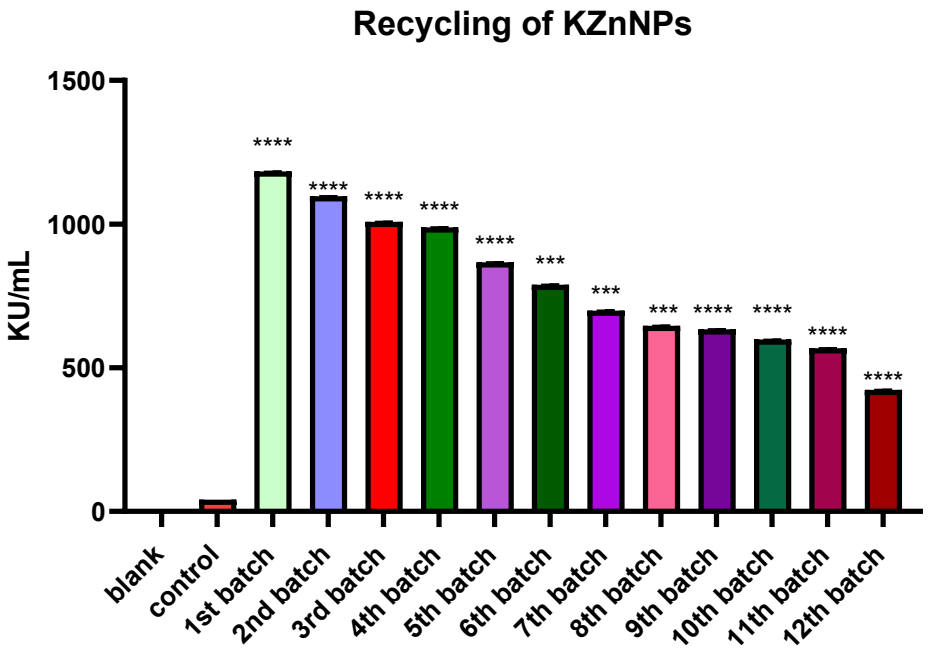


Fig 10: Graphical representation of Recycling of Keratinase Zinc Nanoparticles from purified enzyme.

Effect of KZnNPs on feather degradation levels with significance of ****p < 0.0001 vs ***p<0.001

The prolonged catalytic performance of purified KZnNPs may lead to the immobilization and stabilization of keratinase on the surface, which protects the enzyme from denaturation and enhances operational stability.Thus, the potential of KZnNPs as recyclable biocatalysts for ecological feather waste bioconversion and industrial keratin degradation processes. Nanoparticles synthesized using crude keratinase retained considerable activity for up to three consecutive cycles, whereas those synthesized using purified keratinase remained active for up to fourteen cycles (Nnolim, N.E. et al., 2020).

Storage Stability:

To check its shelf life, keratinase silver nanoparticles were stored at 4°C, and after every 3 months, comparative spectral analysis was done; it was found that there was no significant change in the SPR peak even after 12 months, which indicates the long shelf life of the prepared nanoparticles (Figure 11). It also demonstrates that the enzyme activity of Lyophilized KZnNPs maintained at the storage temperature of 4°C was also unaltered even after 12 months.

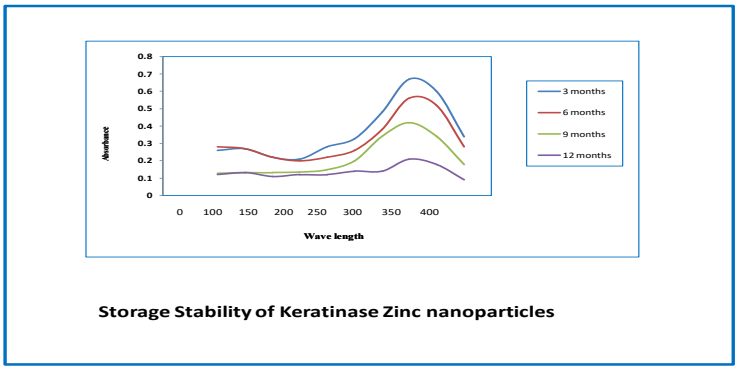


Figure-11: Storage stability of keratinase zinc nanoparticles

Discussion

The production of Zinc Oxide Nanoparticles (ZnO NPs) by using poultry feather waste-derived keratinase as the reducing agent is presented as an eco-friendly process. During the fermentation process, the keratinase activity increased gradually, and the maximum activity was observed on the third day. This is comparable to previous studies where maximum enzyme production was observed within 2–3 days of the incubation period, as there was active microbial growth and efficient utilization of keratin (Gupta and Ramnani et al., 2006 and Brandelli et al*.,* 2010). The purification procedure increased the specific activity of the enzyme, indicating that the unwanted proteins were effectively removed and that the enzyme was more effective. In similar studies, in which traditional purification techniques such as ammonium sulfate precipitation were used, the same results have been obtained (Riffel et al*.,* 2003). This proved that the purified keratinase can be used for other applications. Many studies of green synthesis report the visible colour change that occurs during the process as a sign of the formation of nanoparticles (Iravani et al., 2011; Ahmed et al., 2016). The presence of the peak at 370 nm in the UV-visible spectrum aligned with previous reports, which confirmed the formation of ZnO nanoparticles (Sirelkhatim et al., 2015). The particle size analysis indicated that the particle size distribution was uniform, comparable to other methods of enzyme-mediated nanoparticle production, with an average size of nanoscale. The negative zeta potential indicates that the nanoparticles are stable and do not easily clump together. This stability results from the protein layer surrounding the nanoparticles repelling each other. This was also confirmed by the FTIR analysis, which revealed the presence of functional groups associated with the formation and stabilization of nanoparticles (Singh et al., 2016). The nanoparticles were found to be crystalline with a typical ZnO structure by XRD analysis. There are no impurities, indicating a clean and efficient synthesis process. The SEM image was also found to have well-shaped nanoparticles, like in previous studies. (Ahmed et al., 2016).

Good reusability was also shown by the nanoparticles, which retained their activity after several cycles. This is crucial for industrial use as it decreases expenses and boosts efficiency. Very little loss in activity was observed for the nanoparticles during the 12 months of storage. This long-term stability is probably attributed to the protective effect of the nanoparticles (Riffel et al*.,* 2003).

Conclusion

This study may prove a biogenic method to synthesize the zinc oxide nanoparticles using poultry feather waste-derived keratinase. The findings have shown that keratinase can be obtained, purified, and applied not only for the degradation of keratin but also for the synthesis of nanoparticles. Various characterization techniques clearly prove the synthesis of zinc oxide nanoparticles, demonstrating the reliability as well as efficiency of the method. The nanoparticles were stable, well-structured, and hadexcellent functional properties. Importantly, the activity and stability of the keratinase

were enhanced after it was attached to these nanoparticles as compared to the free enzyme. The nanoparticles also showed excellent reusability and prolonged stability, which makes them appropriate for practical applications. This helps to save money and increase efficiency. In conclusion, this study offers an ecological approach to poultry waste management and the creation of valuable nanomaterials. Compared to traditional chemical methods, this biogenic approach is safer and more environmentally friendly. The results suggest that enzyme technology and nanotechnology have great promise in the future for use in biotechnology and environmental management.

Acknowledgement
We sincerely acknowledge the **DST-FIST**, Dept. of Applied Microbiology, **DST-CURIE** Sri Padmavati Mahila Visvavidyalayam, Tirupati, and Genomix Carl-Pulivendhula for providing research facilities.

Funding: This research received no specific grant from any funding agency.

Conflict of interest: None

References

1. Brandelli, A., Daroit, D.J. and Riffel, A., 2010. Biochemical features of microbial keratinases and their production and applications. Applied Microbiology and Biotechnology, 85(6), pp.1735-1750. DOI: 10.1007/s00253-009-2398-5.
2. Hassan, S., Hassan, F.U., and Rehman, M.S.U., 2020. Nano-particles of Trace Minerals in Poultry Nutrition: Potential Applications and Future Prospects: Hassan et al. Biological trace element research, 195(2), 591-612. DOI: 10.1007/s12011-019-01862-9.
3. Sirelkhatim, A., Mahmud, S., Seeni, A., Kaus, N.H.M., Ann, L.C., Bakhori, S.K.M., Hasan, H. and Mohamad, D., 2015. Review on zinc oxide nanoparticles: antibacterial activity and tocxity mechanism. Nano-microliters, 7(3), 219-242. DOI: 10.1007/s40820-015-0040-x .
4. Sharma, I., Gupta, P., and Kango, N., 2023. Synthesis and characterization of keratinase-laden green-synthesized silver nanoparticles for valorization of feather keratin. Scientific Reports, 13(1), p.11608. DOI: 10.1038/s41598-023-38721-6.
5. Saba, M., Farooq, S., Alessa, A.H., Bektas, K.I., Belduz, A.O., Khan, A.Z., Shah, A.A., Badshah, M. and Khan, S., 2025. Green synthesis of silver nanoparticles using keratinase from Pseudomonas aeruginosa-C1M, characterization, and applications as a novel multifunctional biocatalyst. BMC Biotechnology, 25(1), p. 27. DOI: 10.1186/s12896-025-00959-5.
6. Gopinath, V., Priyadarshini, S., Venkatkumar, G., Saravanan, M., and Mubarak Ali, D., 2015. Tribulus terrestris leaf-mediated biosynthesis of stable antibacterial silver nanoparticles. PharmaceuticalNanotechnology, *3*(1),pp.26-34. DOI:10.2174/2211738503666150626160843.
7. Srivastav, A., Ganjewala, D., Singhal, R.K., Rajput, V.D., Minkina, T., Voloshina, M., Srivastava, S. and Shrivastava, M., 2021. Effect of ZnO nanoparticles on growth and biochemical responses of wheat and maize Plants, 10(12), p.2556. DOI: 10.3390/plants10122556.
8. Yugay, Y.A., Usoltseva, R.V., Silant'Ev, V.E., Egorova, A.E., Karabtsov, A.A., Kumeiko, V.V., Ermakova, S.P., Bulgakov, V.P., and Shkryl, Y.N., 2020. Synthesis of bioactive silver nanoparticles using alginate, fucoidan, and laminaran from brown algae as reducing and stabilizing agents. Carbohydrate Polymers, 245, p.116547. DOI: 10.1016/j.carbpol.2020.116547.
9. Hanna, D.H., Nady, D.S., Wasef, M.W., Fakhry, M.H., Mohamed, F., Isaac, D.M., Kirolos, M.M., Azmy, M.S., Hakeem, G.E., & Fathy, C.A. (2025). Plant-derived nanoparticles: Green synthesis, factors, and bioactivities. Next Materials. DOI:10.1016/j.nxmate.2025.101275.
10. Mpaka, L., Nnolim, N.E., and Nwodo, U.U., 2025. Microbial keratinolytic: eco-friendly valorization of keratinous waste into functional peptides. Microorganisms, 13(10), p.2270. DOI: 10.3390/microorganisms13102270.
11. Gupta, R. and Ramnani, P., 2006. Microbial keratinases and their prospective applications: an overview. Applied Microbiology and Biotechnology, 70(1), 21-33. DOI: 10.1007/s00253-005-0239-8.
12. Vikash, V.L., Kamini, N.R., Ponesakki, G. and Anandasadagopan, S.K., 2025. Keratinous bioresources: their generation, microbial degradation, and value enhancement for biotechnological applications. World Journal of Microbiology and Biotechnology, 41(4), p.118. DOI: 10.1007/s11274-025-04336-4.
13. Jeevana Lakshmi, P., Kumari Chitturi, C.M., and Lakshmi, V.V., 2013. Efficient degradation of feather by keratinase-producing Bacillus sp. International Journal of Microbiology, 2013(1), p.608321. DOI: 10.1155/2013/608321.
14. Vedula Venkata, K.P., JhansiRani, and Potireddy Suvarna Latha. (2025). Efficient Biodegradation of Poultry Feathers Using Keratinase Nanoparticles. International Journal on Science and Technology. DOI: 16. 10.71097/IJSAT.v16.i4.9210.
15. Riffel, A., Lucas, F., Heeb, P., and Brandelli, A., 2003. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Archives of Microbiology, 179(4), 258-265. DOI: 10.1007/s00203-003-0525-8.

16. Brandelli, A., Daroit, D.J., and Riffel, A., 2010. Biochemical features of microbial keratinases and their production and applications. Applied microbiology and biotechnology, 85(6), 1735-1750. DOI: 10.1007/s00253-009-2398-5.
17. Yu, R.J., Harmon, S.R. and Blank, F., 1968. Isolation and purification of an extracellular keratinase of Trichophyton mentagrophytes. Journal of Bacteriology, 96(4), 1435-1436. DOI: 10.1128/jb.96.4.1435-1436.1968.
18. Koutb, M.M., Hassan, E.A., Morsy, F.M., and Bagy, M.M., 2023. Optimization of keratinase production by the keratinolytic fungus Chrysosporiumtropicum and its potential in the biodegradation of chicken feathers. Journal of Umm Al-Qura University for Applied Sciences, 9(1), 50-56. DOI:10.1007/s43994-022-00020-7.
19. Alahdal, F.A., Qashqoosh, M.T., Manea, Y.K., Salem, M.A., Khan, A.M. and Naqvi, S., 2022. Eco-friendly synthesis of zinc oxide nanoparticles as nanosensor, nanocatalyst and antioxidant agent using leaf extract of P. austroarabica. OpenNano, 8, p.100067. DOI:10.1016/j.onano.2022.100067.
20. Jeyabharathi, S., Naveenkumar, S., Chandramohan, S., Venkateshan, N., Gawwad, M.R.A., Elshikh, M.S., Rasheed, R.A., Al Farraj, D.A. and Muthukumaran, A., 2022. Biological synthesis of zinc oxide nanoparticles from the plant extract, Wattakakavolubilis, showed antimicrobial and anti-hyperglycaemic effects. Journal of King Saud University-Science, 34(3), p.101881. DOI:10.1016/j.jksus.2022.101881.
21. Karmakar, S.A.N.A.T., 2019. Particle size distribution and zeta potential based on dynamic light scattering: Techniques to characterize stability and surface charge distribution of charged colloids. Recent Trends Mater. Phys. Chem, 28, pp.117-159.
22. Cullity, B.D. and Smoluchowski, R.J.P.T., 1957. Elements of X-ray Diffraction. Physics Today, 10(3), pp.50-50.
23. Yacouba, A., Bellali, S., Haddad, G., Mavros, N., Fontanini, A., Dubourg, G., Lagier, J.C., Raoult, D. and Bou Khalil, J., 2023. Use of scanning electron microscopy and energy-dispersive X-ray for urine analysis: A preliminary investigation. Microscopy Research and Technique, 86(10), pp.1249-1257. DOI: 10.1002/jemt.24301.
24. Riffel, A., Lucas, F., Heeb, P. and Brandelli, A., 2003. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Archives of Microbiology, 179(4), pp.258-265. DOI: 10.1007/s00203-003-0525-8.
25. Sharma, I., Gupta, P. and Kango, N., 2023. Synthesis and characterization of keratinase-laden green-synthesized silver nanoparticles for valorization of feather keratin. Scientific Reports, 13(1), p.11608. DOI: 10.1038/s41598-023-38721-6.
26. Nnolim, N.E. and Nwodo, U.U., 2020. Bacillus sp. CSK2 produced thermostable alkaline keratinase using agro-wastes: keratinolytic enzyme characterization. BMC Biotechnology, 20(1), p.65. DOI: 10.1186/s12896-020-00659-2.