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Efficient Biodegradation of Poultry Feather Using Keratinase Nanoparticles

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

Department of Applied Microbiology, Sri Padmavati Mahila Visvavidyalayam, Tirupati -517502, Andhra Pradesh, India.
lakshmivedula28@gmail.com

Abstract

Nanoparticles represent an emerging class of biocatalysts with improved stability, activity, and application potential in bioremediation, pharmaceuticals, cosmetics, and material sciences. In the present study, sustainable synthesis of silver nanoparticles (AgNPs) was synthesized using keratinase enzyme was produced from a novel keratin-degrading *Bacillus* species. The biosynthesized silver nanoparticle was characterized through UV–Visible spectroscopy, zeta potential measurement, particle size analysis, and UV–Vis spectra revealed maximum absorbance between 400–450 nm, confirming the formation of AgNPs. Average particle size of 490 nm to 582.2 nm, and zeta potential of −49.3 mV with a single peak. Keratinase Nanoparticles (KNP) showed high keratinase activity and biodegrading poultry feather. KNPs were stable even after 12 months. These findings demonstrate that keratinase-mediated synthesis of AgNPs is an eco-friendly strategy to stabilize the enzyme and extend the application potential of keratinase significantly in biotechnology, medicine, and environmental sustainability.

Keywords: Keratinase, *Bacillus species*, silver nanoparticles, biocatalysts

Introduction

Keratinase is a specialized proteolytic enzyme capable of degrading protein- keratin. Due to its unique ability to hydrolyse highly stable keratin proteins, keratinase has gained important consideration for its potential applications in biotechnology, waste management, and industrial processing [1]. Keratinases produced from microbes are finding important applications in detergent and pharmacological industries. Even though keratin has inflexible nature, it has good essential amino acids, which can be utilized in animal feed manufactures and organic fertilizers [2]. It is not easy to degrade keratin, with common protease enzymes (trypsin, pepsin, and Papain). Hence keratinase producing microorganisms are prominent to degrade these insoluble keratins [3]. Keratin hydrolysates production as major Agro-industrial by-product, has high value for animal feed industry. Earlier studies on isolating keratinolytic organisms soil samples collected from poultry farms of Rama Chandhrapuram, Tirupati, led to development of four bacterial isolates of *Bacillus* species that had ability to degrade feather keratin efficiently (100%) in 4-5 days [4,5].

Silver nanoparticles (AgNPs) have been synthesized using plant extracts [6] and bacterial systems [7]. Their biosynthesis follows a bottom-up strategy involving redox reactions [8], where microbial enzymes and phytochemicals act as reducing and stabilizing agents. This green approach eliminates the need for toxic chemicals.

In this study, AgNPs were synthesized using both crude and purified keratinase enzyme preparations. The nanoparticles were characterized by keratinase assay, UV–Visible spectroscopy, zeta potential analysis and particle size distribution and stability of the keratinase activity.

Material and Methods

Substrate preparation:

Feathers were collected from local poultry sources, washed to remove dust and blood stains, The dried substrate was cut into ~1-inch pieces, air dried and powdered by milling, After that the substrate was washed with distilled H_2O and allowed to air dry. Substrate was sterilized by autoclaving at 10 lbs pressure for 10 min. This work flow of the Sample collection is illustrated in figure -1.

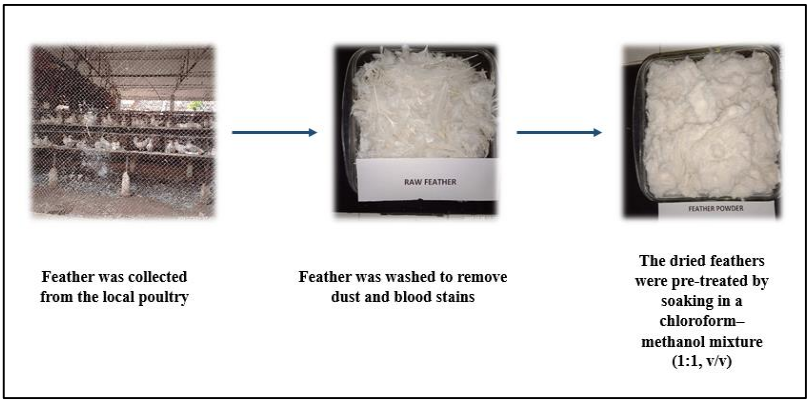

Figure -1: Feather Substrate Preparation

Keratinase Enzyme Production

In this study, production of keratinase enzyme by using a modified method of submerged fermentation of Jeevana Lakshmi, *et al* 2013 [4]. For solid-state fermentation (SSF), the substrate composition was depicted in Table 1, while the composition of the production medium was presented in Table 2 [5].

Table 1. Substrate Composition for SSF

S.no	Component	Quantity (g)
1	Ball-milled feather	20
2	Soybean meal	20
3	Starch	20

Table 2. Composition of Production Medium

S.no	Component	Concentration (g/L)
1	NH_4Cl	0.5
2	NaCl	0.5
3	KH_2PO_4	0.3
4	K_2HPO_4	0.4
5	$MgCl_2$	0.1

The composed media was autoclaved at 121 °C, 15 psi for 15-20 min, cooled, and inoculated with 10 mL of an overnight culture of *Bacillus* species MBF20 (~10^9 CFU/mL). Fermentation was carried out at room temperature on an orbital shaker at 180 rpm for 72 h to ensure complete feather degradation through keratinase production. Every day sample was collected and enzyme assay was done by both spectrophotometric and azokeratin methods. The maximum activity showed on 3rd day as presented in Table -3.

After incubation, cultures filtrate was centrifuged with cooling centrifuge at 3,000 × g for 10-15 min. After that the collected culture supernatant was used as crude keratinase (Figure-2). Enzyme activity was confirmed using both spectrophotometric and Azokeratin assays.

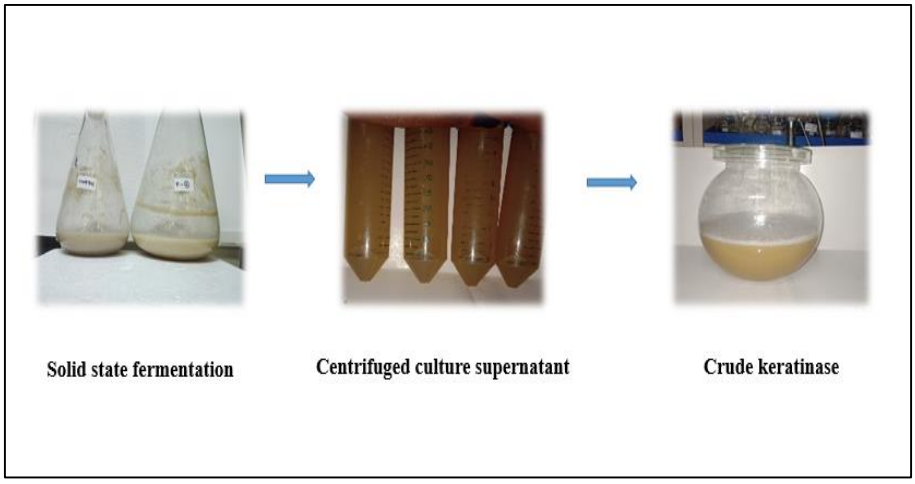

Figure-2: Production of Crude Keratinase

Preparation of Azokeratin

After filtration the Azokeratin was rinsed with deionized water and again suspended in deionized water and stirred at 50 °C for 2 h, followed by filtration. Washing cycles were repeated till the filtrate pH was between 6.0 and 7.0. The absorbance of 0.01 was noted at 450nm [9]. Finally, the product was cleaned twice with 50 mM potassium phosphate buffer (pH 7.5), rinsed with distilled water, dried for 24 hrs at 50 °C, and stored dry until use as presented in figure-3.

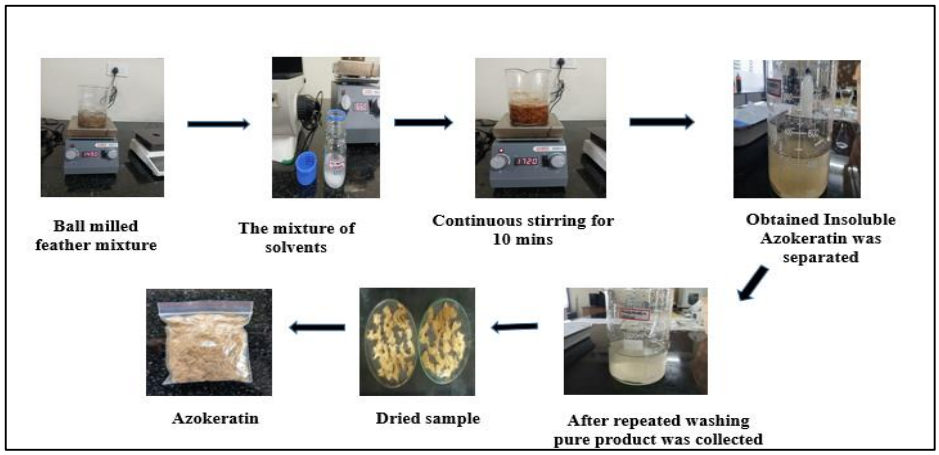

Figure-3: Preparation of Azokeratin substrate

Keratinase Silver Nanoparticles Preparation

Keratinase Silver nanoparticles (KAgNPs) was prepared with crude keratinase enzyme adopting methods of Revathi *et al.* (2013) and Velmurugan *et al.* (2014) [10,11]. 50 mL of silver nitrate solution (2 mM AgNO3) was mixed with 5 microliters of crude enzyme and stored at room temperature for 24–48 h in the dark. After Incubation the samples were centrifugation at 13,000 rpm for 20 min, the supernatant was discarded and carefully the pellet was collected. The collected pellet was again washed with 50mM phosphate buffer, followed by HPLC grade distilled water to remove the unbound particles. Finally, the washed pellet was air dried as shown in figure-4. The dried powdered nanoparticles were stored for further characterization. Formation of silver nanoparticles was confirmed by Ultraviolet–visible.

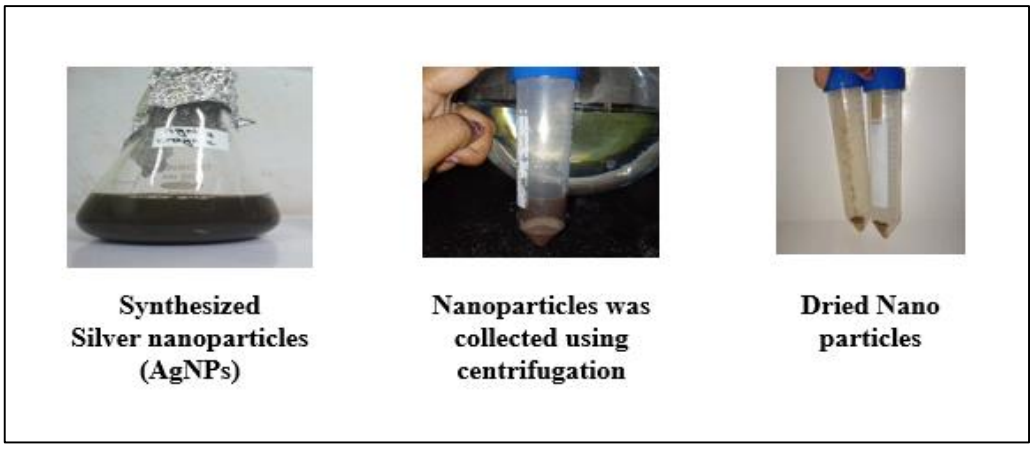

Figure-4: Preparation of Silver Nanoparticles

Characterization of Synthesized AgNPs

Particle Size Analysis

The particle size of the synthesized KAgNPs (keratinase–silver nanoparticles) was determined using a particle size analyser (Horiba SZ-100). The analysis provided mean particle size distribution profiles, confirming the nanoscale range of the synthesized particles [12].

Zeta potential analysis

The surface charges of the synthesized keratinase–silver nanoparticles (KAgNPs) were determined using a zeta potential analyser **(**Horiba SZ-100**)**. Measurements provided insights into the colloidal stability of the nanoparticles, with higher absolute zeta potential values indicating greater electrostatic repulsion and enhanced stability in suspension [13].

UV–Visible Spectral Analysis

The crude keratinase enzyme, Silver Nano particles along with formation of keratinase-mediated silver nanoparticles (KAgNPs) and its degradation activity was done after 12 months of storage and was confirmed by UV–Visible spectrophotometry using a Jasco V-630 spectrophotometer. Spectral measurements were noted in the range of 190–1100 nm [14].

Enzyme assay of crude keratinase

Keratinase enzyme activity was determined by using the Azokeratin method. Azokeratin, an insoluble chromogenic substrate used for keratinase assays, was prepared following the method of Riffel et al. (2003) [15]. Degradation of azokeratin is proportional to colour development, and absorbance was measured by using a UV–Visible spectrophotometer (Jasco V-630) at 450 nm

Feather degradation by using Keratinase Silver nanoparticles (KAgNPs)

For feather degradation testing, in 100ml conical flask, 2grams of feather substrate was taken and 50 ml of Tris HCL buffer was added along with 0.2ml of keratinase enzyme treated silver nanoparticles (KAgNPs) were added. The flasks were incubated in an orbital shaker at 37 °C with 180 rpm for 1 h to achieve complete substrate degradation [16]. After incubation the sample was kept on ice for 10 minutes to stop the reaction. Sample was filtered, the culture filtrate was collected and enzyme activity was measured at 280nm.Finally the enzyme coated nanoparticles was separates by using centrifugation and stored at - 4°C for next batch of feather degradation as shown in table- 4.

RESULTS

Keratinase Activity of crude enzyme

Keratinase enzyme activity was determined by using the Azokeratin method (Jeevana Lakshmi, 2008, Riffel, 2003) [4, 15] and absorbance was measured by using a UV–Visible spectrophotometer (Jasco V-630) at 450 nm. Control samples were prepared by adding TCA before enzyme addition. 1 U (unit) of

keratinase activity was defined as an increase of 0.01 in absorbance at 450 nm after 15 min compared to the control. Enzyme activity remained maximum up to three consecutive batches of feather degradation.

Sample	Keratinase Activity(U/ml)			
	Fermentation period			
	0 hour	1st day	2nd day	3rd day
control	0.00	0.00	0.12	0.29
Flask	0.00	194	345	654

Table-3: keratinase assay of crude enzyme

Feather degradation by using Keratinase Silver nanoparticles

The KAgNPs were synthesised by following the method as mentioned in the methodology. The degradation activity was carried out and frequently measured with the UV spectroscopy. The enzyme activity was confirmed using both spectrophotometric and azokeratin methods. Nanoparticles were separated by centrifugation at 3,000 rpm for 15 min, and the resulting AgNP pellet was stored at −20 °C for subsequent use.

Based on the enzyme activity results it shows that Lyophilized AgNP pellets stored at −20 °C retained enzyme activity even after 12 months, confirming the long-term stability of keratinase when conjugated with AgNPs.

Sample	Batch	Crude Enzyme (U/ml)	Enzyme–AgNPs (U/ml)
Fermentation period – 3rd day	1st batch	456	607
	2nd batch	–	575
	3rd batch	–	456
Fermentation period – 3rd day	1st batch	456	607
	2nd batch	–	575
	3rd batch	–	456

Table 4. Comparative Table for Keratinase activity (U/ml) of crude enzyme and enzyme–AgNPs during fermentation.

Characterization of Synthesized AgNPs

Particle size and zeta potential analysis of KAgNPs

The Analysis of keratinase-silver nanoparticles (KAgNPs) synthesized using keratinase revealed that the particles have of 490.3 nm with a Polydispersity Index (PDI) of 0.432, indicating moderate uniformity in

particle distribution as shown in figure-5. The measurement was performed at 25 °C in a dispersion medium with a viscosity of 0.896 mPa·s. A single dominant peak was detected, showing a mean particle size of 582.2 nm, with the most common particle size (Mode) being 547.4 nm and a standard deviation of 133.2 nm. Based on the results, the particles are relatively well-dispersed with some variation in size. The moderate PDI value indicates that while the nanoparticles are mostly uniform, slight aggregation or polydispersity may be present, which is typical for biologically synthesized nanoparticles.

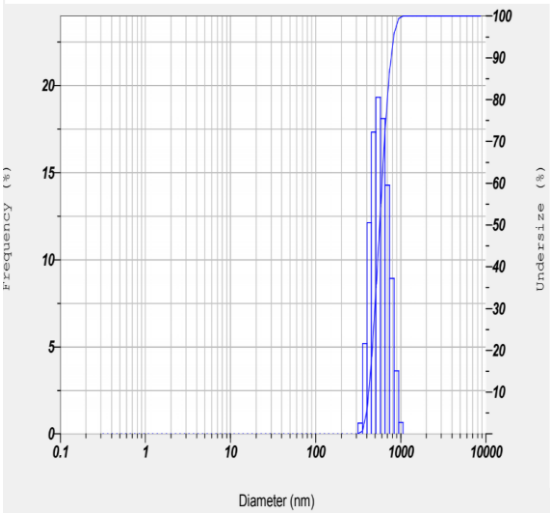

Figure-5: Particle Size of the Synthesized Keratinase-Silver Nanoparticles

The zeta potential analysis value of -49.3 mV indicates that the keratinase-silver nanoparticles are highly stable in suspension was presented in figure-6. This stability allows the nanoparticles to interact efficiently with keratin-containing materials. The strong negative surface charge promotes the rapid degradation of keratin, as the nanoparticles can effectively penetrate and break down the keratin structure without aggregating, ensuring fast and efficient keratin degradation.

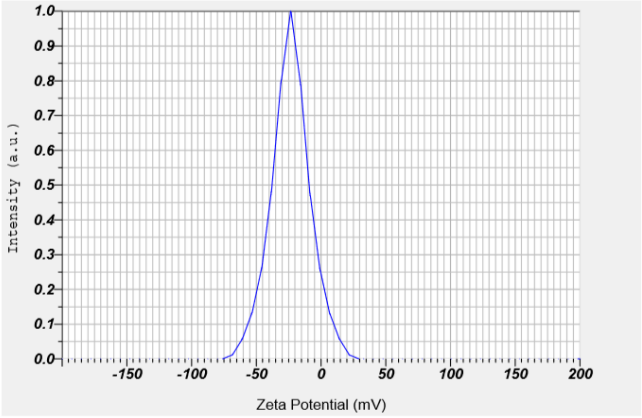

Figure-6: Zeta Potential of Keratinase-Silver Nanoparticle

UV–Visible Spectral Analysis

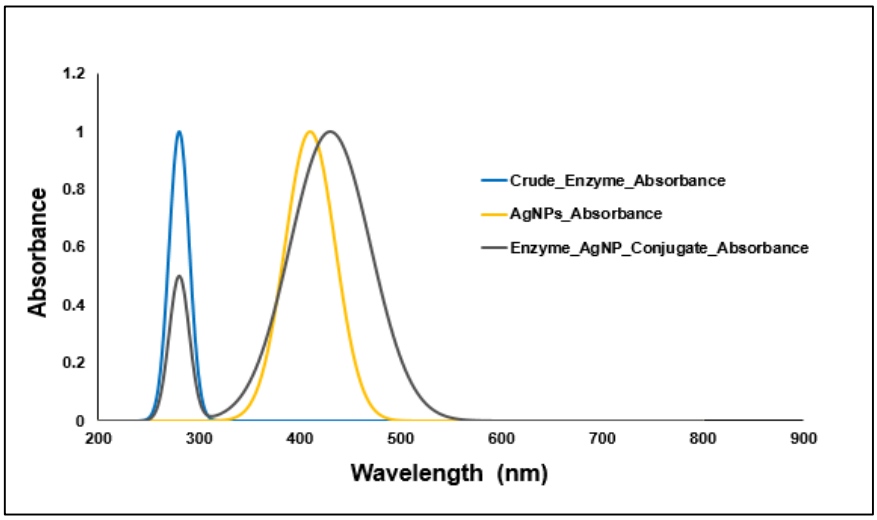

Figure-7: UV–Visible Spectral Analysis of Biosynthesised Enzyme–AgNPs

The spectral analysis shows that the crude enzyme absorbance at 200nm -300nm, Silver nanoparticles at near 430nm and Biosynthesised Enzyme–AgNPs showed absorbance at 280nm and 450nm. A characteristic peak was observed between 400–430 nm, which aligns with previous reports that typically place AgNPs absorbance maxima in the range of 400–450 nm was presented in figure-7. Comparative spectral analysis was also performed up to 12 months with 3 months interval, and the SPR peak was retained without significant shift, indicating the long-term stability of the synthesized nanoparticles as shown in figure-8

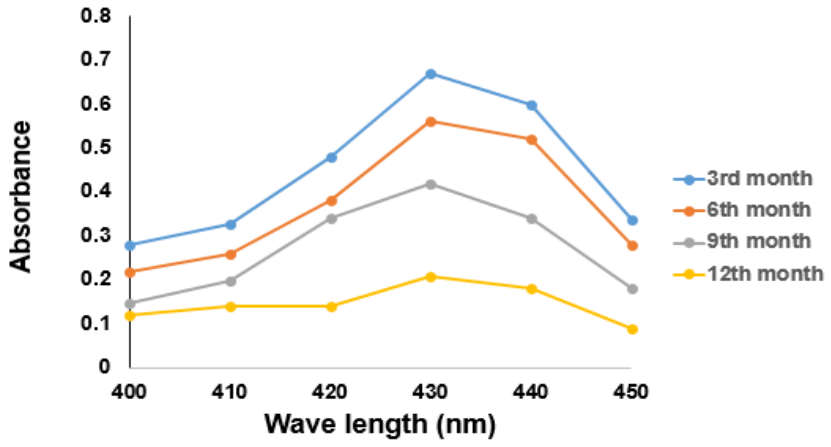

Figure-8: Stability of biosynthesised KAgNPs with time interval up to12 Months

Conclusion

Keratinase enzyme from *Bacillus* species MBF20 was successfully used to synthesize silver nanoparticles (AgNPs) in a biodegradable manner. The synthesized AgNPs were stable, crystalline, and had a usual size of 490 nm to 582.2 nm with a zeta potential of −49.3 mV, representing noble stability. UV–Vis analyses

confirmed the formation of AgNPs and showed that proteins from keratinase acted as natural capping and stabilizing agents. Overall, keratinase-mediated AgNPs offer a green, sustainable approach with potential applications in biotechnology, medicine, and environmental fields.

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