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Keratinase mediated fabrication and partial characterization of gold nanoparticles and its antibacterial potential

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Keratinase is mainly involved in recycling of keratin waste. Of late, researchers extended its application to nanotechnology. In the present study, we have made an attempt to fabricate and characterize gold nanoparticles using crude keratinase enzyme from *Serratia ficaria* and also study their biological application, particularly antibacterial activity. The formation of gold nanoparticles (AuNPs) was first verified by UV-Visible Spectroscopy. FTIR spectra confirmed the presence of responsible secondary metabolites for stabilization of nanoparticles. The morphological characteristics and particle size of synthesized nanoparticles were analyzed. The AuNPs showed significant antibacterial activity against *Klebsiella pneumonia*, *Bacillus cereus* and *Staphylococcus aureus*. The highest radical scavenging activity, 60.62% for AuNPs was observed at 500 µg/mL. Results of this study reveals significance of keratinase application in nano-based biological applications.

Keywords: Antibacterial activity, Antioxidant activity, Bio-nano synthesis, Waste utilization

In the fast-expanding development of nanotechnology, bionanosynthesis is an exciting aspect that gives a tremendous amount of usable biological material. One of the most recent advances is the use of enzymes to fabricate nanoparticles[1]. Keratinase is a group of proteolytic enzymes produced by microorganisms. It has the capability of degrading insoluble keratins present in skin, hair, and feathers[2]. Nanotechnology comprises chemistry, physical, biological, medicinal, and materials science. It also rapidly evolves into a trans-disciplinary area of science that has become a commercially available innovative benefit to society[3].

Nanoparticle synthesis is essential in the fast-growing nanotechnology field to achieve functional nanomaterials for biological applications[1]. Nanoparticle synthesis can be performed by traditional chemical treatment (chemical reduction and precipitation), and physical treatment (microwave-assisted method, pulsed laser ablation method, hydrothermal synthesis), mostly drawback approaches. The chemical procedure uses chemicals that release harmful products, whereas the physical process uses more power and less production[4]. The natural nanoparticle production method has recently received more attention among researchers because of its pollution-free and environmentally friendly approach. Plant extracts, enzymes, and microbes such as fungi, bacteria, yeast, and molds are used as nano-factories for the biological mode of production of nanoparticles. The synthesis of nanoparticles using biocatalysts is one of the latest innovations in the nanotechnology field. The interesting fact behind the mechanism of involving an active enzyme for nanoparticle synthesis was that under favourable conditions, the enzyme would degrade and liberate amino acids, which would act as stabilizing and reducing agents[5]. Among the various types of nanoparticles, AuNPs have time-honoured metals for the synthesis of nanoparticles because of their stability with the least harmful property[6].

Microbial keratinase is a multifunctional biocatalyst with various biotechnological applications such as keratin waste management; production of animal feed, biodegradable films, and biocontrol molecules like pesticides and insecticides; formulation of personal care products and detergents; and manufacturing fabrics[7]. In the present study, we fabricated gold nanoparticles using crude extracellular keratinase enzymes. Synthesized keratinase coupled

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AuNPs were stamped using UV-Vis Spectroscopy, FTIR, a particle size analyzer, and scanning electron microscopy. The antibacterial activity and antioxidant properties of the synthesized AuNPs were assessed.

Materials and Methods

Keratinase enzyme

A keratinase-producing microorganism was isolated from peacock feathers using serial dilution and spread plate technique. The pure isolate was identified using the KB003Hi25TM Enterobacteriaceae identification kit. Our previous study elaborated on the isolation and identification of keratinase-producing microorganisms and the quantification of crude extracellular keratinase enzymes from *Serratia ficaria*[8]. The crude keratinase extracellular enzyme from *S. ficaria* was used for further studies.

Synthesis of gold nanoparticles

With slight modifications from Sanghi *et al*.[9], 1 mL of the crude keratinase enzyme was mixed with 20 mL of 1 mM of aqueous gold chloride ($AuCl_3$) solution for the synthesis of gold nanoparticles, and control was also maintained and incubated the two solutions at 27ºC for 72 h in a dark room for AuNPs formation. The synthesized gold nanoparticles were collected by centrifugation at 15,000 rpm for 20 min. The pellets were collected and dried at 27ºC.

Characterization of keratinase-coated AuNPs

The following techniques were used to characterize the synthesized gold nanoparticles: UV-Vis spectroscopy; FTIR (Fourier Transform Infra-Red spectroscopy); SEM (Scanning Electron Microscopy); and a particle size analyzer. UV–Vis spectroscopy is absorption spectroscopy used to measure the strength of the signal, which is observed after the beam of light passes through the sample or reflection from the surface of a sample. Due to electronic transition, the radiation of UV-Vis interacts with the sample. Perkin Elmer Lambda 35 UV-Vis spectroscopy was used to record the spectrum of the keratinase-coated gold nanoparticles. The Infra-Red absorption identifies molecular band vibration and structure—Perkin-Elmer Spectrum Two equipment documented the frequency absorptions of synthesized gold nanoparticles. CAREL ZEISS EVO 18 model SEM was used to study the morphological examination with direct visualization of a sample, which used a focused electron on the sample to create an image. The particle size distribution in the sample was determined by a particle size analyzer (Micromeritics, Nano Plus).

Antibacterial activity of AuNPs

The antibacterial activity of keratinase-coupled AgNPs was studied following Sudha *et al*.[8]. Nutrient agar slants were maintained with test bacteria, namely *Enterobacter amnigenus*, *Brevibacterium paucivorans*, *Staphylococcus lentus*, *Klebsiella pneumonia*, *Bacillus cereus* and *Staphylococcus aureus*. The microbial culture was subcultured and incubated for 48 h at 5°C. The disc diffusion method was used to assess the antibacterial property of synthesized gold nanoparticles.

The paper disc was made-up of the Whatman No. 1 filter paper. Previously mentioned microorganisms were grown in 10 mL of nutrient broth mixture. The prepared nutrient agar was poured into Petri plates. This was allowed to solidify for 30 min. After that, the test organisms were spread over the plates with L-rod. Paper discs impregnated with 1 mM gold nanoparticle solutions and crude keratinase were kept on the test organisms' plates. The control was set as a paper disc with crude keratinase only. The inhibition zone around the paper disc was considered positive for antibacterial activity. After 24 h incubation at 37°C, the zone of inhibition was evaluated.

Antioxidant activity

The radical scavenging activity of gold nanoparticles of the crude keratinase enzyme was measured based on the scavenging activity of the stable DPPH free radical method with a slight modification of Tahir *et al*.[10]. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations (20-120 μg/mL) of crude keratinase gold nanoparticles solution in the test tube. The mixture was then allowed to stand for incubation. The absorbance was measured at 10 min intervals in the dark five times. One mL of 0.1 mM DPPH solution mixed with 1 mL methanol was used as the control. The decreased absorbance was measured at 517 nm. The percentage of inhibition was calculated as follows:

$$\% \text{ of DPPH radical inhibition} = [(\text{control} - \text{sample})/\text{control}] \times 100$$

Results and Discussion

Synthesis of gold nanoparticles

The gold ion reduction by the enzyme gave colour to this solution, indicating the formation of AuNPs.

Up until the endpoint is achieved, the colour intensity increases to represent the progress of the gold ions and then stabilises. In the fast-expanding development of nanotechnology, bio-nanosynthesis is an exciting aspect that gives a great usable biological material[11]. One of the most recent advances is the use of enzymes to fabricate nanoparticles. Keratinase is a group of proteolytic enzymes produced by microorganisms[2]. In this research, we have synthesized gold nanoparticles with crude keratinase enzymes from feather-degrading microbes. Commonly, active enzymes catalyze nanoparticle formation, but in some cases, enzymes are denatured to release amino acids, which act as capping and reducing agents during nanoparticle synthesis. In the formation of nanoparticles, the enzyme alone may function as a reducing and capping agent[12]. Gold nanoparticles with different enzymes like sulfite reductase[13,14], reductase and keratinase[15], alcohol oxidase[16], serration peptidase[17], and laccase[18] have been documented by researchers.

Characterization of AuNPs

The optimal absorbance due to surface plasmon resonance was recorded. This characteristic absorption spectrum peaked at 550 nm, shown in Fig. 1, and justified the formation of AuNPs with a crude keratinase enzyme. The FTIR spectrum (Fig. 2) for AuNPs showed bands at 3662.82 cm^{-1}, 3104.28 cm^{-1}, 2880.43 cm^{-1}, 2014.27 cm^{-1}, 1725.72 cm^{-1}, 1680.90 cm^{-1}, 1625.03 cm^{-1}, 1503.37 cm^{-1}, 1394.27 cm^{-1}, 1277.28 cm^{-1}, 1182.30 cm^{-1}, 746.94 cm^{-1}, 602.32 cm^{-1}, 556.47 cm^{-1}, 418.83 cm^{-1}, and 467.80 cm^{-1}. The band at 3104.28 cm^{-1} indicated alkenes, the band at 2880.43 cm^{-1} was recognized as carboxylic acids, the band at 1725.72 cm^{-1} indicated aldehydes, the band at 1680.90 cm^{-1} indicated ketones, the band at 1625.03 cm^{-1} indicated amines, the peak of 1503.37 cm^{-1} indicated aromatic compounds, the band at 1277.28 cm^{-1} indicated ethers, the band at 1182.30 cm^{-1} indicated alcohol, the peak of 746.94 cm^{-1} indicated aromatic compounds and the band at 602.32 cm^{-1}, 556.47 cm^{-1}, 418.83 cm^{-1} and 467.80 cm^{-1} indicated alkyl halides. Spherical-shaped nanoparticles of Scanning Electron Micrograph were shown in Fig. 3. Biosynthesized AuNPs using crude keratinase are measured using a diffuse light scattering method to analyze the particle size and their settlement (Fig. 4). The particle size ranged from 132.9 nm, and the diameter was 64.9 nm. There are only few reports available on the use of keratinase for nanoparticle production. Due to surface plasmon resonance, the absorbance found in this study was similar to some other findings[19-21]. The same results showed that the spherical shapes of nanoparticles were characterized and recorded by Moshfegh *et al.*[22]. Some other scientific findings of characterization techniques such as UV-Vis, SEM and FTIR employed for confirming AuNPs supported our present work[5,16,17].

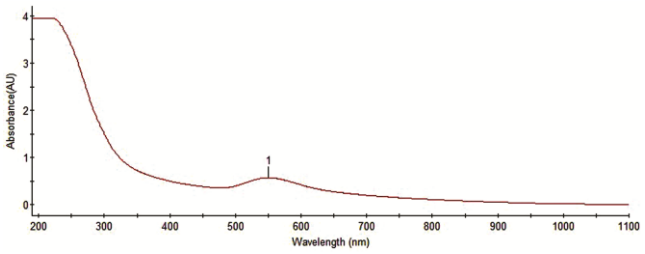

Fig. 1 — UV-Vis spectrum of synthesized gold nanoparticles (AuNPs)

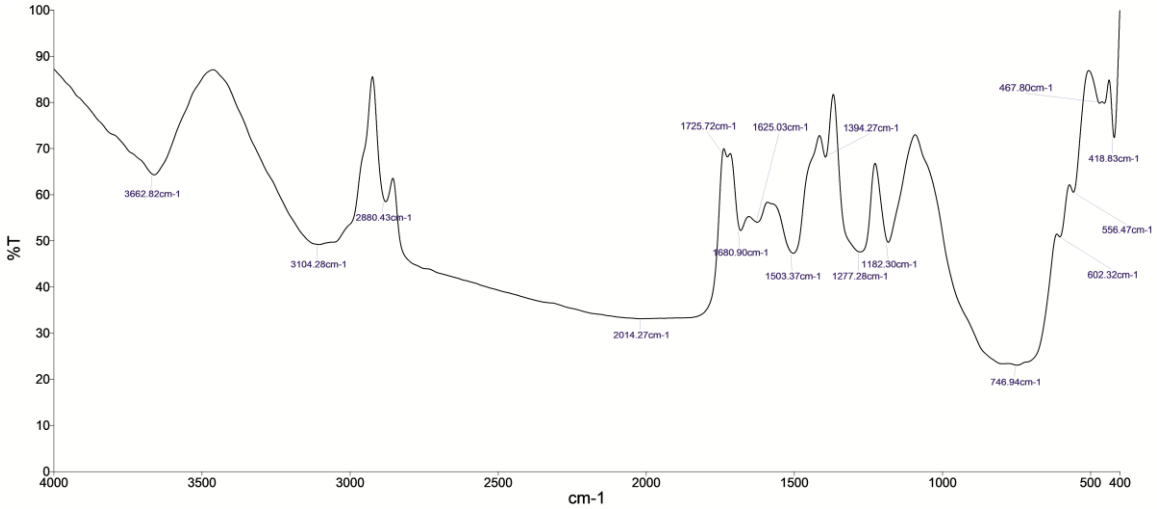

Fig. 2 — FTIR spectrum of synthesized gold nanoparticles (AuNPs)

Antibacterial activity

Compared to *Enterobacter amnigenus*, *Brevibacterium paucivorans* and *Staphylococcus lentus*, the more significant antimicrobial activity was studied with the crude keratinase enzyme-loaded gold nanoparticles against *Klebsiella pneumonia*, *Bacillus cereus* and *Staphylococcus aureus*. The samples enclosing gold nanoparticles revealed an excellent zone of inhibition. Fig. 5 indicates the inhibition zone found against clinical microbes. The area of the clearance zone around the gold nanoparticles was compared with the properties of corresponding customary antibiotics. Table 1 displays the antibacterial activity against clinical pathogens. For decades, gold was used to treat a variety of ailments. Robert Koch was the first to investigate gold's biocidal ability[23]. The antibiotic activity of AuNPs has been mainly utilized in their other applications[24]. Nanoparticles obstruct electrostatic flux across membranes, causing membrane distortion[25,26].

Furthermore, nanoparticles boost gene expression in redox processes, resulting in bacterial death[27]. The antimicrobial potential is due to the surface chemistry, polyvalent nature, smaller scale, and photothermic

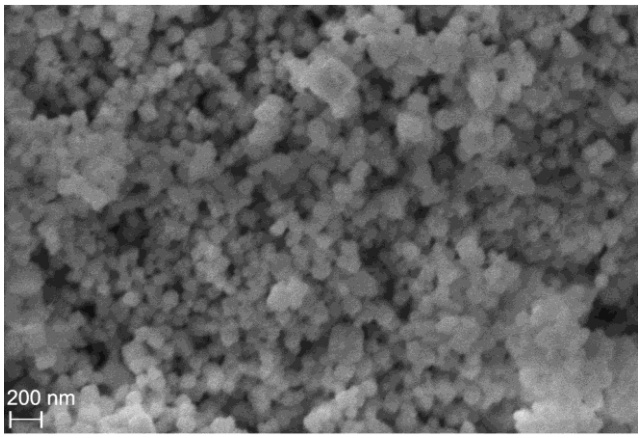

Fig. 3 — SEM micrograph of gold nanoparticles (AuNPs)

Table 1 — Antibacterial activity of gold nanoparticles (AuNPs)

Micro organisms	Antibiotic	Zone of inhibition (mm)		
		Antibiotic	NPs	Keratinase + AuNPs
Klebsiella pneumonia	Nitrofurantoin	2.2	0	2.1
Bacillus cereus	Chloramphenicol	1.4	0	2
Staphylococcus aureus	Ciprofloxacin	3.9	0	4
Enterobacter amnigenus	Chloramphenicol	3.6	0	0
Brevibacterium paucivorans	Ceftriaxone	2.7	0	0
Staphylococcus lentus	Methicillin	2.3	0	0

[NPs, Nanoparticles]

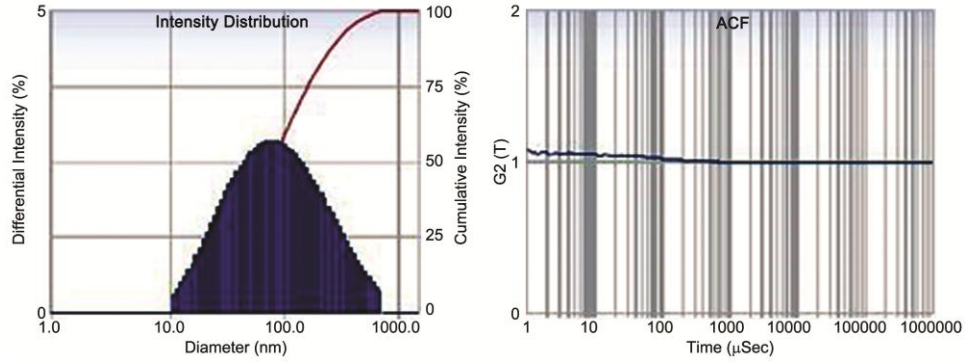

Fig. 4 — Particle size of gold nanoparticles (AuNPs) using crude keratinase by particle size analyzer

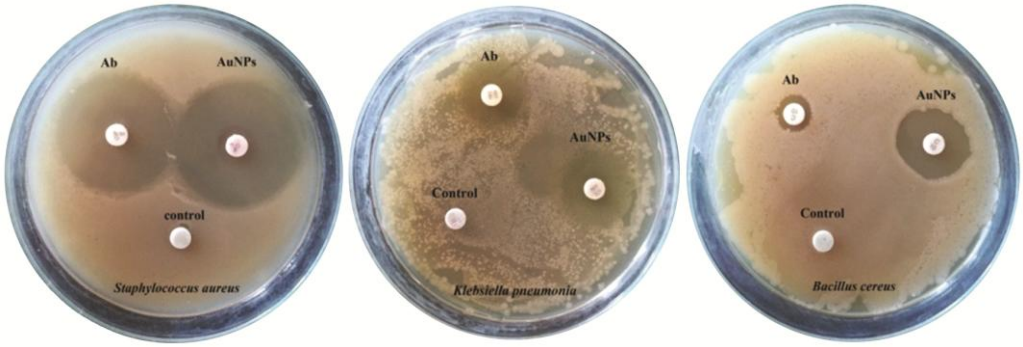

Fig. 5 —Antibacterial activity of keratinase loaded gold nanoparticles (AuNPs)

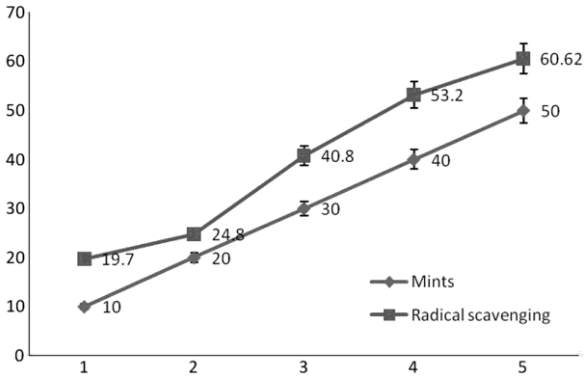

Fig. 6 — Antioxidant activity of gold nanoparticles (AuNPs) using crude keratinase

nature of the molecules[28-30]. However, the exact mechanism is unknown[31]. The nanoparticle's inhibitory capacity can be connected to an attack on the cell membrane to ruin microorganisms, leading to the spillage of cytoplasmic content that reaches a desired cellular damage[32]. Au NPs interact primarily with sulfur or phosphorus-containing bases, the most common targets for Au NPs. When NPs bind to the thiol functional groups of enzymes (nicotinamide adenine dinucleotide (NADH) dehydrogenases), they disrupt the respiratory chains and induce cell death by generating many free radicals [33]. The moderate antibacterial activity of synthesized gold nanoparticles was observed by Elegbede *et al.*[34]. This was the first report that keratinase-mediated gold nanoparticles had antibacterial activity.

Antioxidant activity of keratinase-coated AuNPs

The ability of synthesized gold nanoparticles using a crude keratinase enzyme from keratin producing microorganisms to scavenge free radicals was assessed using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The maximum DPPH radical scavenging activity was 60.62% for AuNPs at 500 µg/mL concentration at 50 min. Synthesized gold nanoparticles using a crude keratinase from keratin-producing microorganisms demonstrated a high capacity for scavenging free radicals by reducing the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) radical to the yellow-coloured 1,1-diphenyl-2-picrylhydrazine and the reducing ability increased with increasing concentration of the mixture. The nanoparticles have been given free radical scavenging capability by responsive bio reductive molecules that can attach to the surface of nanoparticles and thus lead to amplified outer edge zones[35]. The DPPH test is one of the most important and widely used antioxidants against free radicals.

On the other hand, free radicals are damaging and can injure human cells. Antioxidants, such as AuNPs, play a significant role in the fight against free radicals[36]. The active sites of bio-reductant molecules, whose capacity to stick to the substrate of the nanoparticles results in amplified surface areas for activity, have been attributed to the free radical scavenging potency of the nanoparticles[35]. These AuNP activities are similar to those described by Oladipo *et al.*[37].

The present analysis shows that AuNPs produced from crude keratinase have high antimicrobial activity against human pathogens. Depending on the bacterial test strain investigated, the level of antimicrobial activity of AuNPs has been demonstrated. For each bacterial strain deliberated, this antimicrobial performance was extraordinarily greater that of the commercial antibiotics namely nitrofurantoin, chloramphenicol and ciprofloxacin.

Conclusion

The present study has demonstrated synthesis of gold nanoparticles (AuNPs) capped with crude keratinase utilizing a simple and biological method. The organic reductants in the cell-free extract shortened the gold ions into their parallel neutral atoms, forming nanoparticles. Intense antibacterial activity against clinical bacterial isolates and high antioxidant activity have been observed using fabricated gold nanoparticles. The results of this pilot study reveal the potential application of the stabilized and uniform-shaped AuNPs attained from this simple and green synthesis method in the field of pharmacology and biotechnoly.

Conflict of interest

Authors declare no competing interests.

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