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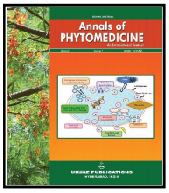

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Enzyme mediated synthesis and characterization of silver nanoparticles using keratinase enzyme producing micro-organisms

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Abstract

Nanobiotechnology is a new multidisciplinary subject with the collaboration of biotechnology, nanotechnology, chemical methodology and physical processing that depends on the nano-size objects with their constitutional properties. Keratinolytic micro-organisms have an enormous role in poultry waste degeneration and its biomodification is used in the production of fertilizer and animal meal. The purpose of this study was to examine the competence of crude extracellular keratinase from bacterial strains for the production of nanoparticles and evaluation of its applications. Two keratinoltyic bacterial strains were isolated from the peacock feather and were identified as *Serratia plymuthica* and *Serratia ficaria.* Among these, *Serratia ficaria* showed high keratinolytic activity. Hence, it was used to synthesize nanoparticles. Silver nanoparticles were synthesised and characterized by UV-visible spectroscopy, FTIR, particle size analyser and SEM. The results indicate that the crude keratinase enzyme produced by *S. ficaria* was found to be a good bioreductant.

1. Introduction

Production with the stabilisation of various types of nanoparticles was achieved by nanotechnology (Shaligram *et al.,* 2009). Of these different types of nanoparticles, silver nanoparticles have verified to be the most effective with excellent antimicrobial potency (Anandaradje *et al.,* 2020). Different kind of methods like laser irradiation, heating technique, radiolysis, ionizing radiation, microwave irradiation, pulsed laser ablation techniques and sonication were widely used for the synthesis of silver nanoparticles, which may expose dangerous effects to the environment. To avoid that, biological way of production is another method, which has shown a very promising solution to these problems. So, biological way of silver nanoparticles have been successfully synthesised using plant extracts (Guimarães *et al.,* 2020); bacteria (Pourali and Yahyaei, 2016); yeast (Fernández *et al.,* 2016) and fungi (Sanguiñedo *et al.,* 2018). In this way, enzymes and phytocompound with reducing potentials have been responsible for the stabilisation and capping of nanoparticles (Hietzschold *et al.,* 2019). This is achieved in an eco-friendly manner, which does not lead to the use of toxic chemicals. AgNPs have been used for so many purposes such as in the prescription of burns and dental works; in textile fabrics manufacturing; for waste water treatment; as sunscreen lotions and in the production of antimicrobial paint; non-linear optics; spectrally selective coating material; intercalation materials; optical receptors; catalysts; antibacterial and bio-labelling agents (Burdu'el *et al.,* 2018). The purpose of this study was to examine the capability of crude extracellular keratinase produced by the bacterial strain that was isolated from peacock feather for the production of silver nanoparticles and evaluate its applications.

2. Materials and Methods

2.1 Sample collection

100 g of peacock feathers were collected from the villages in Virallimalai, Tiruchirappalli district of Tamil Nadu. Half of the collected feathers were cut into pieces and dried for 24 h. After that, feather powder was prepared by burning the peacock feather with ghee flame.

2.2 Isolation of keratinase producing organism

The bacterium used in this study was isolated by following the methods described by Lateef *et al.* (2015) with slight modifications.

The medium composition shown in Table 1.

Table 1: Medium composition

S. No.	Components	Quantity (g/l)
1.	$NaNO_3$	2
2.	NaCl	2
3.	KH_2PO_4	2
4.	$MgSO_4$	0.05
5.	$FeSO_4.7H_2O$	0.1
6.	$CaCO_3$	0.1
7.	Feather powder	10
8.	Agar-agar	20

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The piece of peacock feather was placed on the medium, and allowed for 24 h for the growth of keratinolytic bacterial strain around the peacock feather. Then the bacterial isolation was done by serial dilution and spread plate technique. The pure isolates were identified using biochemical identification KB003Hi25™ *Enterobacteriaceae* Identification kit.

2.3 Keratinase production

A loopful of pure culture was inoculated into a medium containing 1 % keratin substrate (peacock feather powder used as substrate) with 0.2 % yeast extract (pH 7.5). The cultures were incubated at 37°C at 100 rpm for 48 h. 1 ml of inoculum was inoculated into 50 ml of flask containing liquid minimal medium, and the flasks were incubated at 37°C at 1000 rpm for 120 h. After the incubation, the flasks were taken out, and the broth was centrifuged at 5,000 rpm at 10°C for 20 min, and the supernatant was considered as the crude extracellular keratinase (Lateef *et al.,* 2015).

2.4 Keratinase assay

The keratinase activity was determined using the method of Cheng *et al.* (1995) with certain modifications. About 0.5 ml of crude keratinase enzyme was mixed with 2 ml of phosphate buffer (pH 7.5) that contain 0.5 g of feather powder. A control was maintained with buffer and feather powder only. The reaction mixtures were incubated at 40°C for 3 h at 100 rpm. 2 ml of 10 % trichloro acetic acid (TCA) was added to the reaction mixtures to terminate the reaction. The reaction mixture was centrifuged at 5,000 rpm for 20 min at 37°C. The supernatant was taken and the pellet was discarded. The release of protein was measured and converted into keratinase enzyme units (1U = 0.01 absorbance increase for 1 h reaction time (Revathi *et al*., 2013).

2.5 Synthesis and characterization of silver nanoparticle

For the synthesis of silver nanoparticles (AgNPs), 50 ml of 1 mM $AgNO_3$ was added to 1 ml of crude keratinase enzyme and the mixture was incubated at dark for 48 hours. After the incubation, the formation of AgNPs using keratinase were observed by colour change from yellow to reddish brown color. Then the synthesized silver nanoparticles were collected through centrifugation at 10,000 rpm for 10 min. The pellet was collected and dried at room temperature (Lateef *et al.,* 2015).

According to Bhat *et al.* (2011), the synthesised silver nanoparticles were characterized by Fourier transform infrared (FTIR) spectroscopy, UV-Vis, SEM and Particle size analyser. The keratinase coupled AgNP solution was centrifuged at 10,000 rpm for 20 min. The sediment pellet was then dried at room temperature and the powder obtained was used for FTIR measurement. The scanning electron microscopy (SEM) micrograph was obtained for the structural confirmation of nanoparticles. The particle size analysis reflects the reaction of silver particles and also the activity towards agglomeration and settlement. A light source is introduced into the cell and the scattered light is collected and measured for the particle size. Biosynthesized AgNPs using keratinase is measured by diffuse light scattering method to analyse the particle size and their settlement.

2.6 Antibacterial activity of synthesized silver nanoparticle

The test bacteria were maintained in nutrient agar slants. The microbial cultures were sub cultured and the cultured strains were allowed to grow for two days and was stored at 5°C. The test organisms were cultured in nutrient broth medium, incubated at 37°C for 24 h. Antibacterial activity of the synthesised AgNPs was assessed using disc diffusion method as described by Thillaimaharami *et al.* (2013).

Whatman No. 1 filter paper was used as a paper disc. Test organisms were inoculated into 10 ml of distilled water. It was used to prepare 10 fold dilution. The nutrient agar was poured and allowed to solidify. Then the organisms were spread over the plates containing medium. The paper discs were dipped into the silver nanoparticles solutions and placed on the plates that were inoculated with test organisms. However, the control was a set of paper disc with crude keratinase only.

After incubation at room temperature for 24 h, the inhibition zone was measured. Presence of the zone of inhibition around the paper disc was considered as positive for antimicrobial activity (Lateef *et al.,* 2015).

3. Results

3.1 Sample collection and preparation of feather powder

Peacock feather (Figure 1) were collected from Virallimalai, Trichy District, Tamil Nadu, India. Prepared feather powder was shown in Figure 2.

Figure 1: Peacock feather.

3.2 Isolation of the bacterial species

Two bacterial strains (Figure 3) were isolated from the peacock feather. The isolated bacterial strains were identified as (strain A) *Serratia plymuthica* and (strain B) *Serratia ficaria* by KB003 Hi25™ *Enterobacteriaceae* Identification Kit.

Figure 2: Feather powder.

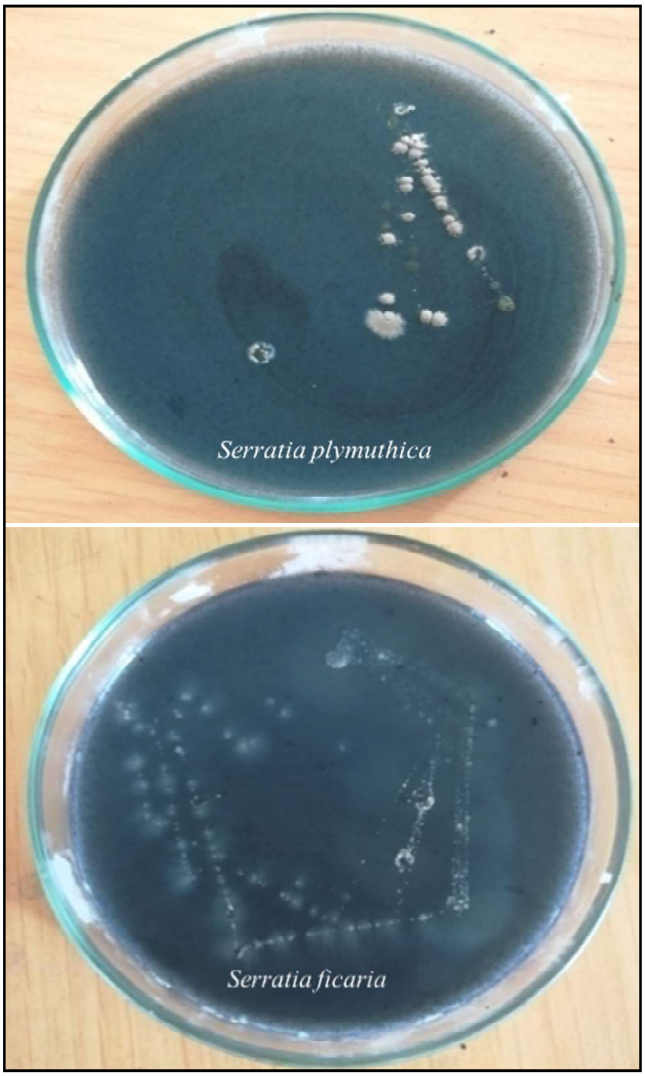

Figure 3: Bacterial isolates.

3.3 Keratinolytic activity

Both two strains *S. plymuthica* and *S. ficaria* produced crude keratinase enzyme. The enzymes were quantified as 2.01-5.6 U ml^{-1} in the *S. plymuthica* and 20.63 - 37.7 U ml^{-1} in the *S. ficaria* during 72 h of incubation using peacock feather powder as keratin substrate.

The enzyme activity reached a maximum of 5.6 U ml^{-1} for *Serratia plymuthica* and 37.7 U ml^{-1} for the *Serratia ficaria* at 72 h of cultivation.

Table 2: Keratinolytic activity of microbial strain

S.No.	Strain	Keratinolytic activity
1.	*Serratia plymuthica*	5.6 ± 0.1
2.	*Serratia ficaria*	37.7 ± 0.5

Among these, *S. ficaria* showed maximum keratinolytic activity. Hence, *S. ficaria* producing keratinase enzyme was used for further studies.

3.4 Biosynthesis of silver nanoparticles

The formation of synthesised silver nanoparticles was monitored through visual observation of the change of colour, and measurement of the absorbance spectrum of the reaction mixture using UV-Visible spectrophotometer. In this study, silver nanoparticles were synthesized using white colored crude extracellular keratinase from *S. ficaria*.

Figure 4 shows the dark brown solution formed from the reaction of crude keratinase and $AgNO_3$ solution after 48 h of reaction, while the $AgNO_3$ solution in the control experiment remained unchanged. The dark brown solution formed is an indication of the synthesis of keratinase coupled AgNPs. The results of the present study showed that the crude keratinase from this strain can readily react with $AgNO_3$ solution to form AgNPs.

Figure 4: Silver nanoparticles solution.

3.5 Characterization of Nanoparticles

Absorption spectroscopy was used to study the optical property of AgNPs. It is the method to observe the bioreduction of silver from silver nitrate to silver nanoparticle. Figure 5 shows the UV-Visible spectra of the nanoparticles obtained by crude keratinase enzyme. The synthesised particle gave an absorption spectrum peaked at 470 nm. This characteristic absorption peak justified the formation of AgNPs.

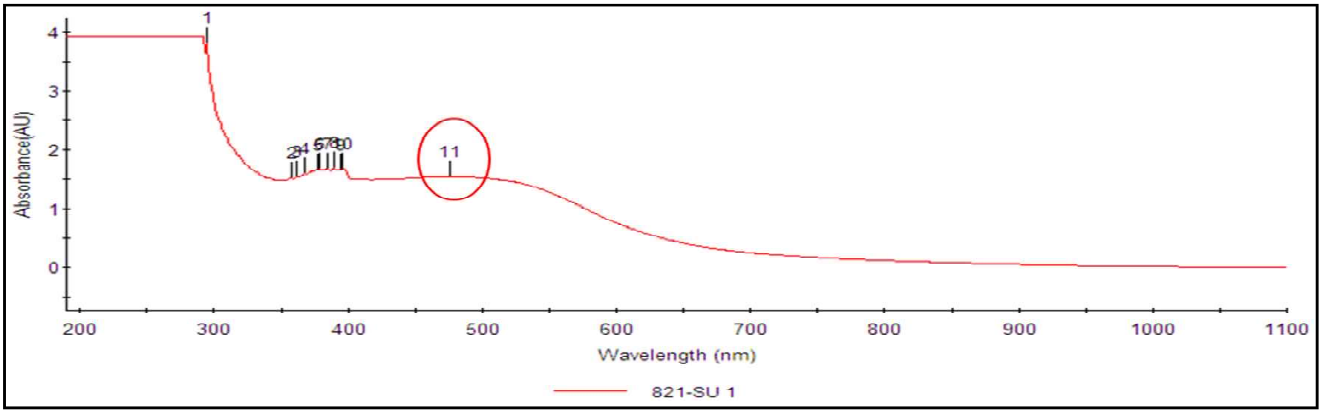

Figure 5: UV-Vis absorption spectrum of AgNPs using crude keratinase.

Functional groups present in the biomolecules are responsible for the silver bioreduction. Figure 6 shows the band intensities in different regions of the spectrum for AgNPs using keratinase. Different peak positions of synthesised silver nanoparticles exhibited bands at 3630.76, 3161.73, 2883.40, 1790.87, 1618.46, 1519.61, 1393.10, 1332.25, 1261.50, 1199.07, 739.43, 601.23, 556.45, 472.97 cm^{-1}. Peak position having spectra with marginal shifts point out the presence of the enzyme in the sample as a capping agent to the AgNPs. The bands at 3630.76 cm^{-1} is assigned as alcohol stretch, the band at 2883.40 cm^{-1} indicates alkanes, the band at 1519.61 cm^{-1} was recognized as amides, the band at 1393.10 cm^{-1} corresponds to alkanes, the band at 1332.25cm^{-1} could be assigned to alkyl halides, the band at 1261.50 cm^{-1} and 1199.07 cm^{-1} indicates alcohols, the 472.97 cm^{-1} indicates alkyl halides.

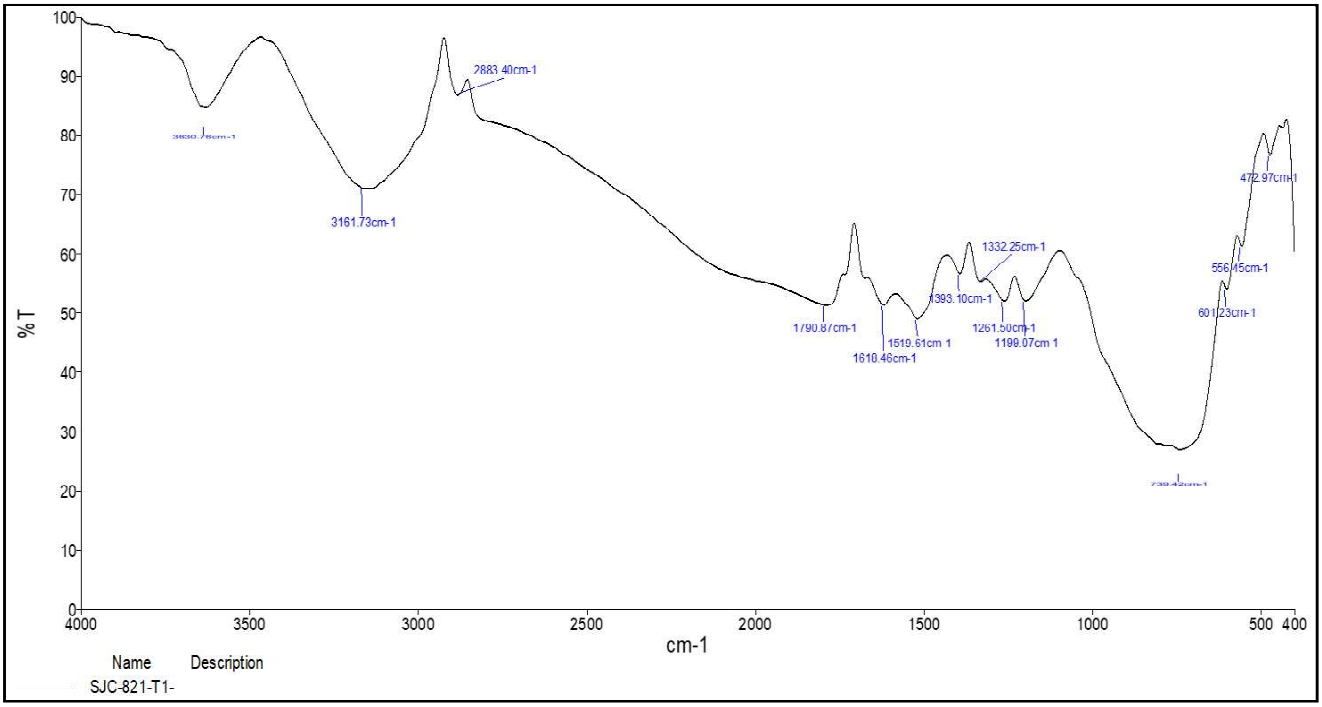

Figure 6: FTIR spectrum of keratinase coated silver nanoparticles.

The SEM micrograph of the keratinase coupled AgNPs is shown in (Figure 7). The morphology of silver nanoparticles was studied through the SEM analysis. Spherical shape of AgNPs has been reported, and the average size was 29 nm (14-44 nm) by scanning electron microscope.

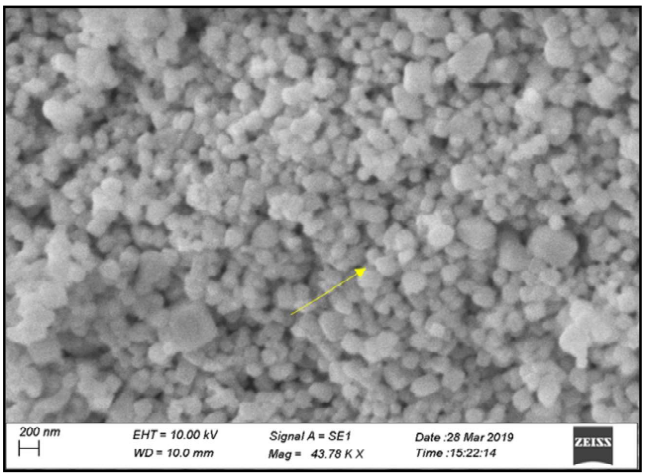

Figure 7: SEM image of keratinase coupled AgNPs using crude keratinase.

The Figure 8 shows size of the diameter of biosynthesised AgNPsat 56.8 nm by particle size analyser.

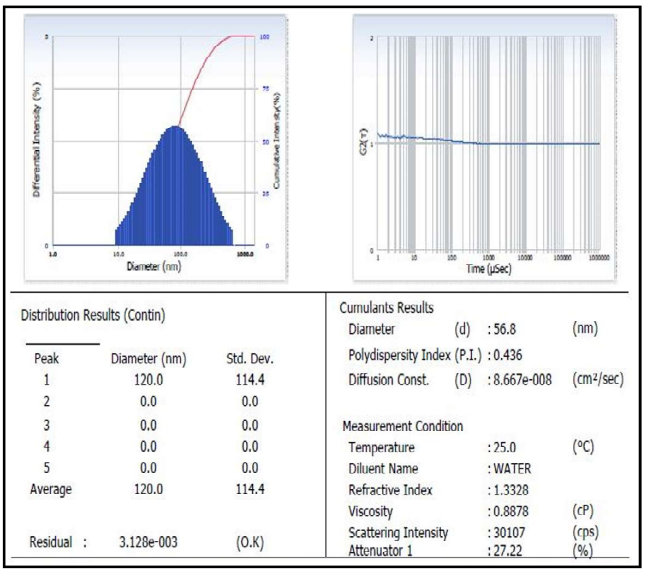

Figure 8: Particle size analysis of AgNPs.

3.6 Antibacterial activity

Keratinase enzyme loaded silver nanoparticles showed higher antimicrobial activity against the bacterial species of *Klebsiella oxytoca* and *Staphylococcus aureus* compared to *Entero bacterium amnigenus, Brevibacterium paucivorans, Staphylococcus lentus and Klebsiella pneumonia*. The samples containing AgNPs showed good inhibition zone. Figure 9 shows the zone of inhibition obtained against clinical pathogens. The zone of clearance was compared with the effects of respective standard antibiotics. Table 3 shows the antibacterial activity against the pathogens. As per the result of this study, a good inhibition activity was observed by silver nanoparticles using keratinase enzyme than the standard antibiotics.

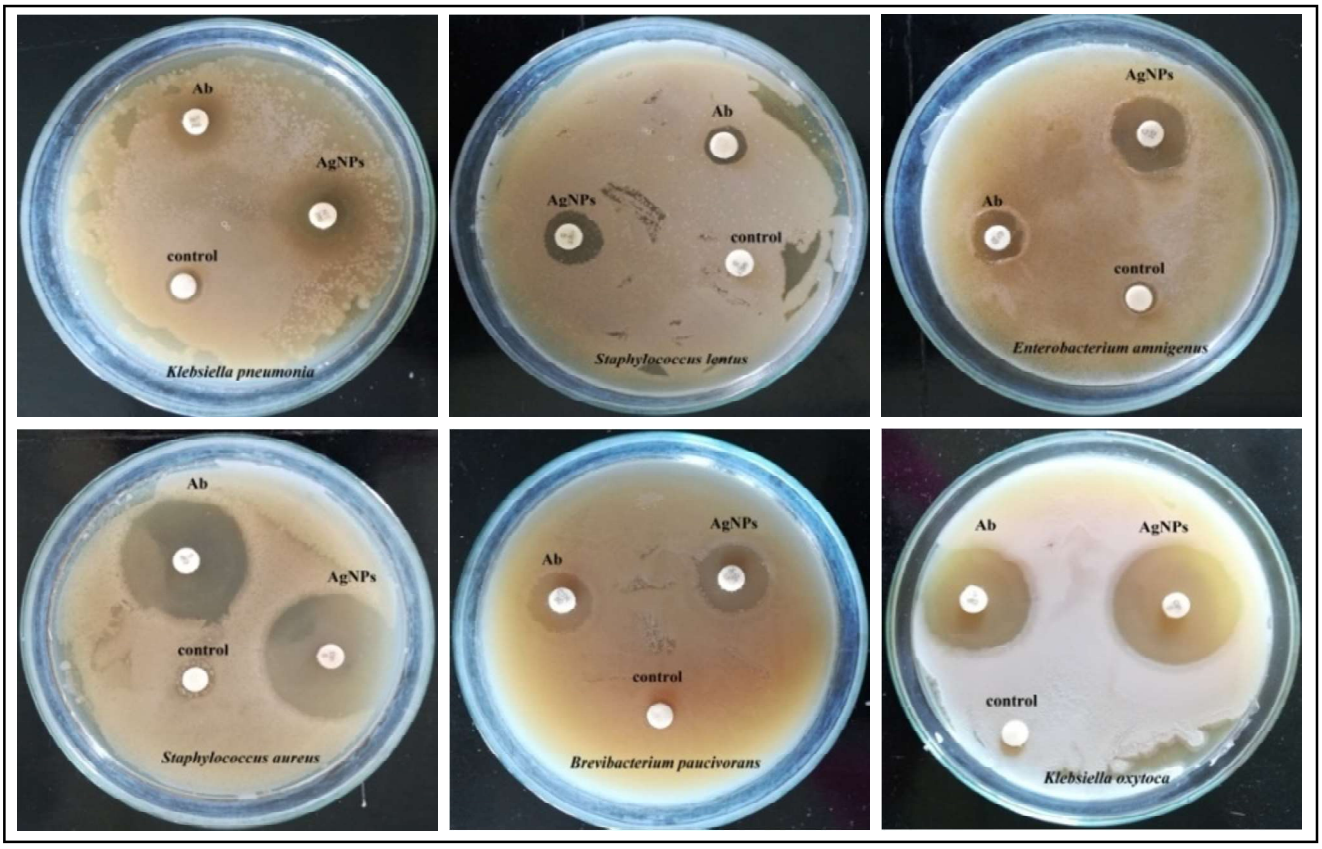

Figure 9: Antibacterial activity of AgNPs.

Table 3: Inhibition zone of AgNPs using keratinase and standard antibiotis

S.No.	Organism name	Inhibitory zone (mm)	Antibiotics (Standard)	Inhibitory zone (mm)
1.	*Klebsiella pneumonia*	20 ± 0.4	Nitrofurantoin	14 ± 0.01
2.	*Staphylococcus lentus*	15 ± 0.21	Methicillin	05 ± 0.23
3.	*Enterobacteriumamnigenus*	21 ± 0.1	Chloramphenicol	16 ± 0.3
4.	*Staphylococcus aureus*	35 ± 0.02	Ciprofloxacin	32 ± 0.1
5.	*Brevibacteriumpaucivorans*	17 ± 0.26	Ceftriaxone	15 ± 0.05
6.	*Klebsiellaoxytoca*	33 ± 0.05	Ciprofloxacin	29 ± 0.14

4. Discussion

Saarela *et al.* (2017) isolated 122 keratinolytic isolates from birds nest. Agrahari and Wadhwa (2010) identified that bacterial strains B. megaterium SN1, B. thuringenesis SN2, B. pumilis from chicken feather dumping site. Like that, we identified *S. plymuthica* and *S. ficaria.* Our findings of keratinolytic activity of microbes associated with Lateef *et al.* (2010) and Kalishwaralal *et al.* (2008).

There are limited reports for the production of nanoparticles using keratinase (Revathi *et al.,* 2013). Silver ion reduction by the enzyme gave a colour to the silver nanoparticles. Based on the progress of silver ion, the colour intensity was increased and when the reaction is completed, it becomes stable. Silver nanoparticle colour was reported byTripathy *et al.* (2010), Roopan *et al.* (2013) and Nahar *et al.* (2020) which may be attributed because of the variations in the biomolecules composition. Kalishwaralal *et al.* (2008) reported that brown colour AgNPs synthesised from culture supernatant of *Bacillus licheniformis.* Revathi *et al.* (2013) revealed that the synthesis of dark brown coloured AgNPs from crude keratinase after 72 h of incubation. Because of the excitation of surface plasmon vibrations in silver nanoparticles, colour formation was observed by Rai *et al.* (2009). These results were similar to our recent findings.

UV-Visible absorption spectrum examined the structural changes and complex formation of synthesised nanopaticles. Bands of surface plasmon resonance play a major role in size, shape and morphology was reported by Pant *et al.* (2013). Absorbance in the range of 400 nm to 450 nm has been used to affirm the silver reduction to metallic silver (Srivastava *et al.,* 2011 and Tripathy *et al.,* 2010).

Measurements of Fourier Transform Infrared Spectroscopy were executed to find the feasible biomoleculs that are responsible for the capping, reduction and potent stabilization of AgNPs (Durán *et al.* 2005). The observed peaks are principally associated due to presence of some secondary metabolites suggested by Pant *et al.* (2013), Roopan *et al.* (2013). Size variation, size distribution and capacity for aggregation, stabilities were observed by SEM analysis Fatema *et al.* (2019). The existence of nanocrsytalline image were also disclosed by Nahar *et al.* (2020) and Lateef *et al.* (2015). These properties may influence the activity of AgNPs especially antimicrobial activity. A study on AgNPs by silver nitrate solution with the culture supernatant of *K. pneumoniae* has also been reported that particle size range was in 52.5 nm (Durán *et al.* 2005). Synthesis of silver nanoparticles using *Morganella* sp., reported that approx. 20 nm size of spherical nanoparticles (Fatema *et al.* 2019). Our present findings about the characterization of keratinase coated silver nanoparticles similar to these results.

The AgNPs disrupts the cell membrane and releases the reactive oxygen species that result in damage of protein and DNA. In the present study, keratinase enzyme - NPs were effective against *S. auerus* and *Klebsiella oxytoca*. So many theories like permeability of cell membrane alteration (Shaligram *et al.,* 2009), leakage of membrane protein and lipopolysaccharide (Klefenz, 2004), production of free radical (Kim *et al.* 2007), collapse of membrane potential due to proton motive force dissipation (Lok *et al.,* 2006) were reported about antibacterial action of AgNPs. Furthermore, silver nanoparticles have high surface area, volume ratio that corresponds to effective antimicrobial action as compared with huge silver metal Lateef *et al.* (2015). Due to close attachment of the nanoparticles surface with the microbial cells determines that the antimicrobial property was to be size dependent Bhat *et al.* (2011). Like that, our results of the recent study cleared that the keratinase loaded silver nanoparticles showed good inhibition activity against the microbes. Hence, the remarkable antibacterial property reported by the AgNPs proved that it could either be combined with antibiotic drugs or used directly as drugs for some topical applications.

5. Conclusion

The results of the present study showed that the two bacterial isolates from the peacock feather was identified as *S. plymuthica* and *S. ficaria*. Among these, the *S. ficaria* showed maximum keratinolytic activity, hence it can be exploited for the synthesis of silver nanoparticles. Biosynthesis of keratinase enzyme loaded AgNPs was successfully done by $AgNO_3$ treated with crude keratinase enzyme. This technique revealed that crude keratinase enzyme can be used as an active stabilizing and capping agent for the silver nanoparticle synthesis. This is a very simple, inexpensive, method for the production of nanoparticles. It shows good antibacterial activity. Therefore, the stabilised and uniform shaped AgNPs obtained from this easy and ecofriendly method of synthesis could be used in drug formulation for topical applications and also for various biotechnological applications.

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Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. All the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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