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Bioengineered silver nanobowls using *Trichoderma viride* and its antibacterial activity against gram-positive and gram-negative bacteria

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Abstract

Myconanotechnology is a study of nanoparticle synthesis using fungi and their application mainly in medicine. Myconanotechnology has great advantage due to wide range and diversity of the fungi. Recently, fungal-mediated synthesis of nanoparticles is a reliable and ecofriendly method. The present work investigates the synthesis of silver nanoparticles using *Trichoderma viride* which is a non-pathogenic fungus. The cell filtrate of *T. viride* was used for the reduction of silver nitrate to silver nanoparticles. The pH of the cell filtrate was changed, and the effect of the pH was monitored on the synthesis of the silver nanoparticles. While changing the pH of the medium, the shape and size of the nanoparticles were controlled. The silver nanobowls were synthesized at first time using *T. viride*. The synthesized silver nanoparticles were characterized using UV spectrophotometer, X-ray diffractometer, Fourier transform infrared spectrophotometer, scanning Electron microscope (SEM), and transmission electron microscope (TEM). The SEM and TEM images showed the shape and size of the nanoparticles, and the synthesized nanoparticles were nanobowl in shape and polydispersed in size. The antibacterial activity of silver nanoparticles was carried out against gram-positive and gram-negative organisms. The maximum zone of inhibition occurred at 50 μ l, while the concentration of the nanoparticles increased the inhibition activity also increased.

Keywords: *Trichoderma viride*, Silver nanoparticles, Extracellular synthesis, Transmission electron microscope, Antibacterial activity

Background

Myconanotechnology is the connecting term of mycology and nanotechnology, and it has a significant potential in the production of nanoparticles. The synthesis of nanoparticles using fungi has gained much importance because they are easy to culture in bulk, and the extracellular secretion of enzymes has an added advantage in the downstream processing. The fungi produce higher amount of protein compared to bacteria, thus resulting in the higher production of nanoparticles [1-3]. Because of their properties, fungi might be extensively used for the rapid and eco-friendly biosynthesis of metal nanoparticles. It has been monitored that the fungal-mediated synthesized nanoparticles have good monodispersity and good dimensions [2,4]. The intracellular and extracellular

methods are being used for the synthesis of nanoparticles via a biological route. The extracellular method is more advantageous than intracellular method because the intracellular method needs an additional step to obtain the purified nanoparticles [5,6].

Synthesis of noble metals has become mandatory issue in the present decade due to the abnormal increase of their market values [7]. Metals such as gold, silver, and copper have been widely used for the synthesis of stable dispersion of nanoparticles, which are being useful in the area of photography, biological labeling, photonics, optoelectronics, and surface-enhanced Raman scattering detection [8-10]. Various physical and chemical methods are used for the synthesis of nanoparticles; these methods are intended at controlling the physical properties of particles. Still, most of these methods are in the development stage, and problems are often solved through the stability of the nanoparticle preparation, control of the crystal growth, and aggregation of the

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particles [11]. As a result of some drawbacks in physical and chemical methods, biological synthesis method has been developed to obtain biocompatible, inexpensive, eco-friendly, and size-controlled nanoparticles [12]. Nowadays, noble metal nanoparticles play a vital role in the fields of medicine, biology, physics, chemistry, and materials science. Among noble metal nanoparticles, silver is of particular interest because of its distinctive properties, such as good electrical conductivity, chemical stability, and catalytic and antibacterial activities [6,13,14].

Trichoderma viride is a rhizosphere competent strain found in all type of soils; it is a non-pathogenic fast-growing organism which is used as a biocontrol agent [15,16]. The present work demonstrated the synthesis of silver nanoparticles using *T. viride*. The synthesized silver nanoparticles were characterized using UV spectrophotometer analysis, X-ray diffractometer to analyze the nature of the nanoparticles, and scanning electron microscope to analyze the surface morphology of the nanoparticles, transmission electron microscope to analyze the size of the nanoparticles, and selected area electron diffraction (SAED) pattern to confirm the crystalline nature of the nanoparticles. Fourier transform infrared spectroscopy (FTIR) is used to identify the functional groups of the silver nanoparticles. The bactericidal activity of silver nanoparticles was carried out using disc diffusion method.

Results and discussion

The UV absorption spectral studies were carried out to confirm the formation of silver nanoparticles using *T. viride* (Figure 1). The absorption peak in UV spectrum equivalent to the surface plasmon resonance, the peak found at 400 nm, and the maximum synthesis of silver

nanoparticles occurred at 96 h of incubation. The UV spectral absorption reading confirmed that the well-dispersed, but not aggregated silver nanoparticles, were formed using *T. viride* cell filtrate [5]. The silver nanoparticles were formed very rapidly within 30 min. Fayaz et al. [16] have reported the extracellular synthesis of silver nanoparticles using *T. viride* after 4 h of incubation. The inset in Figure 1 shows the conical flasks containing *T. viride* filtrate (a) before and (b) after reaction with silver ions. The aqueous solution of the biomass is green in color before the reaction with silver ions, which turned to brown after the reaction with silver ions. The change of color indicates the formation of silver nanoparticles using *T. viride*. The color change occurs due to the excitation of surface plasmon resonance vibration of silver; hence, it confirmed the reduction of silver ions extracellularly [17,18].

Figure 2 shows the UV spectrum of pH effect on the synthesis of silver nanoparticles using *T. viride*. The sharp peak was observed at 400 nm, and the maximum production of silver nanoparticles occurred at pH 9. The inset picture (Figure 2) showed that the color intensity of the aqueous medium increased, while the pH of the fungal cell filtrate increased. The color of the solution varied from yellow to dark brown. The pH of the medium has a great effect on the control of the shape and size of the nanoparticles. The color variation from yellow to dark brown of the solution occurred because of the increase amount of nanoparticle production in the fungal cell filtrate medium [17,19]. Veerasamy et al. [20] explained that the particles are larger at lower pH, and a large amount of smaller diameter particles were formed at higher pH due to the large number of functional groups obtainable for nanoparticle binding. The

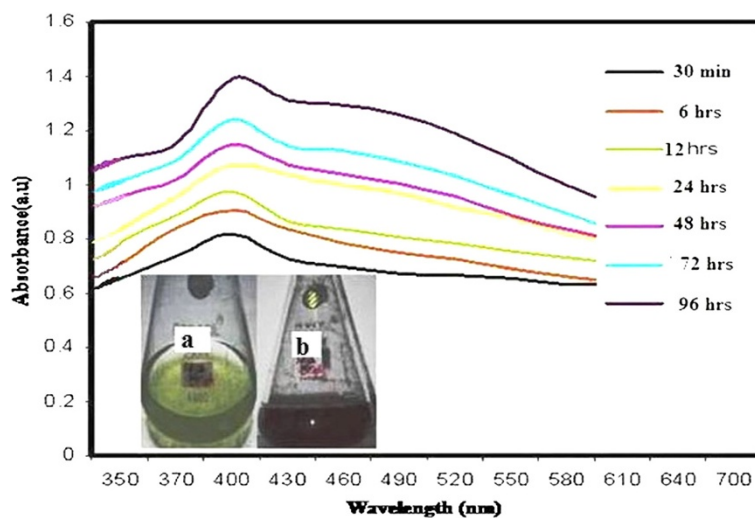


Figure 1 The absorption spectra of silver nanoparticles using *T. viride* cell filtrate at various time intervals. Inset shows (a) *T. viride* cell filtrate without silver nitrate and (b) 96 h of reaction between silver nitrate and fungal cell filtrate.

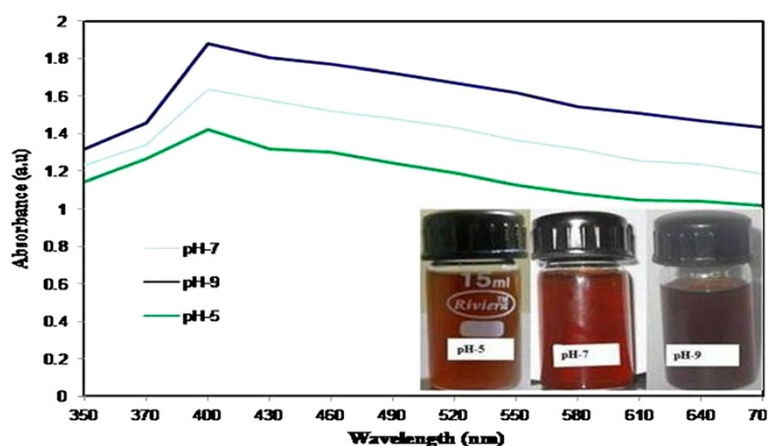


Figure 2 Effect of pH on the synthesis of silver nanoparticles using *T. viride*. The inset shows the color variation of the medium depending on the pH.

nanoparticles which are synthesized using pH 9 were used for further characterization studies.

The X-ray diffraction studies were carried out to confirm the crystalline nature of the nanoparticles, and the XRD pattern (Figure 3) of the synthesized silver nanoparticles shows the crystalline nature of silver nanoparticles synthesized using *T. viride*. The XRD pattern shows strong peaks in the entire spectrum of 2θ values ranging from 20 to 80. The silver nanoparticles synthesized in this experiment were in nanocrystal form as evidenced by the peaks at 2θ values of 38.24° , 44.11° , 64.64° , and 77.47° analogous to (111), (200), (220), and (311) planes for silver. The unassigned peaks present in spectrum indicate the presence of organic matters which are present in the fungal cell filtrate.

FTIR spectrum is used to identify the possible interactions among silver salts and proteins (present in the fungal cell filtrate) which are responsible for the reduction of silver ions and stabilization of nanoparticles. The

FTIR spectrum of the biosynthesized silver nanoparticles using *T. viride* (Figure 4) shows the absorption peaks at $3,396$, $2,925$, $2,855$, $1,740$, $1,651$, $1,548$, and $1,380$ cm^{-1} . The peak at $3,396$ and $2,925$ cm^{-1} reveals the presence of N-H bend, indicating the primary and secondary amine groups of protein. Likewise, the bands at $1,651$ and $1,548$ cm^{-1} correspond to the primary and secondary amine groups of N-H bending and carbonyl stretching vibrations of protein, respectively [21]. Therefore, the FTIR study has shown that the carbonyl group of amino acid residues and peptides of proteins has a stronger metal-binding capability. Most possibly, the proteins could form a coat to cover the nanoparticles and act as capping agents for silver nanoparticle formation to avert the agglomeration of the nanoparticles; therefore, the particles are stabilized [22]. The two bands at $2,855$ and $1,380$ cm^{-1} represent the C-H of symmetric and asymmetric stretching vibrations of aromatic and aliphatic modes, and the C-H stretching vibrations of

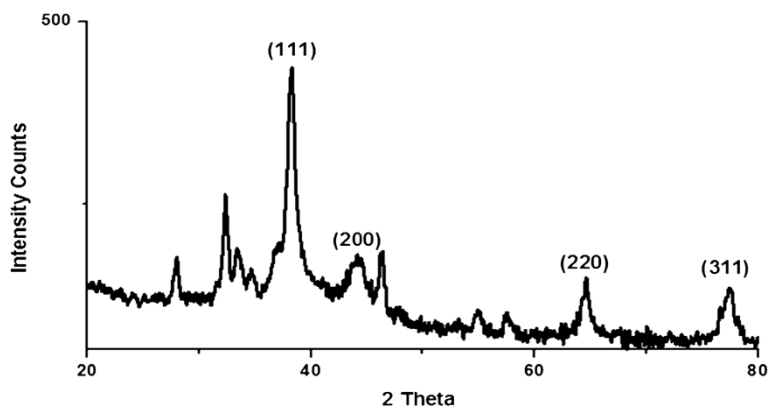


Figure 3 XRD pattern of biosynthesized silver nanoparticles using *T. viride*.

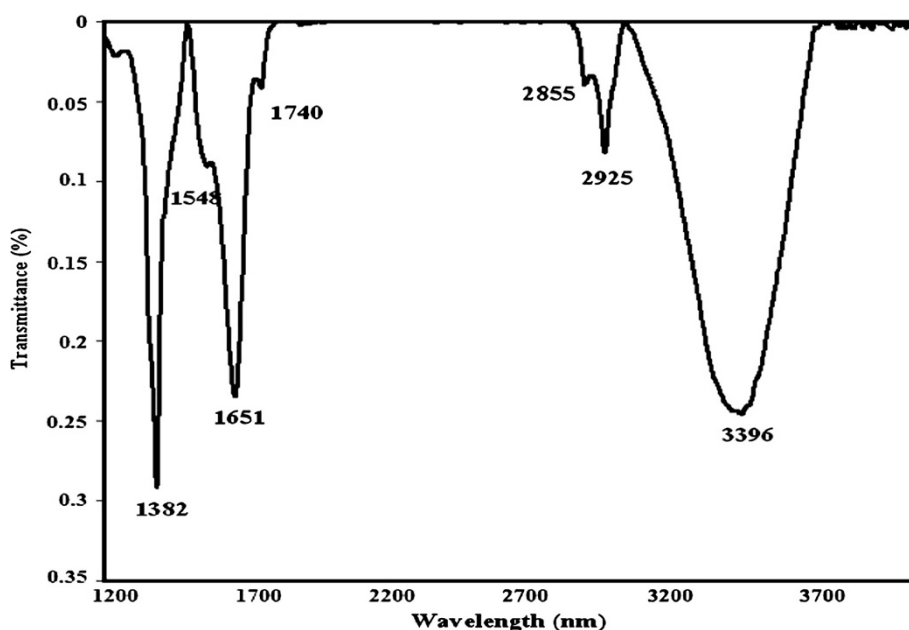


Figure 4 FTIR spectrum of silver nanoparticles.

aromatic and aliphatic amines, respectively. The band at $1,740\text{ cm}^{-1}$ corresponds to C=O stretching vibrations of aldehyde group. Hence, the observations indicate that the bioreductions of silver nitrate are tied to the oxidation of hydroxyl groups in fungal cell filtrate [23]. The whole observation suggests that the biological molecules might possibly be the reason for the formation and stabilization of metal nanoparticles in the fungal cell filtrate.

After 120 h of incubation, the biosynthesized silver nanoparticles were air dried and examined under scanning electron and transmission electron microscopes. The SEM image (Figure 5a,b) of the silver nanoparticles shows the well-dispersed nanoparticles, and the biosynthesized silver nanoparticles have bowl-like shapes. The TEM image

(Figure 6a,b) revealed the size of the synthesized silver nanoparticles. These nanoparticles were polydispersed, and their sizes were in the range from 28 to 59.17 nm. The selected area of electron diffraction pattern (inset in Figure 6) of the silver nanoparticles shows their face centered cubic structure, and the synthesized silver nanoparticles are in crystalline nature. This is the first study to report on silver nanoparticle synthesis using *T. viride* resulting in nanobowl-shaped nanoparticles. The definite reaction mechanism for the synthesis of silver nanoparticles using fungal cell filtrate is not yet to be elucidated. The fungal biomass secretes proteinic compounds in liquid medium which may be accountable for the synthesis of nanoparticles, and consequently, the proteins

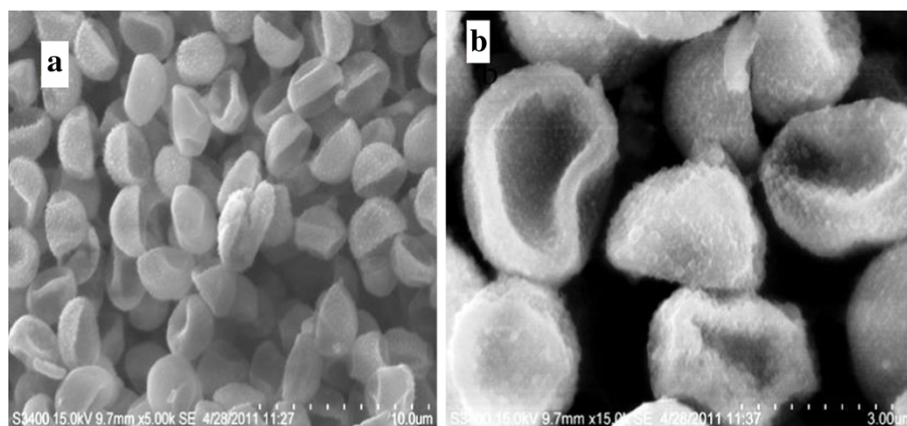


Figure 5 SEM images show the bowl shape of the biosynthesized nanoparticles using *T. viride* (a-b). $\times 15,000$ magnification.

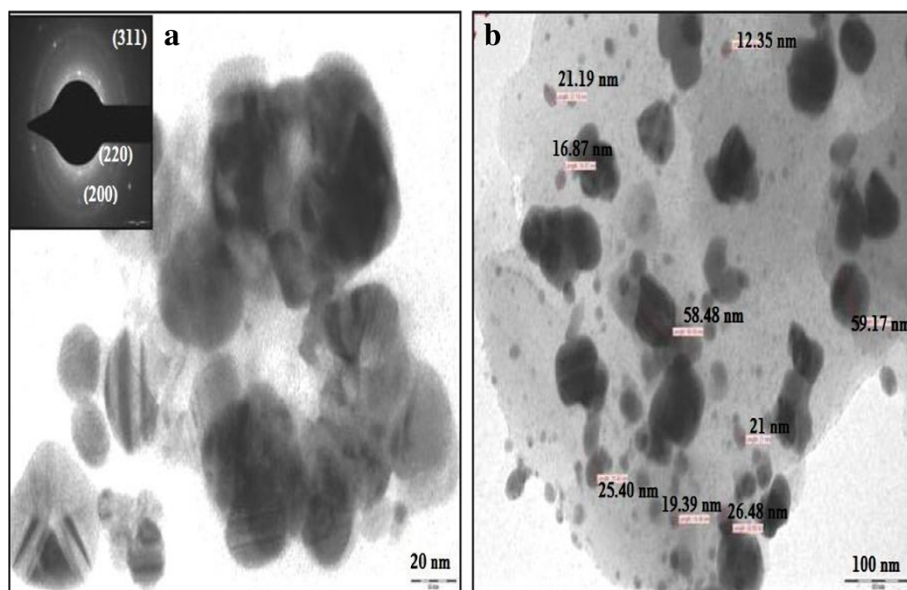


Figure 6 TEM images of the biosynthesized nanoparticles. Scale bars (a) 20 nm and (b) 100 nm. Inset shows the selected area of diffractometer pattern of the synthesized silver nanoparticles.

attach and improve the stability of the nanoparticles [5]. Two protein bands were identified with molecular weights of 45 and 39 KDa, respectively, in silver nanoparticle sample, which may be responsible for the synthesis of silver nanoparticles (Figure 7). The extracellular proteins have great advantage in the synthesis of nanoparticles because they provide large amount of nanoparticles in pure form, free from other cell components [3].

Well diffusion method was used to provide the evidence for the antibacterial activity of biosynthesized silver nanoparticles against *Bacillus subtilis* and *Klebsiella planticola*. Different concentrations (10 to 50 μ l) of silver nanoparticles were used to confirm the antibacterial efficiency of the silver nanoparticles. The maximum zone of inhibition was observed in *K. planticola* followed by *B. subtilis* (Figure 8). The antibacterial activity of silver nanoparticles was indicated by the formation of the zone. The diameter of the inhibition zone was measured in millimeters. The maximum zone of inhibition occurred at 50 μ l concentration of silver nanoparticles. The possible mechanism for the bactericidal activity of silver nanoparticles may be the penetration of the nanoparticles into the cell wall and entry inside the cells, compressing the DNA which simultaneously inactivates the cellular proteins [24,25].

Conclusion

An ecological, cost-effective method was employed for the extracellular synthesis of silver nanoparticles using *T. viride*. X-ray diffractometry revealed the crystalline nature of the nanoparticles. Scanning electron microscopy

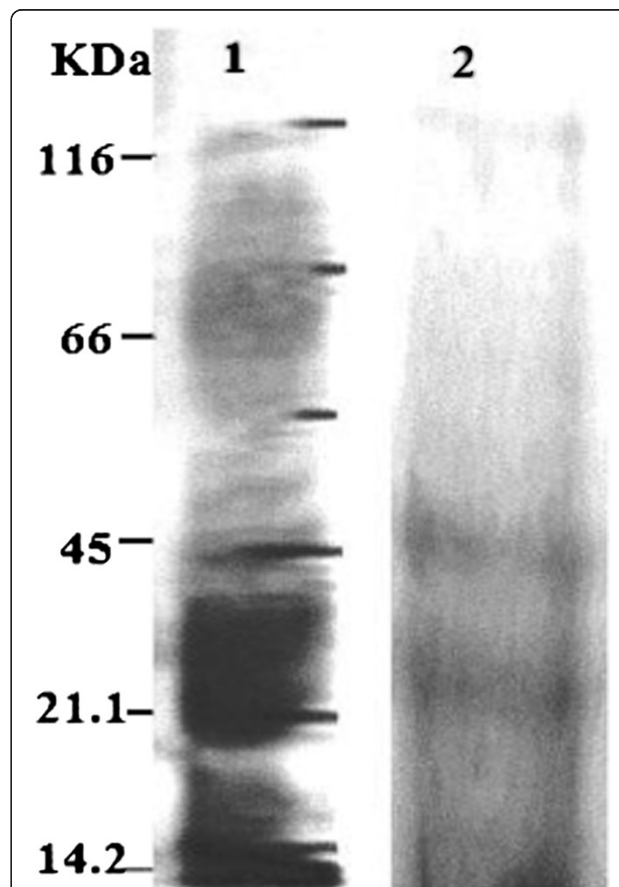


Figure 7 SDS-PAGE gel electrophoresis (1) marker protein and (2) synthesized silver nanoparticles.

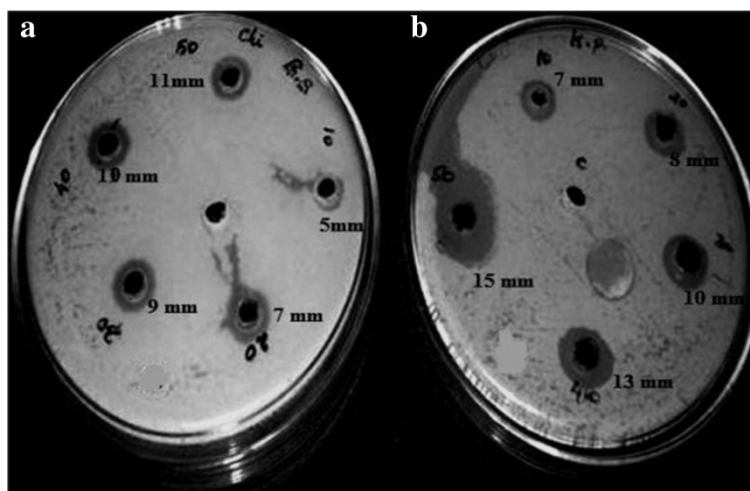


Figure 8 Antimicrobial activity of biosynthesized silver nanoparticles against (a) *B. subtilis* and (b) *K. planticola*.

revealed the bowl-shaped nanoparticles. Transmission electron microscope shows the average size of the nanoparticles to be around 28 nm, and the SAED confirms the crystalline nature of the nanoparticles. The antibacterial activity occurred due to the penetration of the silver nanoparticles into the cell walls, reducing the function of the organisms.

Methods

Preparation of silver nanoparticles

T. viride was obtained from MTCC-800 Mumbai, India. The culture was sub-cultured in SDA media. Then, the *T. viride* was inoculated in a liquid SDA media and grown aerobically at 35°C and agitated at 150 rpm on orbital shaker. The biomass was harvested after 72 h of growth by filtration using Whatman No. 1 filter paper followed by extensive washing with distilled water to remove any medium component from the biomass. The 10 g of biomass was brought in contact with 100 ml of double distilled water for 48 h at 35°C and agitated as described earlier. After 48 h of incubation, the cell filtrate was obtained by passing it through Whatman filter paper No. 1. The silver nitrate (1 mM) was added in 100 ml of cell filtrate, and the reaction was carried out at room temperature. The UV spectroscopic absorbance was taken at various time intervals.

Effect of pH on the synthesis of nanoparticles

The cell filtrate was obtained using the above-stated process, and then the pH (5, 7, and 9) of the cell filtrate was adjusted using 0.1 N of NaOH and 0.1 N of HCl followed by the addition of 1 mM of silver nitrate to the cell filtrate. After 24 h of reaction, the UV spectrophotometric reading was taken at different wavelengths.

Characterization of the synthesized silver nanoparticles

The synthesized silver nanoparticles were characterized using UV (Perkin Elmer Inc., MA, USA) visible absorbance spectra, absorbance taken at various time intervals at different wavelength, powder X-ray diffractometer (Bruker D8 Advance using CuK α radiation, at 40 keV in the range of 10 to 80, Bruker Corporation, Ettlingen, Germany), scanning electron microscope (S-3400N, Hitachi, Tokyo, Japan), Fourier transform infrared spectrometer (6700, Thermo Nicolet, MA, USA), transmission electron microscope (CM200, Koninklijke Philips Electronics N.V., Amsterdam, Netherlands), and selected area electron diffractometer to characterize the nature, shape, functional groups, and size of the synthesized silver nanoparticles.

SDS-PAGE gel electrophoresis

The proteins which might be the reason for the synthesis of nanoparticles were determined using SDS-PAGE gel electrophoresis. Silver nitrate (1 mM) was added to the *T. viride* cell filtrate. After 24 h of incubation, it was precipitated with ammonium sulfate (80% saturation). Then, the pellet was dialyzed against phosphate buffer (0.05 M) using dialysis bag. The sample was analyzed on SDS-Page, and the molecular weight of the protein was determined using marker protein.

Antibacterial activity of silver nanoparticles

The bactericidal activity of the synthesized silver nanoparticles was observed against *B. subtilis* (3053) and *K. planticola* (2277). The bactericidal activity was carried out using well diffusion method. Mueller-Hinton agar medium (MHA) was prepared and poured into plates, and wells were made in the agar medium. Then, 24-h-old test bacterial cultures were swabbed in MHA medium to form a

confluent lawn of bacterial cultures. Various concentrations (10 to 50 μ l) of silver nanoparticles were loaded into the wells. Then, the plates were incubated at 35°C for 24 h. After 24 h of incubation, the plates were observed for zone of inhibition.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KC and GGA provided the same contributions in this article. Both authors read and approved the final manuscript.

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