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Synthesis of silver nanoparticles using bacterial and fungal reduction methods and analytical evaluation of their physical and antimicrobial characteristics

Abstract

Nanoparticles (NPs) have been steadily interesting in Physics, Chemistry, Biology, Biomedicine, etc. In our study, green synthesis of silver nanoparticles by the fungal and bacterial method was investigated. Two different concentrations of $AgNO_3$ viz. 10mM and 1mM were used as precursors, whereas cell- free supernatants from *A. niger* and *E. coli* were prepared and used as source of enzyme for the reduction and stabilization of nanoparticles. The crude enzyme was produced by *A. niger*, using Solid State Fermentation (SSF). The medium used for the fermentation contained wheat bran as the growth substrate and was incubated at 29±1ºC for five days. Whereas for enzyme production by *E. coli*, Luria Bertani (LB) medium was used and it was incubated at 37±1ºC overnight. The culture for the growth of *A. niger* was filtered by muslin cloth and centrifuged at 12000rpm for 20min and the filtrate was used as source of crude enzyme for the synthesis of Ag NPs as well as for characterization. Similarly, the *E. coli* culture was first filtered by Millipore filter paper followed by centrifugation of the supernatant at 12000rpm for 20min. The UV- Vis absorption peak for Ag NPs synthesized by *E. coli* was seen around 416nm; whereas, the UV-Vis peak for Ag NPs synthesized by *A. niger* appeared around 425nm. The next characterization of the synthesized silver nanoparticle was done by Fourier Transform Infrared spectroscopy (FTIR), which was used to reveal the functional groups accompanying the synthesized NPs. Finally the antimicrobial activity of the synthesized silver nanoparticles was tested against three gram negative and two gram positive bacteria by disc diffusion method. Extracellular crude extracts obtained from both microbes did not show any antibacterial effect on the tested pathogens. The Ag NPs showed stronger antibacterial activities on gram positive bacteria than on gram negative bacteria. The results also indicated that a larger value of ZOI was produced for the Ag NPs synthesized at the lowest concentration (1mM) of the precursor $AgNO_3$ used for the synthesis of Ag NPs.

Keywords: *A. niger*, antimicrobial activity, E. coli, FTIR, silver nanoparticles,UV-Vis1

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Introduction

Atoms or molecules bonded together in a conglomeration, typically between 1 and 100nm, whose size is intermediate between a single atom and an aggregate large enough to be treated as bulk, are called nanoparticles.[1] They occur naturally as suspensions in air, water, blood, soil, ash and almost everywhere else. Their dimensions are often not too small to apply quantum mechanics and not large enough to follow classical mechanics. The tiny dimension of nanoparticles (NPs) enhances their surface to volume ratio to high proportions. This and other factors bring about a substantial modification in their physical and chemical properties making them vastly different from that of their bulks. These properties include their catalytic activity, mechanical strength, electrical conductivity, thermal stability, and their response to magnetic, optical and biological signals. Thus all the nanomaterials, especially the nanoparticles offer themselves as a new range of materials which, with or without any further modification, can be made to deliver unconventional functions and results. So they find technological applications such as to toughen a matrix like a ceramic, as cleaning agents, as surface coats, as catalytic agents, as biomarkers and in microelectronics. Nanoparticles could be synthesized using bottoms-up approach or cut from any bulk material using top-down approach. Almost any material in its bulk form such as monoatomic substances, salts, oxides, ceramics, polymers and so forth could be converted to NPs. At those dimensions, they manifest their own properties and accordingly they find avenues of their technological applications. Nanoparticles of metals like Silver (Ag) and Gold (Au) have been extensively investigated as they exhibit properties, which are unique and help in the diagnostics, therapy, and other devices used in medical applications.[2] Nanoparticles of silver (Ag NPs) are known to exhibit antimicrobial potential[3] which is more enhanced than that of the silver ions. It is believed to happen through their ability to limit cell transduction and cause cell lysis. The antimicrobial potential and reactivity of silver gets substantially enhanced at Nano-dimensions through large surface to mass ratio, and it has been used for food preservation[4,5] and packaging.[6] They also find use as catalytic agents, as sensors, in coatings, cosmetics and as therapeutic and bactericidal agents.[7] Attempts have been made to synthesize these NPs by using physical methods such as ablation, sonication, spray, laser ablation, flame-pyrolysis etc. Several chemical methods like sol-gel, co-precipitation, hydrothermal and micro-emulsion procedures have also been used by various scientists to fabricate Ag NPs. However, these synthesizing processes, especially the reduction of silver ions tend to be expensive and involve other problems such as the adsorption of toxic substances on the NPs. To alleviate this problem, other ways of synthesizing Ag NPs like those using biological systems such as bacteria, fungi, and plant extracts are being tried out.[8] The emphasis and the resultant efforts to synthesize

silver nanoparticles and characterize their antibacterial activities have been of growing interest. Some of these deliver NPs in solution and others in their solid phases. However, the chemical reduction methods to prepare NPs of Ag offer better versatility. It manifests through their control on the size in which silver ions are reduced by reductant and stabilizing or protecting agents. It allows a modification of their zeta potential and prevents the agglomeration of nanoparticles. A variety of processes within the chemical avenues have been utilized to produce silver nanoparticles with a distribution of their size and shapes. Biosynthetic and physicochemical approaches have also been used to prepare silver nanoparticles with the aim of improving the conventional methods. The major mechanism of the antibacterial properties of silver nanoparticles is by anchoring to and penetration in the bacterial cell wall. Once inside the cell, silver nanoparticles modulate the cellular signaling by dephosphorylating the putative key peptide. These potential applications increased the needs and they have also accelerated the proliferation in the avenues through which metallic nanoparticles such as Ag NPs could be synthesized. Some NPs as well as their synthesis entail sequences and by-products that are not conducive to the environment. The microbial synthesis method has been chosen for this research because of its eco-friendly aspect and cost-effective. So, a careful and comprehensive comparison of different approaches for preparing the nanoparticles would be crucial. Typically, the emphasis would be on enhancing the reliability, reducing the price and toxicity involved and on the long term sustainability of the process. A comparative view on the chemical and other resources used, quantity and physical characteristics of NPs produced and their antimicrobial potential is stipulated. With these aims in vision, this study idea was formulated with the objectives sated ahead. The basic aim of this study was to synthesize silver nanoparticles through the reduction of silver ions by microbes and to do a comprehensive experimental study of the physical characteristics of these nanoparticles as well as their antimicrobial activities.

Materials and methods

Apparatuses and instruments

The materials and instruments used were digital autoclave, incubators, and refrigerator, rotary shaker, centrifuge, oven, micro plant grinder, hot plate magnetic stirrer, hot water baths, sieves (10, 20, & 30meshes), sensitive electronic balance (max weight 210g, readability 10mg), analytical balance (max weight, 50g; readability, 0.1mg), weighing trays, digital pH meter, vortex tube, Erlenmeyer flask, volumetric flasks, laminar air flow hood, thermometer, digital colony counters, test tubes, quartz cuvette, UV-Visible spectrophotometer, FTIR, beakers, bended glass rod, graduated cylinder, Petri dishes, micropipette, dropper, inoculation loop, forceps, stirring glass rod, ruler, camera, bottles, rack (test tube holder), glass slides, filter paper discs, spatula, plastic bags, ice box, cotton, muslin cloth, aluminum foil, paraffin, Whatman filter paper, tissue papers, Whatman lens cleaning tissue, cleaning brush, detergents, laboratory coat, safety eye glasses (goggles), gloves and mouth mask.

Chemicals and reagents

All chemicals of analytical grade were obtained from Haramaya University Central Laboratory. Silver nitrate ($AgNO_3$, 99.97%) was used as the precursor of silver nanoparticles (Ag NPs), for the biological synthesis methods, while, crude enzymes from *E. coli* and *A. niger* were used as reducing and stabilizing agents. Different types of agar media (such as Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), Luria Bertani Broth (LB), peptone water, MacConkey Agar and Muller Hinton Agar (MHA) were used for culturing, sub-culturing and cultivation of microbes. Wheat bran and salt solutions (ammonium sulphite, sodium hydrogen phosphate, potassium hydrogen phosphate, magnesium sulphate, calcium chloride and sterile distilled water (SDW) were used for solid state fermentation (SSF) to culture *A. niger*, which was used for secreting the crude enzyme, whereas the chemicals NaOH and HCl were used for pH adjustment. Distilled water (DW), deionized water (DIW), sterilized distilled water (SDW) and alcohols were used to prepare the solutions and media and for washing and cleaning purposes, done throughout the process of synthesis of NPs.

Preparation of silver nanoparticles

To achieve the objectives of this thesis, silver nanoparticles (Ag NPs) were synthesized using two microbial methods viz. bacterial synthesis method and fungal synthesis method. For both methods, silver nitrate ($AgNO_3$) was used as precursor; whereas a crude enzyme obtained from the supernatant of both *E. coli* and *A. niger* cultures were used as source of reducing and stabilizing agents for the bacterial and fungal synthesis methods of silver nanoparticles, respectively.

Synthesis of silver nanoparticles using bacteria (*E. coli*) crude enzymes: *Escherichia coli (*ATCC-25922), *Pseudomonas aeruginosa (*ATCC-27853*), Streptococcus* (ATCC-12386*), Shigella flexneri (*ATCC-12022*)* and *Staphylococcus aureus* (ATCC-25223) and *Aspergillus niger* were obtained from Ethiopian Public Health Institute, Addis Ababa, Ethiopia. It was transported to microbiology laboratory, Haramaya University and maintained in refrigerator at 4ºC for further uses. *Escherichia coli* and *Aspergillus niger* were used for bacterial and fungal reduction methods of silver nanoparticles respectively. The other bacteria species including *E. coli* were used for antibacterial activity test of synthesized silver nanoparticles.

Sub-culturing of *E. coli*: The collected bacterial species was cultured and sub-cultured to avoid contamination and to maintain purity for further use. The media prepared for culturing and sub-culturing of *E. coli* and other bacteria were LB agar media and they were prepared as follows: Materials used were digital electronic balance, spatula, DW, 500ml graduated cylinder, 4 bottles of 250ml capacity each, 1000ml flask, sterile distilled water (SDW), sterile plates, funnel, weighing trays, magnetic stirrer, cotton and aluminum foil. The components of LB media were yeast extract, 5g/l; peptone, 10g/l; and sodium chloride, 5g/l.

Procedures of media preparation: Mass of all the composition of LB media was measured on clean dry weighting tray using digital electronic balance calibrated at mass=0.00gram. These measured components of LB media were added to 1000ml of Erlenmeyer flask using clean funnel and mixed with 500ml of SDW. Magnetic stirrer was inserted into the solution and boiled using hot plate at an appropriate temperature and stirring speed. After boiling, agar was added and stirred properly to homogenize the media. This medium was poured into autoclaving bottles and loosely closed with caps and the neck was covered with aluminum foil to protect the entrance of moisture from outside. Then the medium was autoclaved at 121ºC, 15psi for 15minutes. After autoclaving, it was cooled to around 45ºC and put into hot water bath at 45ºC until inoculation.

Inoculation: Materials used were inoculating wire loop, bacterial inoculum, spirit lamp, 70% alcohol, cleaning tissue paper and cotton, sterilized petri dishes, incubator and MacConkey agar, and hot water bath. The procedures followed for prior to inoculation were cleaning and disinfecting the safety hood by UV-light, and alcohol; wiping

with sterile cotton. Around 20ml of autoclaved LB media were poured into the center of 90mm diameter petri dishes followed by swirling the petri dishes slowly to distribute the media uniformly and were allowed to wait until it coagulated. By using sterile wire loop, a loopful inoculum of *E. coli* was inoculated into LB medium by the streak plate technique and incubated at 37±1ºC for 24hours.

Maintaining of the pure culture: Materials used were inoculation wire loop, bacteria samples, flame, sterilizing alcohol, cleaning tissue paper and cotton, sterilized test tube, incubator and MacConkey agar, and hot water bath. For sub-culturing MacConkey agar solution was poured into the test tubes up to halfway after which the tube was slanted at around 30º to 45º until it coagulated. The *E. coli* sample was inoculated to the position by sterile wire loop, incubated aerobically for 24hours at 37±1ºC. Then the culture was stored in refrigerator at 4ºC for further use.

Source of crude enzyme used for the synthesis of Ag NPs: The source of crude enzyme that was required for the synthesis of silver nanoparticles was clear supernatant obtained after centrifugation of grown culture of *E. coli* for 24hours. To do this, *E. coli* was cultured in an Erlenmeyer flask of 250ml containing LB medium (composition : trytone, 10g/l; yeast extract, 5g/l and NaCl, 5g/l) at a pH of 7.03 ± 0.01 and incubated in an orbital shaker at 37±1 ºC and agitated at 80 rpm in triplicates. The resulting LB culture medium after 24hours of growth was filtered by Whatman number-1 filter paper followed by centrifuging at 12,000rpm of 4ºC for 15minutes, twice. The clear supernatant was collected in sterilize 500ml (Schott, Duran, West Germany) flask and kept in refrigerator at 4ºC for Ag NPs synthesis and characterizations.

Synthesis of Ag NPs by using *E. coli* crude enzyme: The crude enzyme from *E. Coli* were used for the synthesis of silver nanoparticles. It was prepared by mixing 10ml of supernatant with 5ml of different concentration of silver nitrate ($AgNO_3$, 99.97%) solutions (10mM and 1mM). Another reaction mixture of equal volume but without silver nitrate was used as a control. These prepared solutions were incubated at 37±1ºC for 24h. All solutions were kept in dark to avoid any photochemical reactions during the experiment. After 24hours, the colors of the solutions were visually observed and the color of clear supernatant was unchanged. But the color of the solution after mixing with 1mM $AgNO_3$ aqueous solution and incubated at dark condition for 24hrs was changed from pale yellow to orange. The synthesized silver nanoparticles (Ag NPs) were purified by centrifuging at 12,000rpm for 15min twice. The precipitate was collected for UV-Vis spectroscopic and FTIR characterization, and also for tests of antibacterial activities.

Synthesis of silver nanoparticle using *A. niger*: Pure culture of *Aspergillus niger* which was collected from Ethiopian Public Health Institute, Addis Ababa, was transported to the PhD Plant Protection Research Lab, faculty of Plant Sciences, Haramaya University. The samples were stored in refrigerator at 4ºC for future uses.

Sub culturing of *A. niger*: Materials used were Bunsen burner, alcohol**,** inoculation wire loop, Petri dish with *A. niger* sample**,** three flasks of volume 250ml each with 150ml of autoclaved PDB solution. The procedure followed were sterilizing inoculating wire loop, cleaning and disinfecting the safety hood and the bench with alcohol, inoculating aseptically PDA plates and PDB with *A. niger* inoculums, inoculating the medium containing wheat bran with *A. niger* inoculum.

Preparation of Solid State Fermentation (SSF) medium: Materials used were micro plant grinder, sensitive electronic balance, DW, sieves of different pore sizes (10, 20 and 30meshes), wheat seed, salts, muslin cloth, pH meter, centrifuge, drying oven, and 60ml volume sterile syringe. The procedures followed for the preparation of solid state fermentation were as studied by for the production of crude enzyme by *A. niger* through solid state fermentation. The wheat seed collected from market was rinsed with distilled water and dried at 40ºC overnight. This wheat seed was ground by micro plant grinder in the microbiology Lab of Haramaya University. This ground floor was sieved using 10, 20 and 30-meshes and only 20-mesh sieve was taken to get uniform size range and the wheat bran was collected as solid substrate for the production of crude enzyme extract by SSF by *Aspergillus niger.* For the production of crude enzyme extract by SSF from *A. niger*, 10g of sieved wheat bran was put in three identical Erlenmeyer flasks of 250ml and Sprinkled with 1ml of salts solution (ammonium sulphite, 5g/l; sodium hydrogen phosphate, 2.0 g/l; magnesium sulphate, 3.0g/l and calcium chloride, 3.0g/l) then moisture to 70% with DW and autoclaved at 121ºC for 15min at pressure of 15Psi.[9]

Inoculum preparation: A loopful of fungal spores was transferred to 10ml of sterile double distilled water. A uniform spore suspension was obtained by mixing vigorously, and the absorbance was measured under white light. One milliliter of spore suspension (10^7 to 10^8spores/ml) was transferred to 10g of wheat bran solid substrate in each of three identical sets. Then the inoculum in wheat bran media was incubated for 6days at 29±1ºC. After six days of incubation it was treated with 50ml of SDW and was stirred for quarter of an hour using sterilized glass rod and followed by filtration using muslin cloth. Cell-free crude extract was collected in sterile 500ml flask and the residue was retreated with 50ml SDW and filtered by muslin cloth. These crude extract was then centrifuged at 12,000 rpm for 15min at 4ºC twice, collected in the sterile 500ml Brussels flask and kept in the refrigerator at 4ºC for further use.[10] This supernatant was filtered by 0.45µm Millipore membrane filter and followed by 0.2µm Millipore membrane filter. The pH of clear supernatant measured was to be at 7.0.

Synthesis of silver nanoparticles: For the biosynthesis of silver nanoparticles, 10ml of clear supernatant from *A. niger* was mixed with 5ml silver nitrate ($AgNO_3$) solutions with the concentrations of (10mM and 1mM) separately and another reaction mixture without silver nitrate solution was used as control. The prepared solutions were incubated at 29±1ºC for 36hours. All solutions were kept in dark to avoid any photochemical reactions during the experiment. After 36 hours, the solution turned from brown to dark brown. The Ag NPs was purified by centrifuging at 12,000rpm for 15min two times.

Characterization of silver nanoparticles: Characterization was done after freeze-drying of the purified silver nanoparticles. The absorption peak and composition of the synthesized silver nanoparticles were analyzed by UV-Visible spectrophotometer and Fourier transforms Infrared (FTIR) spectral analysis methods.

UV-Vis spectrophotometry analysis: The Materials used for UV-Visible analysis were UV-Vis spectrophotometer (UV-1800), DW as reference sample, two quartz cuvette with light path 10mm, for the absorbance measurement of blank sample and crude enzyme samples. Digital pH meter was used to measure pH of the sample solutions. The pH of LB medium was measured to be 7.00±0.01 and the pH of SSF culture medium of *A. niger* after a treating with 100ml

of DW was 6.02±0.01. The UV-Vis spectroscopic absorption for the crude enzyme from *E. coil* and *A. niger* and Ag NPs synthesized by the action of those microbes crude enzyme were observed from the spectral analysis of spectrophotometer. Optical absorption spectra of microbial supernatant were observed through UV-Vis spectrometer, UV-1800, in the wavelength range from 190nm to 450nm. To avoid absorption spectrum of the background, the equipment was first calibrated with a DIW. The sample was placed into the quartz cuvette and was inserted in the spectrophotometer sample chamber. A pulse of wide-spectrum light, generated by a tungsten bulb, was passed through the sample and the resulting absorption was collected and the blank sample was subtracted out.

FTIR spectra analysis of silver nanoparticles: The silver nanoparticle solution thus obtained was centrifuged at 12,000rpm for 15min, after which the supernatant was discarded whereas the pellet was re-dispersed in DIW to get rid of any uncoordinated biological molecules. The purified pellets were then freeze-dried, powdered, and used for FTIR analyses. Fourier transform-infrared (FT-IR) analysis was performed to identify the possible biomolecules responsible for the reduction of the Ag^+ ions and capping of the reduced Ag NPs synthesized using crude enzyme extracts from *E. coli* and *A. niger*. The functional groups of synthesized silver nanoparticle were analyzed by FTIR spectral peaks using origin 7.0software applications.

Test for Antibacterial Activities of Ag NPs: Antibacterial activities of biosynthesized silver nanoparticles were evaluated by using standard disc diffusion method.[11] The antibacterial activities testing photogenic bacteria were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Shigella flexneri* ATCC 12022, *Staphylococcus aureus* ATCC 259323, and *Streptococcus spp. (ATCC-12386)*. The Ag NPs solution synthesized from 10mM $AgNO_3$ and 1mM $AgNO_3$ by the action of crude enzymes from *E. coli* and *A. niger* were used for the tests of antibacterial activities. The bacterial suspension of 24hours grown strains was swabbed on MHA media plates using sterile micropipette. Double sterilized 6mm paper discs were loaded with Chlorampenicol (20µl/6 mm disc=0.002mg) solution and solutions containing silver nanoparticles were loaded with 20µl/6disc. Then the discs were air dried in sterile condition and placed on MHA media plates and incubated at 37± 1ºC for 24hours. Finally, the maximum ZOI were measured.[12] Comparisons of antibacterial activity were analyzed by SAS software version 9.0.

Results and discussion

Optical absorption of microbial crude enzymes

As indicated in the appendix Figures 1A–1C, the visually observed color of the supernatant from *E. coli* grown in LB medium was pale yellow and the color of supernatant produced by *A. niger* in the SSF medium was brown. These colors were changed from pale yellow to orange for Ag NPs synthesized by the crude enzyme from *E. coli* and, from brown to dark brown for supernatant from *A. niger.* These color changes indicated the bio-reduction of silver ions to Ag NPs in 24hours by the action of supernatant from *E. coli* as shown in Appendix Figure 1C and *A. niger* Appendix Figure 1D respectively. The UV-Vis spectra of the crude enzyme extract from *A. niger* showed a peak at the wave length around 223nm (Figure 2). It indicated that the presence of Ethyl ether/Isopropyl alcohol and another peak around 268nm refers to the bioactive organic molecules used for the biosynthesis of NPs. The absorption at the wave length 231nm, which represents 4-methyl-3-penten-2-one (mesityl oxide) in hexane. Another peak at 265nm indicated the presence of aromatic amino acids of proteins in the cell free clear supernatant from *E. coli*. The overall UV-Vis spectra analysis indicated that the supernatant extracted from *A. niger* and *E. coli* contained bioactive protein and enzymes which may be responsible for the synthesis and stabilization of Ag NPs. UV-Vis spectroscopy is one of the most widely used techniques for structural characterization of silver nanoparticles.[13]

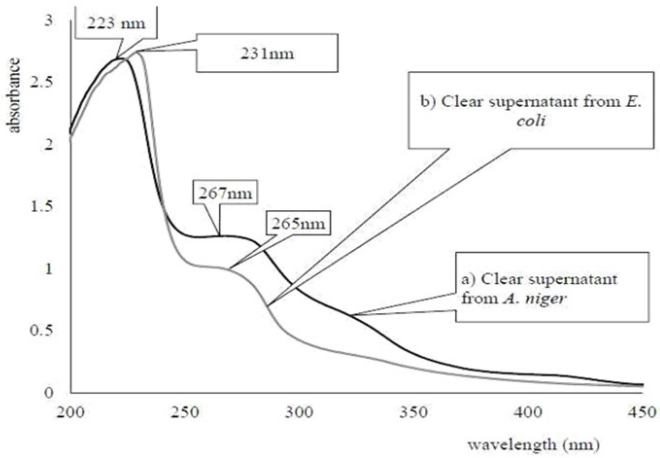

Figure 1 UV- Vis absorbance of microbial clear supernatant A) *A. niger* and B) *E. coli.*

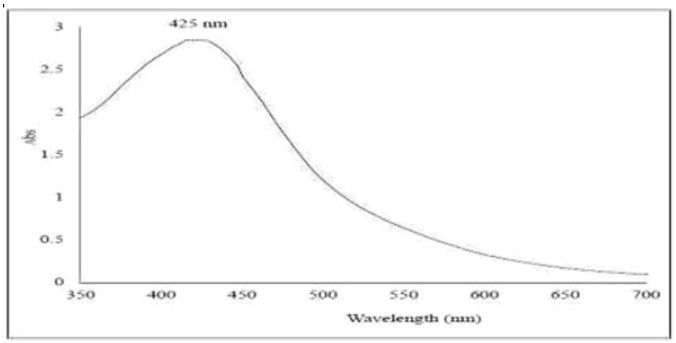

Figure 2 UV-Visible absorption of Ag NPs by *A. niger* cell-free supernatant.

UV–visible spectra analysis of silver nanoparticles

Detection of synthesis of Ag NPs was primarily carried out by visual observation through color changes of the culture supernatant from *E. coli* and *A. niger* after treating with silver nitrate solution. As shown in Appendix Figure 1A & 1B, the initial color of clear supernatant from *E. coli* was pale yellow, but after treating with 1mM $AgNO_3$ solution and incubating at 37±1ºC for 24hours, the color was changed to orange. But there was no change in color on the reference supernatant of equal volume which was incubated without $AgNO_3$ in the same condition. Similarly, the color of clear supernatant from *A. niger* after being mixed with 1mM $AgNO_3$ in the ratio of 2: 1, was changed to dark brown when it was incubated at 29 ± 1ºC for 36hours. So those color changes were considered as visual evidences for the formation of Ag NPs in the reaction mixture and a confirmation for the occurrence of reduction of $Ag^+$to Ag^0. Formation of Ag NPs can also be easily detected by UV-Vis spectroscopy, since the NP suspension in the solution showed a peak in specified nanometer range. The UV-Vis spectral peak in Figure 2 was observed in the UV-Vis spectrum at the wave length of 425nm for Ag NPs synthesized by the supernatant from *A. niger*. The peak in the UV-Vis absorption result corresponded

to the maximum absorption peak obtained at 425nm. Similar result was reported by **Sing *et al*.**[14] (2014) for the Ag NPs synthesized by endophytic *Penicillium* species. In Figure 3, it can be seen that the maximum absorption peak for the Ag NPs synthesized by *E. coli* crude enzyme, was at around 416nm. After 24hours of incubation, nearly there was no further increase in the absorbance. It implies that there was a complete reduction of Ag^+ to Ag NPs. This result was in line with,[15] who reported the change in the color of the crude enzyme of *A. niger* to brown after treatment with 1mM $AgNO_3$. Silver nitrate solution was added to the supernatant from *E. coli* and this mixture was incubated at 37±1ºC for 24hours to synthesize Ag NPs in the solution. Another similar sample of *E. coli* was incubated without adding any silver nitrate solution to it. The color of the former silver nitrate solution changed from pale yellow to orange. But the control sample exhibited almost no change in color. Even after 24hours, no significant change in the color was seen in the control sample.[16] This result was in consonance with the result obtained by Natarajan et al.,[17] for their synthesis of Ag NPs through microbial method. The corresponding UV-Vis absorption spectra of Ag NPs by supernatant from *E. coli* were shown in Figure 4. It may be noted that the control sample (without silver nitrate solution) showed no evidence of absorption in the wavelength range 300 to 700nm. However, the solution which included $AgNO_3$ in it showed an absorption peak around 416nm. The observation of peak in the UV–Vis absorption in this range is known to be associated with the Surface Plasmon Resonance (SPR) of Nano-sized silver metal presence of Ag NPs by this method. Larger particles show Plasmon absorbance at longer wavelengths.[18]

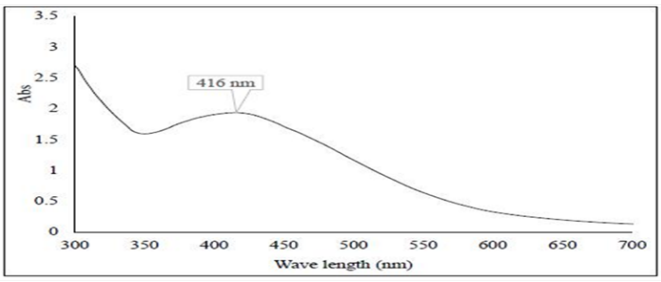

Figure 3 UV - Visible Absorption of Ag NPs by *E. coli* cell free filtrate.

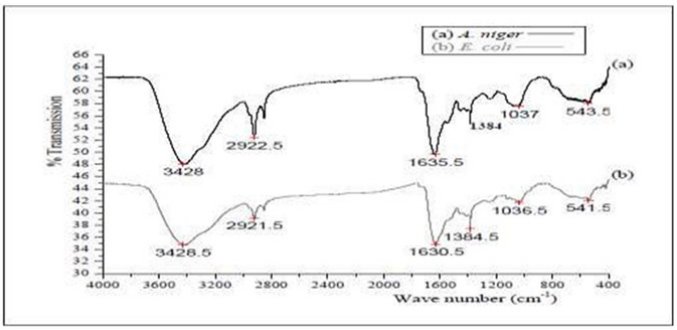

Figure 4 FTIR spectra of silver nanoparticles synthesized by using: A) *A. niger* and B) *E. coli*.

Fourier transform infrared (ft-ir) analysis

After the reduction of silver nanoparticles got completed, any free biomass residue or compound, which had not already adhered to the nanoparticles as its capping ligand, was removed by repeated centrifugation of the reaction mixture at 12,000rpm for 20min. For this purpose, the supernatant was replaced by distilled water each time. Thereafter, the purified suspension was freeze-dried to obtain dry powder. These dried nanoparticles solid samples in powder form were also mixed with an IR transparent material such as KBr or KCl to make it visible in the infrared and molded into pellets under high pressure. Finally, the FT-IR Spectra analysis were done for the detection of different functional groups by analyzing various peaks observed between the region of 4000cm[1] and 400 cm^{-1} with the resolution of 4 cm^{-1}. FTIR measurements of the freeze-dried samples were carried out to identify the possible interactions between silver and microbial bioactive molecules, which may be responsible for synthesis and stabilization (capping material) of silver nanoparticles. Similar study for the green synthesis of silver nanoparticles by plants viz. *P. amarus* and *T. cordifolia* was studied by Singh et al.,[14] In Figure 4A, the FTIR spectra for the silver nanoparticles synthesized by using clear supernatant from *A. niger* is shown. The number of functional groups presented in the nanomaterial can be determined by the size of the peaks of the spectrum.[12,19] The figure has six distinct peaks appearing at 541.5, 1036.5, 1384.5, 1630.5, 2921.5 and 3428.5 cm^{-1}. Figure 4B shows FTIR spectra for the Ag NPs synthesized by supernatant from *E. coli*. The FTIR spectral peaks for Ag NPs synthesized by mixing clear supernatant from *A. niger* appeared at 543.5, 1037, 1384, 1635.5, 2922.5 and 3428 cm^{-1}. The low band at 543.5 cm^{-1} was typically known to present C–Br bond corresponding to alkyl halide. This peak has apparently been caused by the presence of bromine used during FTIR characterization. The peak at 1037 cm^{-1} indicated the presence of C–N stretch bond corresponding to the functional group of aliphatic amine. The medium intense band at 1635.5 cm^{-1} arose from the C=O stretching mode in amine I, group which was commonly found in the proteins. It indicated the presence of proteins as capping agent; the protein itself increases the stability of the synthesized nanoparticles. The two peaks at 2921.5 and 2922.5 cm^{-1} refer to C–H symmetrical stretch vibration of alkanes. The strong and broad peak observed at 3428 cm^{-1} has its origin in the O–H stretching vibration and H–bond of alcohols and phenols.

Let us look at Figure 4B viz. the FTIR spectra for silver nanoparticles synthesized by *E. coli* bio extract clear supernatant. Starting from the smallest wave number, the first two peaks at 543.5cm^{-1} and 541.5cm−1 refer to the C–Cl stretch of alkyl halide. The peak at 1036.5cm^{-1} indicated the presence of C–N stretch vibrations of aliphatic amines. The strong absorption peak appearing at 1384.5cm^{-1}, represents the presence of NO_2 which may be from AgNO3 solution, the metal precursor involved in the process of synthesizing Ag nanoparticles. The peak at 1630.5cm^{-1}refer to the carbonyl stretch, which was assigned to the amide I bond of protein.[20] The narrow strong peak at 2921.5cm^{-1}refers to the C–H stretch of alkanes. The strong and broad peak at 3428.5cm^{-1} indicated O–H stretching vibration and H–bond of the alcohols and phenols. From the FTIR spectra analysis it clear that the functional groups existing on the silver nanoparticles synthesized by fungal crude enzyme as well as those synthesized by the bacterial crude enzyme were of the same types. That means the distinct peaks observed in the FTIR spectra were at almost the same place for Ag NPs synthesized by the extracellular clear supernatants from *A. niger* and *E. coli*. These results indicated that the enzymes and biomolecules that were in of the fungus *A. niger* were also found in *E. coli* crude enzyme. Those proteins and enzymes were actually responsible for the reduction of silver ions to silver nanoparticles and the protection of these nanoparticles from agglomerating to macro-particles. The proteins present over the silver nanoparticle surface act as capping agent responsible for its stabilization. The carbonyl groups of the amino acid residues and the peptides have strong ability to bind to the silver. Thus our FTIR spectra analysis in Figure 4, clearly

confirmed that there were enzymes and biomolecules especially the proteins present in the clear supernatant, which were responsible for the synthesis and stabilization of Ag NPs in colloidal form, and this result agreed with the studies by Jeevan *et al.*,[21]

Antibacterial activities of Ag NPs

The antibacterial activity of these Ag NPs, which were synthesized microbial viz. using extracellular crude enzymes from *E. coli* and *A. niger* were tested comprehensively. For this purpose, two concentrations of silver nitrate solutions were used. These were 10mM and 1mM and both were added to the same volume of clear supernatants from *E. coli* and *A. niger* separately. The purpose was to compare the antibacterial activities of Ag NPs synthesized from different concentrations of the same precursor. The synthesized Ag NPs were used for the tests of antibacterial activities. The clear supernatants from *A. niger* and *E. coli* were used as negative control, whereas the antibiotic, Chlorampenicol, was used as positive control. The antibacterial activities of all the samples were tested against each of the following five pathogens, three gram negative bacteria; namely, *Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella*; and two gram positive bacteria i.e. *Streptococcus spp.* and *Staphylococcus aureus***.** The standard disc diffusion method as per studied by Ravendra et al.,[11] was used for testing the antibacterial activities of Ag NPs. The antibiotic, two extracts and four Ag NPs were allowed to act on the pathogens and the resulting zones of inhibitions were observed after 24hrs. The maximum ZOI for each of these 5 experiments were carefully measured to determine the efficacy of the synthesized Ag NPs in inhibiting each kind of test microorganism. This experiment was repeated and the averaged values of the measured zone of inhibition were listed in Table 1. The results of tests of antibacterial activities were shown in the Table 1 Tests of antibacterial activities were conducted using negative controls (supernatant from *E. coli* and *A. niger),* did not shown any clear ZOI for any of the tested pathogens. These results indicated that the enzyme extracts did not possess any antibacterial potential against the tested pathogens. The antibacterial activities of the antibiotic (Chlorampenicol) and Ag NPs suspensions against all the five bacteria samples were examined through their corresponding zones of inhibition around the disc loaded in the bacterial medium this inhibition indicated that the effect of inhibition was not due to supernatant, but it was due to Ag NPs loaded on the disc. Therefore the antibacterial activities were discussed from Table 1 as follows. E1- 10mM $AgNO_3$ + supernatant from *E. coli* E0 – supernatant from *E. coli*; E2- 1mM $AgNO_3$ supernatant from *E. coli* A0 – supernatant from *A. niger*; A1- 10mM$AgNO_3$ + supernatant from *A. niger* Antibiotic - Chlorampenicol; A2- 1mM$AgNO_3$ + supernatant from *A. niger* Silver nanoparticles synthesized by reduction of silver ion and capped by crude enzyme from *E. coli* and *A. niger* showed antibacterial effect in all the tested sample pathogens. To compare the antibacterial activities of the synthesized Ag NPs the ZOI were measured by using meter ruler. The zone of inhibition for the pathogen *E. coli* when tested with Ag NPs synthesized by mixing supernatant from *E. coli* with 10mM of $AgNO_3$ and also by 1mM of $AgNO_3$ were 11.50mm and 12.50mm, respectively as shown in Table 1. This pathogen was also treated by Ag NPs synthesized by mixing 10mM of $AgNO_3$ and 1mM of $AgNO_3$ with crude enzyme from *A. niger.* Zone of inhibition measured for Ag NPs synthesized from 10mM of $AgNO_3$ was 11.50mm, whereas it was 12.50mm in case of 1mM of $AgNO_3$. From the quantitative results presented in Table 1, it can be concluded that the antibacterial activity of Ag NPs synthesized by the reaction of supernatant from *E. coli* against the pathogen *E. coli* was stronger than the antibacterial activity exhibited by Ag NPs synthesized by enzyme extract from *A. niger*. The clear zone (ZOI) measured around the test disc by the action of Ag NPs prepared by *E. coli* tested against the pathogen *Pseudomonas aeruginosa* were 10.50mm and 11.00mm, respectively for nanoparticles prepared using 10mM of $AgNO_3$ and 1mM of $AgNO_3$ as indicated in Table 1 & Figure 2B. When *P. aeruginosa* was treated by Ag NPs which was synthesized by reacting 10mM $AgNO_3$ and with the supernatant from *A. niger*, the ZOI was 10.00mm and a wider ZOI i.e. 11.00mm was obtained when Ag NPs prepared from 1mM $AgNO_3$ were used. Inhibition zone against the pathogen *P. aeruginosa* in case of Ag NPs prepared by supernatant from *E. coli* was relatively larger than when that obtained for Ag NPs synthesized by *A. niger* supernatant. Thus we found that NPs show better antibacterial activity if they are synthesized by the reduction of silver nitrate with lower concentration (1mM of $AgNO_3$) than when prepared from higher concentration (10mM of $AgNO_3$). Like Rajashree etal.,[22] we also observed that the highest ZOI was observed against *P. aeruginosa* when compared against other gram negative bacteria. As indicated in Table 1 & Figure 2, the antibacterial activity of Ag NPs prepared by *E. coli* tested on the pathogen Streptococcus *spp.* Treated by 10mM of $AgNO_3$ and 1mM of $AgNO_3$ were 12.50mm and 14.00mm, respectively. When the pathogen was treated by Ag NPs synthesized by reacting 10mM $AgNO_3$ and 1mM $AgNO_3$ with *A. niger* supernatant, the ZOI values were 11.00mm and 12.00mm, respectively. This result shows that for the pathogen *Streptococcus spp.,* the antibacterial activity of Ag NPs synthesized by the mixing supernatant from *E. coli* was better than that of Ag NPs synthesized by supernatant from *A. niger.* The ZOI in both Ag NPs were maximum for the reaction of silver nitrate with 1mM of $AgNO_3$ than with 10mM of $AgNO_3$.

Table 1 Comparison of antibacterial activities of Ag NPs on five pathogens: A) *Escherichia coli,* B) *Pseudomonas aeruginosa,* C) *Shigella,* D*) Streptococcus aureus,* and E) *Staphylococcus aureus*

S. No	Test Pathogens bacteria	Triplicated antimicrobial activities test average zone of inhibition in (mm)						
		E1	E2	A1	A2	E0	A0	Antibiotic
1	*E. coli(ATCC-25922)*	11.00	12.50	11.50	12.50	0.00	0.00	21.00mm
2.	*P. aeruginosa(ATCC-27853)*	10.50	11.00	10.00	11.50	0.00	0.00	20.00mm
3.	*Shigella (ATCC-12022)*	11.75	12.17	11.83	14.00	0.00	0.00	19.00mm
4.	*Strep(ATCC-12386)*	12.50	14.00	11.00	1212.0.**0**0	00.00.	00..00	2626.0.**0**mm0 mm
5.	*Staphylococcus aureus* (ATCC-25223)	13.33	14.75	14.00	17.00	0.00	0.00	29.00mm

As indicated in Table 1 the ZOI measured for the pathogen *Shigella* when treated by Ag NPs synthesized by mixing supernatant from *E. coli* with 10mM AgNO3 and 1mM of $AgNO_3$ were 11.75mm and 12.17mm, respectively. But ZOI showed by Ag NPs synthesized by reacting

supernatant from *A. niger* with 10mM $AgNO_3$ and 1mM $AgNO_3$ were 11.83mm and 14.00mm, respectively. This result showed that the antibacterial activity of Ag NPs synthesized by mixing silver nitrate solutions with the supernatant from *E. Coli* were better antibacterial activity as compared to Ag NPs synthesized by supernatant from *A. niger*. This result may be due to high surface area of the synthesized Ag NPs by the lower concentration of $AgNO_3$ solutions reduced by microbial crude enzymes. As it was studied by biologically synthesized silver nanoparticles have immense use in medical textiles, food industries, cosmetic industries and other industries for their efficient antibacterial and antimicrobial properties. The antibacterial activities of silver nanoparticles, which were synthesized through green route, showed interesting antimicrobial activities against multi-drug resistant bacteria. This eco-friendly method can potentially be used in various areas, including pharmaceuticals, cosmetics, foods, and medical applications the chemical and physical processes mostly involve hazardous chemicals, high energy requirements and other strict conditions. The sizes and morphologies of silver nanoparticles synthesized from these two methods are quite variable depending on the conditions and methods applied. In case of biological method, also known as the bottom-up approach has been able to biosynthesize silver nanoparticles with better sizes and morphologies. Most of the NPs produced were reported to have a predominantly spherical shape. Other benefits of the use of the green approach are the use of biological reductants, low to zero energy requirements and better characteristics of the metallic silver nanoparticles, with the advantage of elimination of the need for toxic chemicals to be used as surfactants or stabilizers since various proteins present in the extracellular enzymes act as reducing as well as capping agents for silver NPs. Synthesizing nanoparticles via biological entities acting as biological factories offers a clean, nontoxic and environment-friendly method of synthesizing nanoparticles with a wide range of sizes, shapes, compositions, and physicochemical properties.[23]

Conclusion

This study experimentally demonstrated a simple biological and low-cost approach for the preparation of stable silver nanoparticles by reduction of silver nitrate solution bio-reduction executed by the aqueous extracellular crude enzyme from *E. coli* and *A. niger*. In conclusion, this synthesis method is a simple, cost effective, and ecofriendly method to synthesize silver nanoparticles in a suspension. Agro-industrial residue wheat bran was used for SSF in order to obtain crude enzyme from *A. niger,* which was used for synthesis of Ag NPs. There was no need to use high pressure, energy, temperature, toxic chemicals, different reducing and stabilizing chemicals etc. In most cases, the chemical synthesis methods lead to some chemically toxic substances being absorbed on the surface and can hinder their usage in medical applications. Based on this study, some other Nano-materials may be prepared in future. From the point of view of nanotechnology, this is a significant advancement to synthesize silver nanoparticles through green route. Characterizations of the synthesized NPs have been successfully done using UV-Vis spectrometer and FTIR spectroscopic techniques. UV-Vis results indicated that the absorption peak for the Ag NPs synthesized by *A. niger* appeared around 425nm, whereas for E.coli it appeared around 416nm. The UV-Vis spectra results confirmed that the reduction of silver nitrate to silver nanoparticles with high stability and without using expensive and toxic chemicals like sodium borohydride for reduction of Ag^+ to Ag^0. Examination on the antibacterial effect of Nano-sized silver against *Escherichia coli (*ATCC-25922), *Pseudomonas aeruginosa (*ATCC-27853*), Streptococcus aureus* (ATCC-12386*), Shigella (*ATCC-12022*)* and *Staphylococcus aureus* (ATCC-25223) microbes reveals high efficacy of silver nanoparticles as strong antibacterial agent. A biological synthesized silver nanoparticle using fungi and bacteria provides advancement over chemical and physical synthesis as it was cost effective and environmental friendly. In general synthesis of nanoparticles and nanomaterials through green route using fungal and bacterial methods benefits the users as well as the environment in multi directions. Finally, we recommend that our work was specifically focused to synthesize Ag NPs and demonstrate their antimicrobial activity. As an extension, it can be applied to synthesize other nanomaterial, try them for newer pathogens and use them in different industries. It would extend this stream of nanotechnology.

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Conflicts of interests

The authors declare that there is no competing of interest.

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