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RESEARCH ARTICLE - BIOLOGICAL SCIENCES

Antibacterial Activity of Silver Nanoparticles Isolated from Cow's Milk, Hen's Egg White and Lysozyme: A Comparative Study

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Abstract
The aim of the present study is to evaluate the antibacterial activity of biosynthesized silver nanoparticles (AgNPs) with the dietary, nontoxic, eco-friendly biological materials such as raw and pasteurized cow's milk, egg white and lysozyme. The chosen organisms are clinically important, and hence its *in vitro* evaluation gains significance in the field of medicine. The AgNPs were characterized by UV–visible spectroscopy which revealed surface plasmon absorbance peaks, ranging between 400 and 450 nm. Fourier transform infrared spectroscopy showed the presence of characteristic C=O and O–H bonds. Transmission electron microscopy revealed spherical particles ranging between 20 and 200 nm. Scanning electron microscopy–energy-dispersive spectroscopy revealed peak at 3 keV confirming the presence of AgNPs. SDS-PAGE analysis further ascertained this with the absence of some protein bands in AgNPs solution as against their respective controls which could indicate its role during the synthesis. The comparative antibacterial activity was determined by well diffusion method. Effective inhibition zones obtained by AgNPs synthesized from the pasteurized milk were 12 mm ± 0.7 against *Escherichia coli* DH5 α and *Bacillus subtilis*, 14.5 mm ± 0.5 against *Pseudomonas alcaligenes* and *Staphylococcus aureus*, 15 mm ± 0.7 against *Bacillus cereus*. The percentage inhibition displayed by AgNPs from pasteurized cow's milk was 48%, 58%, 65.9%, 85.7% and 68.2% against the growth of *E. coli* DH5α, *P. alcaligenes*, *S. aureus*, *B. subtilis* and *B. cereus*, respectively, which was significant when compared to the inhibition profiles of AgNPs obtained from other sources such as raw milk, lysozyme and egg white.

Keywords Silver nanoparticles (AgNPs) · Characterization · SDS-PAGE · Antibacterial · Egg white · Lysozyme · Cow's milk

1 Introduction

Nanoscience and nanotechnology is one of the advancing fields employed to explore the darkest avenues of medicine. The key challenge in this field involves the synthesis of the nanoscale particles or structures of required size, shape and functionality. These nanoparticles are intermediate sized, between the bulk materials and atomic structures, and possess unique physicochemical, optical and biological properties compared to their bulk equivalents, and thus they can be manipulated for desired applications. Based on the source of synthesis, nanoparticles can be broadly classified as organic (micelles, dendrimers, liposomes etc.) and inorganic (metals and their oxides). Among the metal nanoparticles, silver nanoparticles have been greatly used owing to their distinct properties like chemical stability, conductivity, nonlinear optical behavior, high surface to volume ratio and catalytic activity [1]. Synthesis of these nanoparticles can be achieved by either conventional, physical and/or chemical methods but they are expensive, toxic and non-eco-friendly. In the recent times, green synthesis of AgNPs is achieved by using microorganisms, plant extracts, enzymes, cow's milk, egg white, etc. [2–12]. Several research works have been carried out to explore the potential of AgNPs as antifungal, antibiofouling, anticancer, antioxidant, antibacterial, etc. [11–19].

Very few studies have been reported with AgNPs obtained from cow's milk (obtained from the market). Among them, Lee et al. have reported the significant antifungal application AgNPs from cow's milk obtained from local market against phytopathogens [11]. Hegazi et al. have reported the effec-

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tive antibacterial property of AgNPs conjugated with cow and camel milk against the pathogenic bacterial strains [18]. Lu et al. have reported that AgNPs obtained from egg white have excellent biocompatibility and thus can be potential candidates for cancer radiation therapy [12]. Antibacterial activity of the lysozyme AgNPs has been reported in earlier studies where the synthesis of AgNPs is through various chemical methods and also with several modifications in lysozyme [9]. The following have been reported for the first time in this study: (a) using raw milk as a source to synthesize the AgNPs and its application toward antibacterial activity. (b) Exploring the comparative antibacterial potential of AgNPs obtained from hen's egg white, raw and pasteurized milk and lysozyme. (c) Biosynthesis of AgNPs has been done from all sources with necessary modifications from the earlier methods. (d) Further to this, efforts have been initiated toward understanding the protein profile variations with the synthesized silver nanoparticle solutions which may form a basis for identification of specific protein bands in the reaction that can be isolated and purified for advanced research. AgNPs synthesis from various sources provides an insight as to which is the easy, reliable and effective source for exhibiting the antibacterial activity.

2 Materials and Methods

2.1 Synthesis of AgNPs

AgNPs were synthesized from different sources, viz. cow's pasteurized milk (4 ml) procured from local market, fresh raw milk (4 ml) collected from nearby cowshed, purified lysozyme (5 ml of 1 mg/ml) obtained from Aristogene Biosciences, Bangalore, and hen's egg white isolated from egg obtained from local shop. These sources were set into protocols of reference works by Lee et al., Ashraf et al. and Lu et al., respectively, for AgNPs synthesis with modifications in reaction conditions. Chemicals, antibiotics and reagents used were of analytical grade procured from Sigma-Aldrich, India.

AgNPs from raw and pasteurized milk: The experimental flask was set up by adding raw/pasteurized milk (4 ml) and 96 ml of 1 mM silver nitrate solution making up the total reaction volume to 100 ml. Two control flasks: one with raw/pasteurized (4 ml) and 96 ml autoclaved distilled water ($D.H_2O$) and the other control with 96 ml of 1 mM silver nitrate solution and 4 ml of autoclaved distilled water.

AgNPs from purified lysozyme: Experimental flask containing 5 ml of 1 mg/ml purified lysozyme and 50 ml of silver nitrate (1 mM) was set up making up the total reaction volume to 55 ml. Two control flasks: one with 5 ml of autoclaved distilled water and 50 ml of 1 mM silver nitrate ($AgNO_3$) and the other control with 5 ml of 1 mg/ml purified lysozyme with 50 ml of autoclaved distilled water.

AgNPs from egg white: Experimental flask was set up by adding egg white (1 ml) to 97 ml of autoclaved distilled water. This mixture was continuously stirred on magnetic stirrer for 20 minutes. The cloudy solution obtained was filtered through the Whatman filter paper until the clear solution was obtained. Further, 2 ml of 10 mM silver nitrate ($AgNO_3$) solution was added to the clear solution obtained making up the reaction volume to 100 ml. Two control flasks: one with 2 ml of 10 mM silver nitrate ($AgNO_3$) and 98 ml of autoclaved distilled water and the other with 1 ml egg white and 99 ml autoclaved distilled water.

Experimental and control flasks in all the above cases were incubated in orbital shaker at 37 °C, 120 rpm for 72 h for all sources except in case of lysozyme which was 5–8 h. The flasks were observed for color change from white to dark brown. Further flasks showing the color change were centrifuged for 2–3 times at 12,000 rpm for 20 min to separate the pellet. Pellets were lyophilized and collected for further characterization studies.

2.2 Characterization of AgNPs

Confirmation of AgNPs obtained from different sources was done by checking its absorption maxima between 300–700 nm using Perkin-Elmer Lambda 750 UV/VIS spectrophotometer. The morphology of colloidal sample with elemental compositions was examined using SEM-Hitachi su1510, scanning electron microscopy (SEM) and energy-dispersive spectroscopy (EDS). Fourier transform infrared spectroscopy (FTIR) where infrared spectra were recorded in the range of 400–4000 cm^{-1} using Perkin-Elmer Fourier transform spectrophotometer was used to determine possible functional groups responsible for bioreduction of Ag+ and capping or stabilization of AgNPs. High-resolution transmission electron microscopy (TEM) at 200 Kv using Jeol/JEM 2100, LaB6 at STIC (Sophisticated Test and Instrumentation Center, Cochin, India) was used to detect particle size and shape of synthesized AgNPs.

SDS-PAGE was performed according to the standard protocols to determine protein profiles responsible for synthesis of AgNPs.

2.3 Antibacterial Activity Studies

Gram-positive and gram-negative bacterial strains, namely *Escherichia coli* DH5 α (accession no. CP017100), *Pseudomonas alcaligenes* (GU447236.1), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (MTCC 441) and *Bacillus cereus* (ATCC 14579) culture, were procured from Aristogene Biosciences Pvt. Ltd, Bangalore, and used for the study.

The antibacterial activity was determined by well diffusion method. Individual cultures of bacteria (200 µl) were spread on MHA (Mueller-Hinton agar) plates, and 6 mM

wells were punctured by using sterile microtips. Synthesized AgNPs from each of the sources, namely cow's pasteurized and raw milk, hen's egg white and purified lysozyme, were put into the wells along with standard antibiotic (cefazolin, 30 mg) and respective controls. Further, plates were incubated at 37 °C for 24 h and zone of inhibitions were recorded.

3 Results and Discussion

3.1 Synthesis of AgNPs

The experimental flasks (flasks 'a' in Fig. 1-B, D, F, H) showed the color change from milky white to dark brown due to reduction reaction indicating the presence of AgNPs as against the controls which showed no color change (flasks 'a' in Fig. 1-A, C, E, G).

3.2 Characterization

3.2.1 UV–VIS

The AgNPs synthesized when studied under UV–vis spectrophotometer showed varied absorption maxima with respect to sources. The egg white and purified lysozyme displayed peak between 425 and 430 nm and 400–430 nm, respectively (Fig. 1-i, ii), whereas cow's pasteurized and raw milk displayed peak between 430 and 450 nm (Fig. 1-iii, iv). The peak value obtained in the case of egg white was similar to the study reported previously [12]. This is first of its kind where complete characterization of AgNPs from cow's raw milk is performed. Surface plasmon resonance peak indicated in all cases confirms the presence of AgNPs.

3.2.2 EDS and SEM

The EDS study generated the energy-dispersive spectrum of the synthesized nanoparticles for individual sources. In all the cases, it exhibits strong absorption peaks for silver between the range of 2.5–3.5 keV (Fig. 2-a, b, c, d). Several research reports suggest that peaks obtained approximately at 3 keV are due to the absorption ability of metallic crystallites, mainly silver due to surface plasmon resonance [11,20,21]. Along with the peak obtained at 3 keV for silver, the spectrum also provides a quantitative information of AgNPs synthesized from the studied sources. The presence of elements such as carbon (C), oxygen (O), aluminum (Al), chlorine (Cl), calcium (Ca), sulfur (S) and copper (Cu) was also noticed. These trace elements could perhaps correspond to the protein capping over the synthesized AgNPs. The presence of other elements in EDS spectra and their possible function are in coherence with Ahmad et al. who have studied the synthesis of AgNp from *Desmodium triflorum* [22].

The surface morphology of AgNPs synthesized from studied sources was obtained using SEM at the resolution of 100–500 nm. On careful observation, nanoparticles were found aggregated in the form of flakes, tubes and fibers as shown in Fig. 2-a1, a2, b1, b2, c1, c2, d1, d2. This could be because of the organic moieties like lipids and proteins surrounding them.

3.2.3 FTIR

To know the interactions of biomolecules with metal nanoparticles, further FTIR spectra were recorded in the range of 400–4000 cm^{-1}. Figure 3A—a, b displays the FTIR spectra for AgNPs synthesized from hen's egg white. High peak was recorded at 2841 cm^{-1} and 2981 cm^{-1} which can be assigned to C–H stretch. At 2621 cm^{-1}, another strong peak was seen which is assigned to the OH stretch of the proteins found in the egg white. N–H and C–N stretches could be assigned to the peak formed at 1538 cm^{-1} and 783 cm^{-1}, respectively. C=0 peak was recorded at 1881 cm^{-1}. Figure 3A—c, d displays the FTIR spectra for AgNPs synthesized from lysozyme. C–N and N–H stretches were recorded at 1638 cm^{-1} and 1500 cm^{-1}, respectively. OH stretch was represented by the peak formed at 2623 cm^{-1} and 3065 cm^{-1}. At 1888 cm^{-1}, C=O stretch was visible. Strong peaks were obtained at 3065 cm^{-1}, 2623 cm^{-1} followed by 1638 cm^{-1} which predicts that amino acids might be responsible for bioreduction of Ag+ and capping or stabilization of synthesized AgNPs.

Figure 3A—e, f shows the FTIR spectra for AgNPs synthesized from raw milk with highest peaks recorded at 1538 cm^{-1}, 2915 cm^{-1} and 2633 cm^{-1} corresponding to N–H, C–H, OH stretches, respectively. C=N stretch was assigned at 720 cm^{-1}. Further C=O stretch was observed at 1875cm^{-1}. In all cases, C=0 stretch marked the characteristic peak for the synthesized AgNPs. Figure 3A—g, h shows the FTIR spectra for AgNPs synthesized from pasteurized milk. Here, C=0 stretch was obtained at 1883cm^{-1} and OH stretch was assigned at 2617 cm^{-1} and 3279 cm^{-1}. Further at 1636 cm^{-1}, N=H stretch was seen and at 2924 cm^{-1} there was a peak which signifies the C=H stretch. As stronger peaks are obtained for carboxylic acids, they could be the major biomolecules followed by proteins, fats and minerals for bioreduction of Ag+ and capping or stabilization of synthesized AgNPs. The peaks and stretches obtained for FTIR were in agreement with previous studies reported for AgNPs obtained from several sources [9,12,19,23,24].

3.2.4 TEM

To obtain size and surface morphologies of the AgNPs, TEM analysis was carried out. Figure 3B—i, ii, iii, iv represents the images of AgNPs obtained from egg white, purified

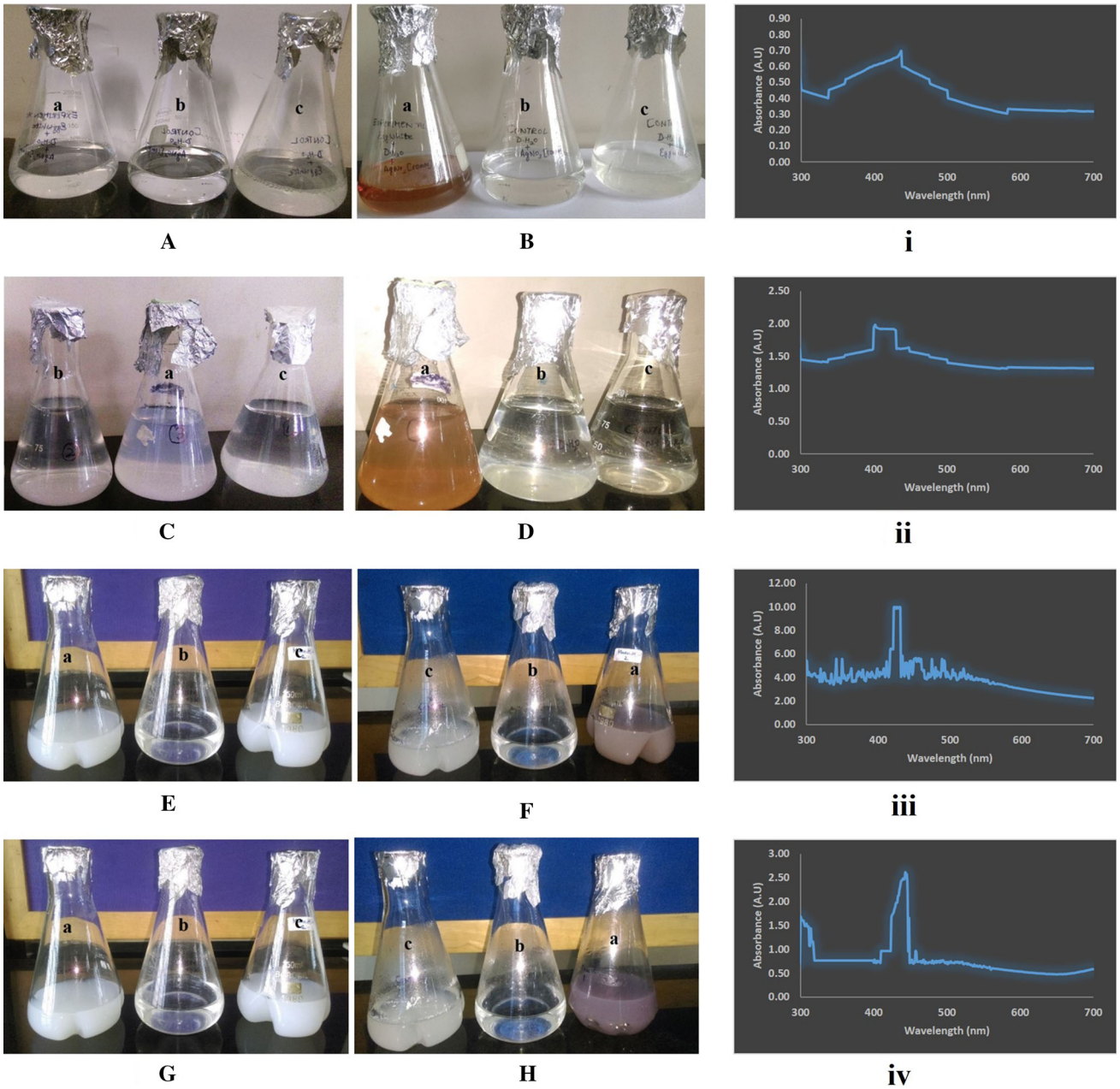


Fig. 1 **A**, **C**, **E**, **G** initial set up and **B**, **D**, **F**, **H** after completion of the reaction. Flasks 'c' and 'b' (controls—no color changes); flask 'a' in **B**, **D**, **F**, **H** (experimental flasks—color changes observed); i, ii, iii, iv: surface plasmon resonance peaks obtained for AgNPs obtained from egg white, purified lysozyme, raw milk, pasteurized milk, respectively

lysozyme, raw and pasteurized milk, respectively. According to this, AgNPs from egg white measured the size of the particles ranging from 20 to 200 nm and were spherical to oval in shape. Few particles had smooth edges and few had uneven edges. Also, the edges of the particles were lighter than the center of the particle and this observation was consistent with the earlier report [12]. Most of the nanoparticles were in isolated conditions but some were found aggregated. This could be because of several proteins present in the egg white. AgNPs obtained from purified lysozyme showed the spherical particles measuring 10–100 nm. AgNPs produced by raw milk and pasteurized milk were circular in shape and mostly present in aggregates with particle size of 20–200 nm. Aggregation could be because of the organic moieties present in the milk samples.

3.2.5 SDS-PAGE Analysis

Once the AgNPs were synthesized and characterized, PAGE analysis was carried out to know whether any protein profile

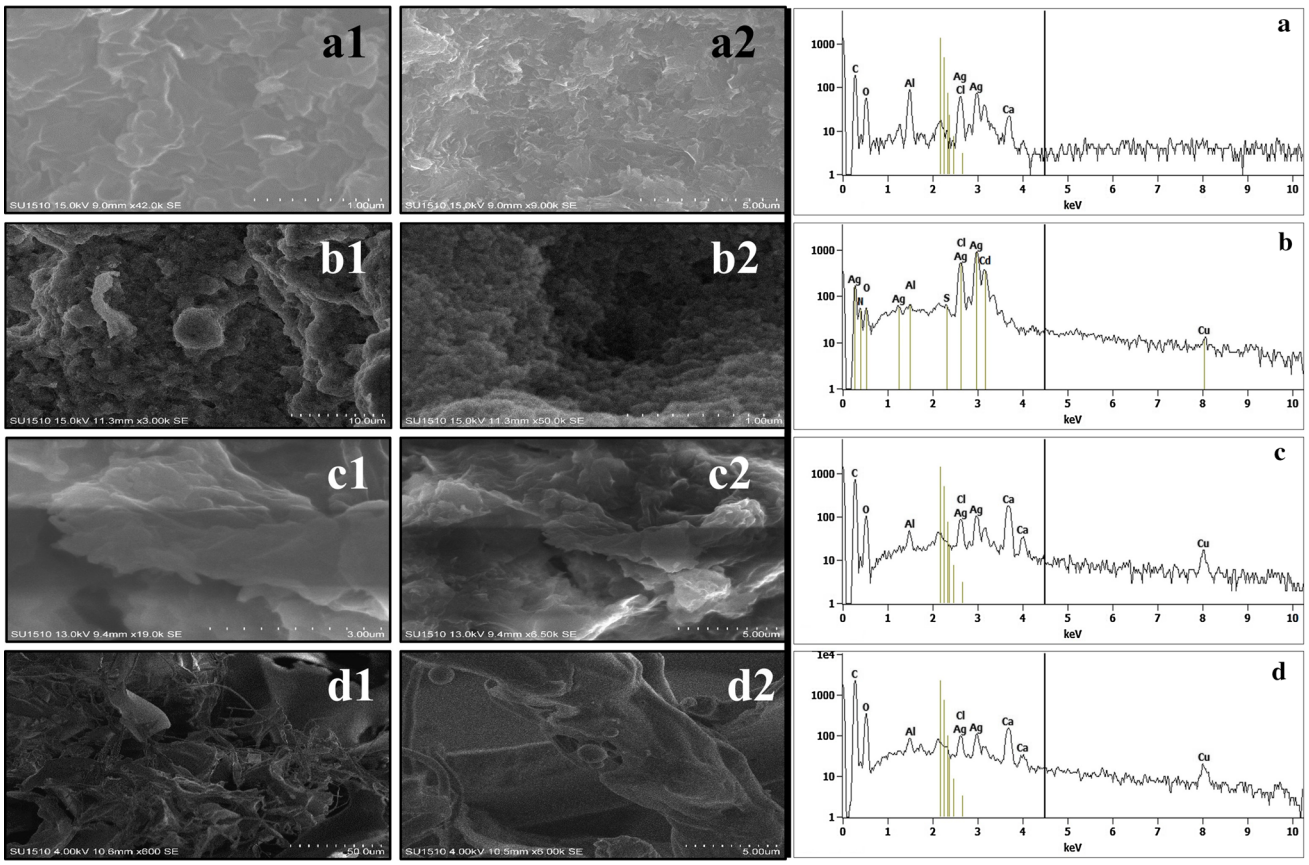
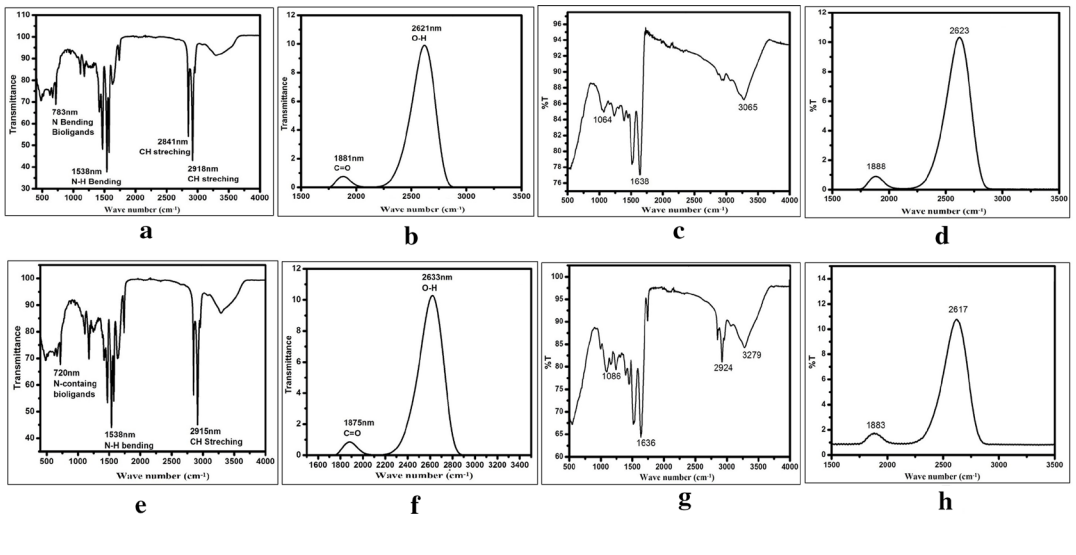


Fig. 2 SEM images of AgNPs obtained from egg white (**a1**, **a2**), purified lysozyme (**b1**, **b2**), raw milk (**c1**, **c2**), pasteurized milk (**d1**, **d2**); EDS spectrum for AgNPs obtained from egg white (**a**), purified lysozyme (**b**), raw milk (**c**), pasteurized milk (**d**)

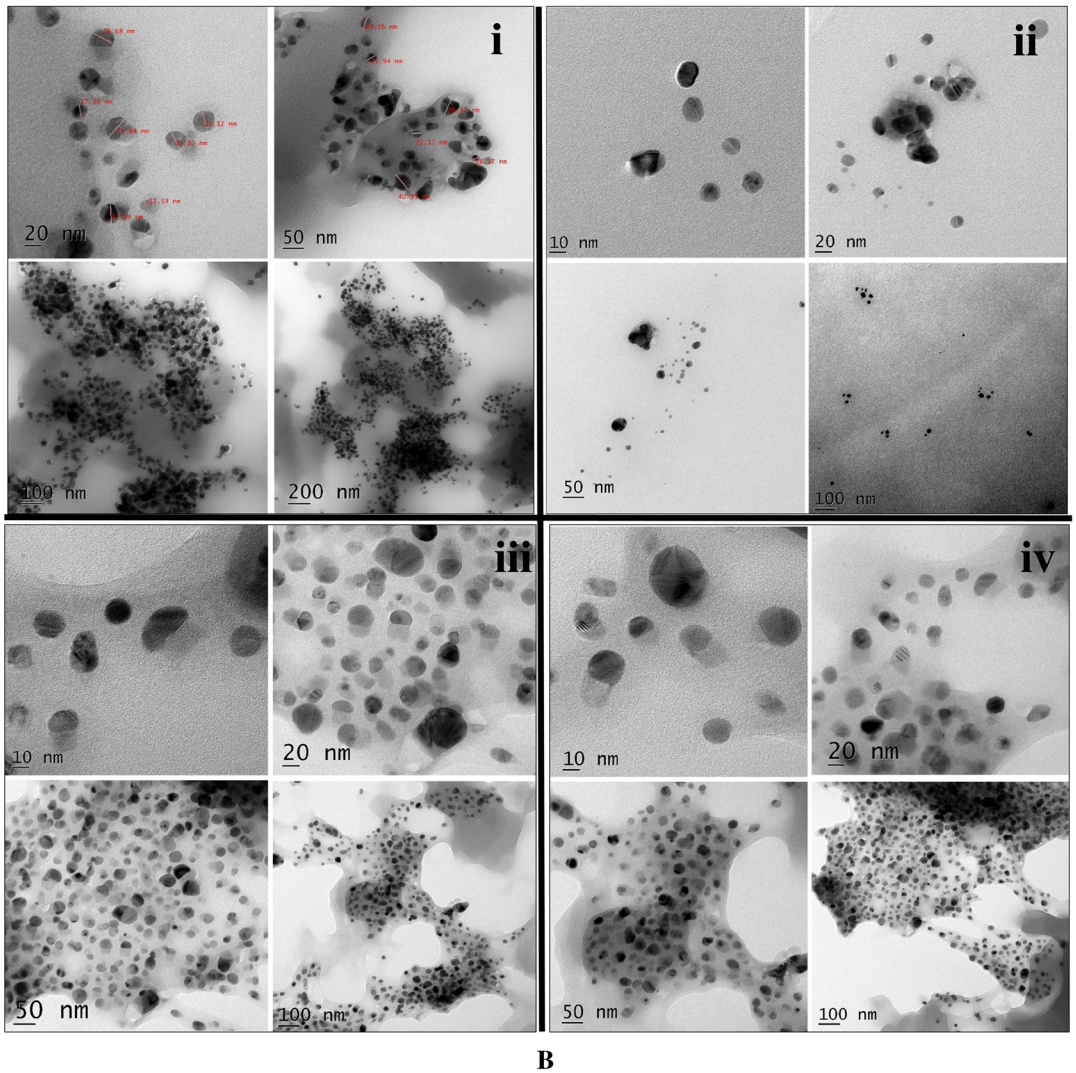
is responsible for reduction of $AgNO_3$. Previous studies have reported that AgNPs synthesized from bacteria and fungi showed the appearance of an extra protein band which is responsible for capping and stabilizing the AgNPs [25–28]. Interestingly, in this study, it is noted that some of the protein bands disappeared in the AgNPs synthesized solutions unlike the earlier reports. This could be because of the sources such as milk, egg white and lysozyme which already have plenty of proteins present, unlike the earlier studies in which the proteins had to be secreted from bacteria and fungi. SDS-PAGE analysis with respect to these sources is reported for the first instance in this study. Figure 4 represents the protein profiles of controls and AgNPs synthesized from all four studied sources. Figure 4A depicts the protein profiles of egg white- synthesized AgNPs. Lane 3a represents the control (egg white + $D.H_2O$) in which 36.5 kDa and 14.3 kDa bands were seen. These bands were absent in the silver nanoparticle synthesized solution [lane 3b, 3c]. Figure 4B depicts the protein profiles of AgNPs obtained from purified lysozyme. Lane 4a represents the control (purified lysozyme + $D.H_2O$) in which a high-intensity band was seen at 14.3 kDa. Intensity of this band is reduced in the solution containing the AgNPs which could be observed in lanes 4b and 4c. Figure 4C represents the protein profiles of AgNPs synthesized from raw milk (obtained directly from cow) where 1a represents the control lane (raw milk + $D.H_2O$). Two thin bands were present in the control lane between 97.4 kDa and 66 kDa. These are absent in lane 1b and 1c, which represents the solution containing the AgNPs. Figure 4D represents the protein profiles of AgNPs synthesized from pasteurized milk. Control (pasteurized milk + $D.H_2O$) lane was represented by 2a. Around 66 kDa region, two thin bands were present in the control lane, which were absent in lane 2b and 2c containing the AgNPs. From the above observations, it could be noted that protein profiles of solutions containing the AgNPs had some protein bands missing as compared to the controls. This may also suggest that these missing protein bands could be involved during the synthesis of AgNPs. Also, it could signify that these proteins if identified could play a pivotal role in bringing about the reduction of macrosilver to nanosilver.

3.3 Antibacterial Activity of AgNPs

Antibacterial activity of AgNPs obtained from four studied sources was tested against five bacteria, viz. *E. coli*, *P. alcaligenes, S. aureus*, *B. subtilis* and *B. cereus* by well dif-



A



B

Fig. 3 **A** FTIR spectrum for AgNPs obtained from: a and b egg white, c and d purified lysozyme, e and f raw milk, g and h pasteurized milk; **B** TEM images of AgNPs obtained from: i egg white, ii purified lysozyme, iii raw milk, iv pasteurized milk

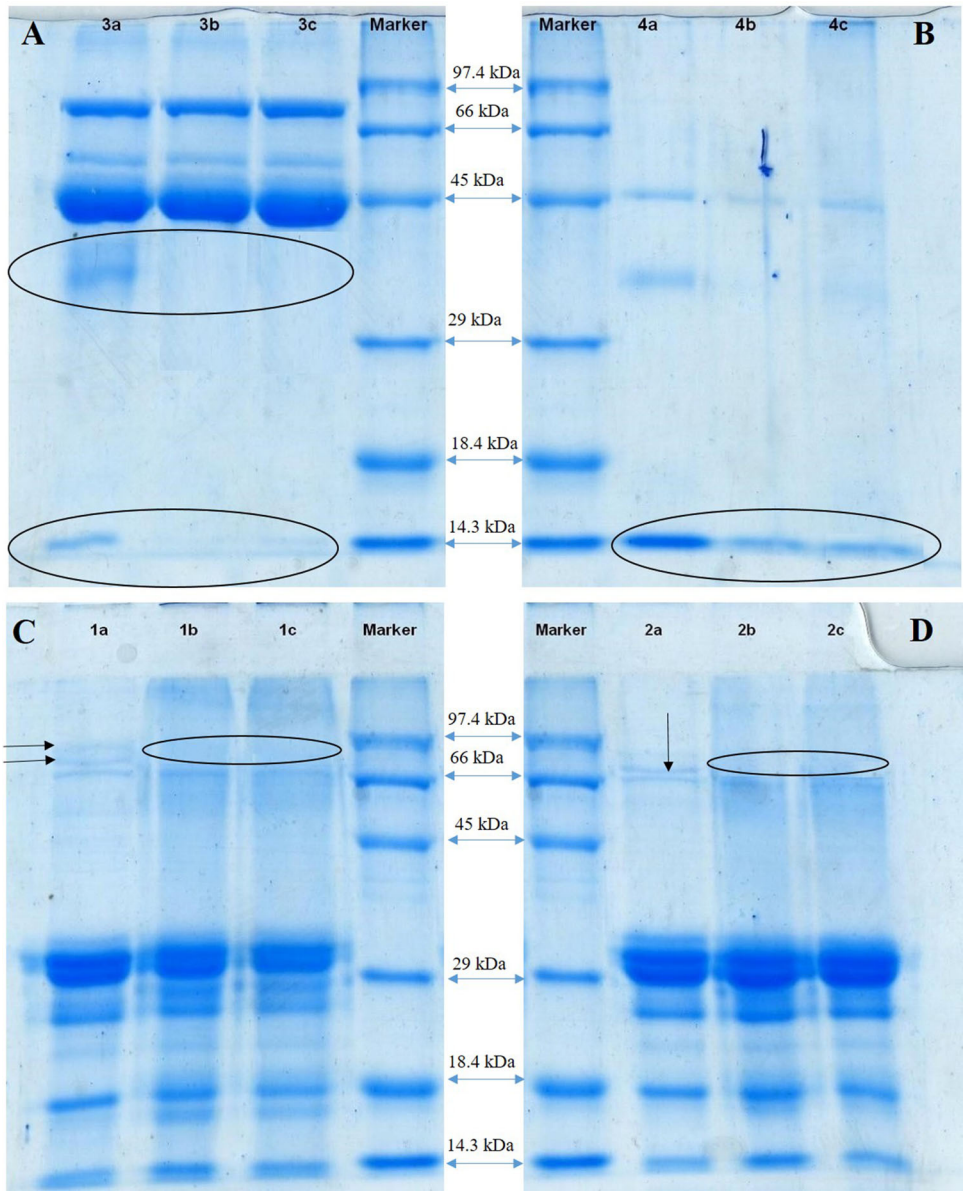
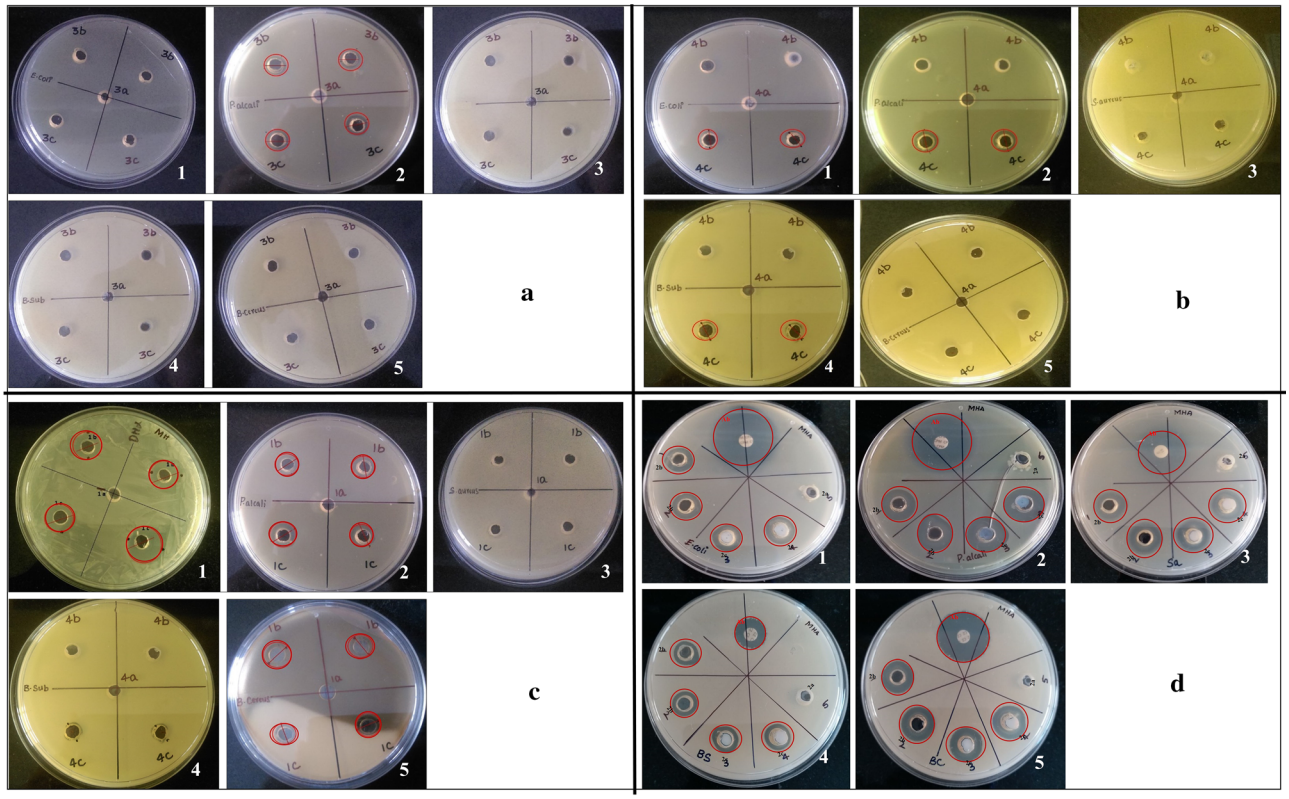
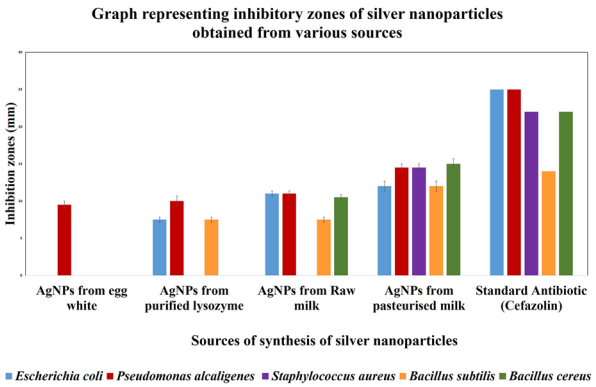


Fig. 4 **A**, **B**, **C**, **D** SDS-PAGE analysis of AgNPs synthesized from egg white, purified lysozyme, raw milk and pasteurized milk, respectively. 3a Control (egg white + $D.H_2O$); 3b, 3c egg white synthesized AgNPs. 4a Control (purified lysozyme + $D.H_2O$); 4b, 4c lysozyme-synthesized AgNPs. 1a Control (raw milk + $D.H_2O$); 1b, 1c raw milk-synthesized AgNPs. 2a Control (pasteurized milk + $D.H_2O$); 2b, 2c pasteurized milk-synthesized AgNPs

fusion method. Zone of inhibitions (encircled with red in Fig. 5A:a,b,c,d) were recorded for AgNPs obtained from each of the sources and standard antibiotic (cefazolin, 30 mg) against the five organisms mentioned.

AgNPs obtained from pasteurized milk displayed inhibition zones of 12 mm ± 0.7 against *E. coli* and *B. subtilis*, 14.5 mm ± 0.5 against *P. alkaligenes* and *S. aureus* and 15 mm ± 0.7 against *B. cereus*. The most effective antibacterial activity was shown by AgNPs synthesized from cow's pasteurized milk against all bacteria. This result gains significance as the source is easily available and nontoxic, and in the future, AgNPs from the same could be a possible answer for combating multidrug-resistant bacteria (MDR) at higher levels of research. Cow's raw milk AgNPs indicated its antibacterial activity against *E. coli* (11 mm ± 0.35),*P.alkaligenes* (11 mm ± 0.35),*B.subtilis* (7.5 mm ± 0.35) and *B.cereus* (10.5mm ± 0.35) but did not yield any zone of inhibition against *S. aureus*. AgNPs from purified lysozyme also showed less zone of inhibition against *E.coli* (7.5 mm ± 0.35), *P.alkaligenes* (10 mm ± 0.7), *B.subtilis* (7.5 mm ± 0.35), with no activity against *S. aureus and B. cereus.* AgNPs from egg white displayed the lowest antibacterial activity with average zone of inhibition of 9.5mm ± 0.5 against *P. alkaligenes* alone. No activity was recorded against the rest four bacteria. Low antibacterial activity in egg white and lysozyme AgNPs could be because of the chemical modifications that occur in the cell walls of the organisms that leads to development of resistance for the proteins present in egg white such as lysozyme, proteases, ovotransferrins and others according to the study reported





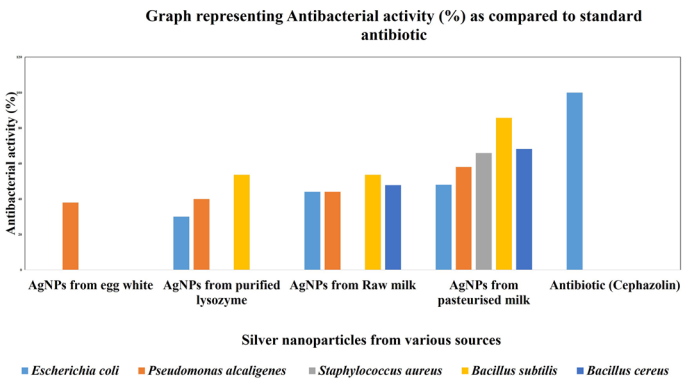


Fig. 5 **A** Inhibition zones recorded for 1 *Escherichia coli*, 2 *Pseudomonas alcaligenes*, 3 *Staphylococcus aureus*, 4 *B. subtilis*, 5 *Bacillus cereus*; a, b, c, d antibacterial activity of AgNPs obtained from egg white, purified lysozyme, raw milk and pasteurized milk, respectively. Ab Standard antibiotic (cefazolin, 30mcg); **B** graph representing the inhibition zones; **C** graph representing percentage of inhibition of AgNPs as compared to the standard antibiotic. Calculation: [(inhibitory zone obtained by AgNPs − inhibitory zone obtained by antibiotic)*100]

by Abergel et al. [29]. Their findings also suggest that the antibacterial activity is restricted to several gram-positive and gram-negative organisms like *S. aureus*, *B. subtilis*, *E. coli*, etc. which is in coherence with the present study.

In all cases, control (i.e., egg white solution, purified lysozyme solution, raw milk solution and pasteurized milk solution) did not show any zone of inhibition, indicating AgNPs formed were responsible for antibacterial activity rather the source by itself.

4 Conclusions

The AgNPs successfully synthesized from all studied sources followed safe, eco-friendly and nontoxic approach. Obtained

particles were polydisperse in nature and were roughly spherical indicative of good nanotransporter. Further, protein profile study revealed that some protein bands were missing in the silver nanoparticle synthesized solutions vis-a-vis controls. This base work suggests that these might be the proteins, if identified and purified can be best source for silver nanoparticle synthesis. Results from comparative analysis of the antibacterial activity exhibited by the AgNPs synthesized from pasteurized milk displayed significant antibacterial activity against all the five selected strains of bacteria suggesting it to be one of the reliable source for AgNPs. This could perhaps be used against clinically challenged pathogens and MDR bacteria in future works.

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