Anti-pathogenic efficacy of biogenic silver nanoparticles through adherence and biofilm inhibition in multidrug resistant ESKAPE pathogens

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Research article

Keywords: ESKAPE pathogens, biogenic AgNPs, anti-pathogenic activity, Live/dead assay, biofilm formation

Posted Date: August 28th, 2019

DOI: <https://doi.org/10.21203/rs.2.13646/v1>

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Abstract

Background The current study aimed to produce AgNPs through a biogenic approach and assessed for their significant anti-pathogenic activities against multi-drug resistant ESKAPE pathogens. The biogenic AgNPs were synthesized through non-toxic manner and characterized by using UV-vis, XRD, DLS, TGA-DTA, FTIR, SEM along with EDX and UHRTEM were used to determine the absorption spectra, shape, size, thermal behaviour, functional groups, morphology, elemental constituents and defined particle size distribution profile, respectively. The AgNPs were evaluated for their anti-pathogenic effects against eleven strains of multi drug resistant ESKAPE pathogens by growth inhibition, biofilm adhesion, growth kinetics and Live/dead assays. **Results** The inhibitory range of AgNPs concentration was investigated as higher zones at escalating concentration (50 to 200 µg/ml). The growth kinetics of inhibition of all tested pathogens occurred after 4 hrs of treatment with AgNPs. Adherence assay exhibited highest inhibition in *E. faecium* (MCC 2763), *P. aeruginosa* (MTCC 1688) and *E. species* (MCC 2296) at 100µg/ml of AgNPs. The exposure of AgNPs increased the dead cell and consequently reduced cells density with AgNPs comparable with the effect of commercial antibiotics. The selected pathogens were found more sensitive to AgNPs than Cefotaxime/AgNO₃ with the statistically significant ($P < 0.05$). **Conclusion** The emergence of drug resistance in ESKAPE pathogens are the extending reason for nosocomial infections, limiting the choice of antibiotics. Nanomaterials have been considered potential agents to prevent infections. Therefore, present study showed the broad spectrum potential and anti-pathogenic potency of biogenic AgNPs as an alternative to conventional antimicrobial agents.

Background

Most of the chronic and opportunistic infections arise through health care units included pneumonia, bacteremia, urinary tract/gastrointestinal infections, osteomyelitis, meningitis and endocarditis [1]. The ESKAPE pathogens are the main agents in a nosocomial infection to spread in hospital settings, compelling the patient for a longer stay [2]. These pathogens are highly susceptible to genetic modifications leading to resistance and able to survive in harsh conditions. Most of them are having a tendency to form biofilms on various surfaces including catheters, eye lenses, thermometers and artificial heart valves [3, 4]. The formation of biofilm on the medical devices leads to the development of chronic infections and more suitable with immune-compromised patients. Therefore, actions to reduce opportunistic infection or prevent the formation of biofilm should be directed by the alternative of the anti-adherent agents. The development of a biocompatible and cost-effective method are being considered to treat multi drug resistant (MDR) human pathogens, and are indispensable to save various lives [5]. The technology of extremely small things in the form of nano has been an unavoidable boon to humanity in therapeutics, pharmaceuticals and biocatalysis [6]. The availability of natural resources for the green synthesis of nanoparticles is very common like plants and their products, bacteria, algae, yeast fungi and viruses [7]. The most efficient method is bacterial mediated synthesis in which the genetic manipulation is also possible for the production of nonmaterial. In addition to this the high yield, low toxicity, less time consuming, cost-effective and its biocompatibility synergizes to its values [8, 9].

Recent decade, AgNPs have led focus of the nano investigators and regarded as an emerging field of nanomedicine in targeting drug resistant pathogens to treat chronic infections/inflammations [10]. In the field of medicine, experimental reports showed that silver representing a broad-spectrum potency and effective against more than 650 pathogens [11]. The use of silver in the form nano enhances their multiple properties in a ubiquitous range of effectiveness. Generally silver nitrate is also effective against microbes but at higher concentrations while silver nanoparticles are enough to show its efficacy, owing to their low volume and a large surface area that are available to expose and bind with microbial populations [12]. The appropriate rating of AgNPs as antimicrobial agents can form one of the potential alternative strategies towards combating drug resistant microorganisms especially ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) describes a group of pathogens which are most difficult to treat with available antibiotics and responsible for hospital raises infections [13]. Based on the present scenario, AgNPs considered one of the most viable alternatives to available antibiotics because it seems to have a high potential with the possible mode of actions to solve the problem of multidrug resistance in several strains of the pathogens [14].

In this context, we reported a conventional method for the synthesis of AgNPs and evaluated their antimicrobial impression on ESKAPE pathogens. The efficacy of AgNPs was quantified and tested by well diffusion assay, anti-adherence assay, time kinetics growth inhibition and Live/dead assay against multi drug resistant ESKAPE pathogens. The biologically synthesized nanoparticles were characterized by UV–Vis spectrophotometer, FTIR, XRD, DLS, TGA, HRSEM with EDX and HRTEM analysis. Therefore, the present study is a naive attempt to develop a non-toxic based approach to cure the diseases caused by drug resistant pathogens.

Methods

2.1. Materials and ESKAPE strains: Silver nitrate and the media used in all experiments were purchased from Himedia Laboratories Mumbai- India. Sterile ultra pure water (UPH₂O) was used to prepare all the aqueous solutions throughout the experimental work. The strains of ESKAPE pathogens including *Enterococcus faecium* (MCC 2763); Methicillin–resistant *Staphylococcus aureus* (ATCC 33591, MTCC 1430); *Klebsiella pneumonia* (ATCC 35657, MTCC 432); *Acinetobacter baumannii* (ATCC 19606, MTCC 1920); *Pseudomonas aeruginosa* (ATCC 27853, MTCC 1688); *Enterobacter aerogenes* (MTCC 111) and *Enterobacter* species (MCC 2296) were purchased from ATCC (American type culture collection), MTCC (Microbial type culture collection) and MCC (Microbial culture collection) for the evaluation of AgNPs.

2.2. Screening of bacteria and biogenic production of AgNPs: Most silver nitrate salt tolerant bacteria (AgTB), isolated from pond water, contaminated and polluted with the sewage and garbage. The screened bacteria were grown on assorted concentrations of AgNO₃. A pure colony of AgTB (*Bacillus cereus*) was selected for the non-toxic biogenic synthesis of AgNPs [15]. The healthy colony of *Bacillus cereus* inoculated at 37⁰C, 100 rpm for 36 hrs in incubator Shaker (New Brunswick [™] Innova® 40,

Eppendorf New York USA). Then, the culture was centrifuged at 10000 rpm for 10 mins (Eppendorf 5804R Refrigerated Centrifuge). Whatman No.1 filter paper was used to obtain the final filtrate collection.

Bacterial supernatant and AgNO_3 was utilized to standardize the reaction parameters. The supernatants were taken in the different volumes (1, 5, 10, 20, 30, 40, and 50%) and mixed with freshly prepared 1.5 mM AgNO_3 solution maintaining the total volume 100 ml in sterile flasks. In this non-toxic synthesis method, the supernatant- AgNO_3 reaction mixture was subjected to incubator shaker at 37°C . Different set of experiments were carried out and the aqueous extracellular material along with AgNO_3 solution was used as control up to the desired reaction period (12 hrs). The experiments were performed under visually observation and photographed for changes in colour from light yellow to dark brown [16, 17]. The colored solution containing AgNPs was centrifuged at 10000 rpm, 6°C for 10 mins followed by washing (thrice) with sterile UPH_2O to remove biological interactive molecules. Finally, dry powder form of AgNPs was stored for characterization techniques and the antimicrobial applications.

2.3. Characterization of biogenic AgNPs by using UV-vis, XRD, DLS, TGA-DTA, FTIR, SEM along with EDX and UHRTEM:

The UV-vis analysis was determined by sampling from the reaction mixture periodically, the measurement of spectra was recorded at the wavelength ranges between 350- 800 nm. Double beam UV-vis spectrophotometer (Jasco V-730 from Jasco Corporation Tokyo, Japan) was used to record the spectra at different time points (0, 0.5, 1, 3, 6, 9 and 12 hrs) with varying conditions and parameters. The sterile UPH_2O was used as a blank. The UV-vis spectra of the supernatant and AgNO_3 solution were also recorded [18].

For XRD measurements the crystalline nature of the AgNPs were subjected to XRD analysis by coating the dried powder on XRD grid. The spectra were recorded by XRD system (Rigaku Corporation, Tokyo, Japan) by using Scherrer's formula: $D = 0.9\lambda / \beta \cos\theta$ operating at 40 kV and a current of 30 mA with Cu Ka radiation. The diffracted pattern was scanned in 2θ ranges from 20° to 80° [19]. Dynamic light scattering measurements were carried out by using DLS (Zetasizer Nano ZSP, Malvern- Worce, UK). The reference dispersive medium, ultrapure water used as a dispersive medium with a refractive index of 1.330, a viscosity of 0.8872 cP, and PDI were analyzed in acquisition time 60 s, at 25°C . Three measurements were performed and the average particle size of AgNPs calculated [20].

Thermal gravimetric analysis (TGA) was used to determine the thermal stability of surface capped AgNPs and its fraction of volatile components by monitoring the weight loss that occurs as the sample is heated. The sample was subjected to TGA using (TG/DTA 6200 SII Nanotechnology, Japan) instrument over a temperature range of $30-800^\circ\text{C}$ at a heating rate of $20^\circ\text{C}/\text{min}$ under nitrogen gaseous atmosphere [21]. FTIR analysis carried out find the possible functional groups responsible for surface capping of AgNPs in biomolecules present in bacterial supernatant. The measurement of spectra at a scanning speed 2 mm/sec in transmittance mode from 400 to 4000 cm^{-1} at 4 cm^{-1} resolution with 32 scans by using (JASCO-FTIR 6300 Type A, Tokyo, Japan) instrument.

The morphological evaluation of biosynthesized AgNPs was carried out by using FEI Quanta 200 SEM with EDX system (Thermo Fisher Scientific, USA), accelerating at the voltage of 20 KeV with high vacuum mode and recorded the micrographic images at lower to the higher range of magnifications. The elemental composition of the bio-inspired material was analyzed by the use of EDX spectra recorded simultaneously with the SEM analysis. The UHRTEM was used to visualize the size and morphological characteristics of AgNPs were evaluated for their composition by the use of High resolution transmission electron microscope (JEOL JEM 2100, Japan). The samples were sonicated for 20 mins in ethanol and spotted on the carbon coated copper grid with 200 meshed and dried at ambient temperature. The operating energy unit of TEM was 200 KeV at its high resolution mode with the filament LaB6. The size of the particles was considered as an average counts from the TEM micrographs after counting more than 100 particles images.

2.4. Anti-pathogenic effect of biogenic AgNPs against ESKAPE pathogens:

2.4.1. Well diffusion assay- The biogenic AgNPs were evaluated for their antibacterial activity against 11 strains of ESKAPE pathogens. Briefly, 100µl of exponentially phase cultures ($1-5 \times 10^6$ CFU/ml) *E. faecium* (MCC 2763), Methicillin-resistant *S. aureus* (ATCC 33591, MTCC 1430), *Klebsiella pneumonia* (ATCC 35657, MTCC 432), *A. baumannii* (ATCC 19606, MTCC 1920), *P. aeruginosa* (ATCC 27853, MTCC 1688), *E. aerogenes* (MTCC 111) and *Enterobacter* species (MCC 2296) were uniformly spread with the help of L-rod on LB plates. The wells (6 mm) were cut on plates with the help of the cork borer [22]. AgNPs were sonicated in increasing concentrations. (10, 50, 100, 200µg/ml) and 100µl suspension were transferred immediately to each well. Cefotaxime and AgNO₃ solution of similar concentrations was used as a control to compare the efficacy of AgNPs. The loaded plates were gently incubated at 37°C for 18 hrs. Measurements of zones of growth inhibition were determined as radius of the clear zones with the help of Antibiotic Zonescale (Himedia) in triplicate and calculated the sizes as a mean of four zones with $\pm$ SD.

2.4.2. Bacterial growth kinetics: The efficacy of AgNPs as a growth inhibitor was determined against ESKAPE pathogens by performing growth kinetics assay [23]. The growth kinetic patterns of bacteria were measured by optical density at 490 nm (Multi-detector microplate reader- VICTOR™ X3, PerkinElmer, Inc. Waltham, USA). Freshly grown cultures (5%) of all strains were added to increasing concentrations (7.5, 15, 30 and 60µg/ml) of AgNPs in 96 well plates. Cefotaxime a broad-spectrum antibiotics was used as a positive control to compare the results. The same concentrations of nanoparticles were mixed with LB broth without adding bacteria were read as a blank in parallel wells, another untreated cells were used as a negative control. The treated and untreated samples were transferred to a microtiter plate (100 µl in each well) and incubated at 37°C. The absorbance of all samples was measured at 490 nm at regular time intervals (0, 1, 2, 4, 8 and 16 hrs) by using the multimode plate reader. To validate the results, at least three independent experiments were performed for each set of conditions and mean of duplicate readings with $\pm$ SD were considered as significant values. In each case, the mean of absorbance values of the blank control were subtracted from the readings of all treated samples.

2.4.3. Adherence inhibition assay- The assay was performed to determine the adherence pattern inhibition of ESKAPE pathogens by AgNPs on the glass surface. Young cultures, grown over night containing 10^7 CFU/ml were added to glass test tubes at different concentrations of AgNPs (5, 25, 50 and 100 μ l/ml) and maintained a total volume 5 ml, in each test tube including positive (Hydrogen peroxide) and negative (sterile UPH₂O) control to compare the results of anti-adherence activity of pathogens on the surface [24, 25]. Shaking of solutions in test tubes were done and incubated at 37°C for 24 hrs in an inclined plane position at 30°. After the completion of incubation period, adhered cells were fixed by using 5 ml 100% methanol per tube for 15 mins. Fixed bacterial layers were remained on the glass surface followed were dried and then stained with 5 ml of 0.5% (w/v) crystal violet for 5 mins. The stained samples were then washed and air dried. For the quantification of stained adherent cells 5 ml of 33% (v/v) glacial acetic acid per tube was used to remove the dye bound cells. Three independent experiments were performed for each experimental condition to conclude the results. For quantification of adhered bacteria was done at 590 nm. Ultra pure water was used as a blank to subtract the reading from all the samples.

2.4.4. Live and dead staining assay: The strains of ESKAPE pathogens were grown as a biofilm to test their viability. The young cultures (5% inocula) were seeded in 96 well plates and a total of 200 μ l volume was maintained in each well at 37 °C and 100 rpm. Media (LB broth) were changed at a regular interval of 12 hrs upto 72 hrs. The grown biofilms were treated with increasing concentrations of AgNPs (10, 50, 100, 200 μ g/ml) along with positive (Cefotaxime) and negative (sterile UPH₂O) control for 1 hr to compare the results of viable cells. After the treatment the wells were emptied and washed once with sterile water. The biofilms were stained with LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific), for quantitative assays [26]. The wells were filled 200 μ l of PBS (1X) by adding 0.1 μ l stain in each well and kept for 1 hr in an aseptic condition to quantify the live/dead cells. Multi-detector microplate reader - VICTOR™ X3 (PerkinElmer) was used to measure the fluorescence intensity. The wells containing untreated cells were considered as a control.

2.5. Statistical analysis: In the present study, the results of all experiments were carried out in triplicate and the means along with $\pm$ SD of the data sets were calculated in duplicates and employed. The appropriate statistical proficiencies were adopted by using Graph pad prism 7.0 and SPSS 17.0 analytical software packages. To examine the efficacy of AgNPs, the multiple comparisons were applied with AgNPs verses positive and negative control. In case of Well diffusion assay, Anti adherence activity and Live/dead, the significant variances among the means of samples with $\pm$ SD were analyzed to determine the level of significance by using Tukey's multiple comparisons test within each row, compare columns (simple effects within rows). The *P*-values > 0.05 were considered statistically significant and the analysis of variance was executed by two-way ANOVA method.

Results

3.1. Biogenic synthesis and characterization of AgNPs: In present study, a facile procedure has been exploited by using the supernatant of AgTB for the synthesis of tiny functionalized AgNPs. The bacterial

mediated aqueous suspension (40%) added with AgNO_3 at 1.5 mM which showed a gradually change in colour from light yellow to dark brown **Figure 1(A)**.

UV-vis spectroscopy analysis represents the pictures and graphs which are showing that the supernatant and AgNO_3 mixed in a set (1, 5, 10, 20, 30, 40, and 50%) of experiment displayed a gradual change in their appearance. A UV-vis spectra shows a strong peak at 428 nm is imputed to the surface plasmon resonance of AgNPs. The intensity of the functionalized AgNPs change in colour was found in an increased manner with the contact time, the sharper peak was observed. The reduction gradually started after 0.5 and 1 hr, and small peaks started to appears at 424 nm. A shift in peaks was also reported from 426 nm to 448 nm but after the reaction period of 3 and 6 hrs. The maximum colour intensity was attained after 12 hrs and the maximum absorbance reported at 428 nm and there was no shift observed in peaks upto ending of incubation period. Bacterial supernatant and AgNO_3 solution was also scanned individually but there no any peak observed **Figure 1(B)**.

XRD pattern of AgNPs main peaks at 2θ **Figure 2** clearly shows as 27.84, 32.24, 38.12, 44.33, 46.25, 54.82, 57.48, 64.45, 77.39, and 81.52 which are corresponding to the (210), (122), (111), (200), (231), (142), (241), (220), (311) and (222).

The dynamic light scattering (DLS) analysis showed sharp peak of the graph explains mean diameter size of nanoparticles is 91.3 nm nm with Polydispersity index (PDI) value 0.453 **Figure 3 (A)**. For TGA-DTA thermogram studies the temperature range of 30°C-800°C and ceramic (Al_2O_3) crucible was used to record TG and DTA spectra **Figure 3 (B)**. The results displays the DTA-TG thermogram of biogenic AgNPs which gives a clear picture about the weight loss of the sample occurred at two different points in the temperature region between 200-300°C and 500-600°C. The FTIR spectrum of sample **Figure 3 (C)** showed band intensities in different regions at 702.92, 1011.48, 1159.00, 1573.62, 1875.43, 2103.95, 2218.70, 2645.85, 3094.22, 3298.64, 3410.49, 3463.52, 3617.80, 3724.83, and 3791.36.

SEM analysis of AgNP showed in micrographs of the dried silver nanoparticles which expresses morphology and size distribution of nanoparticles **Figure 4 (A)**. In SEM analysis, the images elucidating the particles are predominantly irregular, cuboids and spherical in shape and aggregates into larger particles with no well-defined morphology was observed. The estimation of elemental composition and the purity was determined by EDX analysis. The strong signals of capped AgNPs were observed from the silver atoms at 3 KeV, which indicates the crystalline property of silver nanocrystals **Figure 4 (B)**. The determination of the mass of total product was estimated as the strong signal for Silver (88.62%) and weak signals of Carbon (6.14%) Oxygen (2.57%) and Sulphur (2.67%) **Figure 4 (C)**.

In UHRTEM analysis, the microgram of UHRTEM exhibit pleomorphic morphology including rectangular and oval shape but the majority of particles are in the spherical shape with a smooth surface **Figure 5 (A, B, C, D, E and F)**. The frequency distribution histogram of particle size represent that the majority of nanoparticles were at the maximum count (37%) with an average size 26.00 ± 0.41 nm and all other particles showed an average size ranges from 15.5 to 55.5 nm **Figure 5 (G)**.

1.2. Evaluation of anti-pathogenic potential of biogenic AgNPs against ESKAPE pathogens:

1.2.1. The well-diffusion assay against ESKAPE pathogens- The diameter of zone of inhibition was measured in four replicates and the mean of the zones considered as an antimicrobial factor. In the present investigation, the antibacterial activities of four different concentrations of AgNPs with eleven microorganisms including MDR pathogens were studied. The mean zone of inhibition measured as a mean of zones after 18 hrs of incubation. The inhibitory range of AgNPs concentration was investigated between 50-200 µg/ml against all ESKAPE pathogens including drug resistant bacteria. *E. faecium* (MCC 2763), MRSA(ATCC 33591), *S. aureus* (MTCC 1430), *K. pneumoniae* (ATCC 35657 and MTCC 432), *A. baumannii* (ATCC 19606 and MTCC 1920), *P. aeruginosa* (ATCC 27853 and MTCC 1688), *E. aerogenes* (MTCC 111) and *Enterobacter* species (MCC 2296) all exhibited lesser zone at 10 µg/ml while higher at significance ($P < 0.05$) increasing concentration (50 to 200 µg/ml) **Figure 6 (A, B, C and D)**. After a particular concentration, the size of zones were not increased and showed equal size with previous decreasing concentrations.

1.2.2. Time kinetics of AgNPs against ESKAPE pathogens- The results of growth kinetics **Figure 7** demonstrate a differential growth inhibition pattern of all ESKAPE pathogens with increasing concentrations of AgNPs in growth medium. In case of *E. faecium* (MCC 2763), *K. pneumonia* (ATCC 35657 and MTCC 432), *A. baumannii* (ATCC 19606 and MTCC 1920) and *E. aerogenes* (MTCC 111) at higher to lower treatment, the log phase was negligible or absent **Figure 7 (A, D, E, F)** and **Figure S1 (G, K)** but in case of, *S. aureus* (ATCC 33591 and MTCC 1430), *P. aeruginosa* (ATCC 27853 and MTCC 1688) and *Enterobacter* species (MCC 2296) the slightly log and stationary phase was present **Figure 7 (B, C)** and **Figure S1 (H, I, J)** that was observed at 4 hrs completion when growth pattern was compared after 10 hrs of incubation with untreated control sets of experiment. Indeed, significant growth inhibition of all tested pathogens occurred after 4 hrs of treatment with AgNPs and not much gain in killing ensued upon extended incubation up to 16 hrs.

1.2.3. ESKAPE pathogen adherence inhibition assay: The inhibitory effect of AgNPs on adherence of cells to the glass surfaces exhibited adherence inhibition of the growing cells and eliminated the attachment with including drug resistant pathogens and noticed that the inhibitory effective was concentration dependent **Figure 8 (A-D)**. The level of adhesion on a glass surface was significantly reduced in AgNPs treated group when compared with Positive control (H_2O_2) in case of *E. faecium* (MCC 2763), *S. aureus* (MTCC 1430), *K. pneumonia* (MTCC 432), *A. baumannii* (MTCC 1920), *P. aeruginosa* (ATCC 27853 and MTCC 1688) and *E. aerogenes* (MTCC 111), but when compared with negative control (sterile UPH_2O), the attachment of all strains of ESKAPE pathogens was very significantly reduced on the glass surface. The highest inhibition was found in *E. faecium* (MCC 2763), *P. aeruginosa* (MTCC 1688) and *E. species* (MCC 2296) at 100µg/ml of AgNPs. Similarly, the low concentrations of silver nanoparticles (5µg/ml) also significantly ($P < 0.05$) inhibited bacterial attachment of the all tested pathogens.

1.2.4. Live and dead staining assay- The biofilm treated with increasing concentrations of AgNPs, cefotaxime and no treatment, evaluates the cells density in the treated medium. The graphical

representation of live/dead cells depicted **Figure 9 (A-F)** and **Figure S2 (G-K)** showed a gradual reduction of populations in the increasing manner. As a result it was observed that, at concentration 10 µg/ml, the pattern of dead cells of KP-1 → PA-1 → PA-2 → AB-1 → EA → KP-2 → SA-2 → SA-1 → EF → ESp and at 200 µg/ml AB-1 → PA-1 → PA-2 → KP-1 → EF → EA → ESp → SA-1 → KP-2 → SA-2 → AB-2 respectively and the remaining cells were reported live cells as vice versa. Consequently the exposure of AgNPs increased the dead cells significantly ($P < 0.05$). The step-down of cells density with AgNPs impression was comparable with the effect of cefotaxime and was found significantly higher than the reduction caused by the antibiotics.

Discussion

Bacterial mediated AgNPs applications is a relatively growing field and have a great attention to rid off exiting toxicological consequence and also to increase attention towards the nanotherapy. The procedure for biogenic production of AgNPs was standardized with the UV-vis analysis, in which the spectra were recorded time to time to represent the approaches. The change in colour was due to the excitation of surface plasmon resonance by newly formed nuclei within 30 mins in the mixed solution reveals the reduction of silver ions into silver nanoparticles [27]. The formation of AgNPs in an aqueous medium was confirmed by the UV–visible spectra and the maximum colour intensity was attained after 12 hrs. The change in colour was due to reduction of silver ion by the biomolecules and enzymes present in the cell-free extracts [28]. The presences of free electrons in silver nanoparticles are responsible to raise absorption band with the combined vibration of electrons of metal nanoparticles [29]. The reaction time completed in 12 hrs and spectra were becomes sharper which indicate the formation of mono dispersed nanoparticles [30]. Based on enzyme kinetics action it is clear that the highest optical density found upto at 1.5 mM by attending high intensity of reduction while beyond that of the concentrations of silver nitrate shows gradually decreased bio-reduction of particles [31].

The synthesized AgNPs were characterized by XRD to determine the translational symmetry-size and shape incurred from the peak positions of diffractograms pattern. The XRD confirmed the synthesized particles as metallic silver and Braggs's reflections of silver nanocrystals which are already reported [32]. The XRD results showed typical pattern of planes found to report the face centred cubic (fcc) silver, respectively [33]. Thus the XRD analysis strongly suggested the crystallinity of AgNPs [34]. In DLS analysis, the high intensity distribution at a lower range of particle size and a single peak indicates the lower range of size and the quality of the particles respectively [35]. The TGA results shows loss of 8.4% up to 300°C and 10.6% up to 800°C. DTA plot gives information about the simultaneous occurrence of complete thermal decomposition and crystallization of the sample. An approximate of no weight loss was observed below 200°C which can be largely linked to the evaporation of water and organic components [36]. The presence of an exothermic peak between 200°C and 400°C in the DTA plot can be associated with the crystallization of silver nanoparticles [37]. The FTIR analysis of the powder sample used to investigate the action of biomolecules and responsible factor for capping and stabilization of nanoparticles. In current study, FTIR peaks correspond to alkyl halides (C-Cl and C-F) responsible to make compounds bioactive in a medium, Amine (C-N) responsible for increase polarity, reactivity and labeling of peptides/proteins [38]. Aromatics (C=C) substances contain alternating single and double bonds in its

chemical structure so these bonds may break with the extracellular materials and produces odor in medium and forms a stability with the particles [39]. Ketonic group (C=O) the group refers to amino and amino-methyl stretching groups of protein. The presence of carbonyl groups of the amino acid residues and the peptides have a strong ability to bind to the silver [40], alkynes (C≡C-) contributing in tagging of biomolecules including proteins and lipids [41], nitriles (C≡N) helping in making up a proper structural format of the synthesized nanoparticles with vibrational probes of proteins [42], alkanes (C-H) responsible for saturation and instauration of molecules and also reported that the proteins can bind to AgNPs either through free amine or cysteine groups in proteins present in medium [43], basically these are enzyme-mediated and produced by bacteria [44], amides (N-H) responsible for increase polarity and labelling of peptides/proteins [38] alcoholic group (O-H) and carboxylic acid (O-H) an active role played by the groups in reduction of metal ions and oxidation of biomolecules, followed by formation of nanoparticles [45] respectively. Finding of these groups in medium confers the stability of the synthesized AgNPs for a period time and keeps functional in both wet and dried conditions [46]. Intermolecular forces are another factor may involve in prevention of nanoparticles to aggregate in the medium. The possible interactions between silver and bioactive molecules and capping agents could be responsible for the synthesis and stabilization of AgNPs [47]. Biomolecules present on the surface of nanoparticles leads to agglomeration structure. In SEM measurements, on the surface of slide the larger view of silver particles may be due to the aggregation of the smaller ones. Similar image illustrations were also reported by *Vibrio alginolyticus* mediated synthesis of AgNPs [48]. The weak signals were also observed during the EDX analysis, strongly suggested the capping of the particles with indication for the presence of biomolecules released in bacterial supernatant contributed in stabilization of the particles [49]. SEM images shows pleomorphic morphology of particles and expressing some difference in size with DLS results, the variation in size and shape may be due to the dispersion and various biological reductants used in the synthesis process [50]. Through TEM images, biological component as a capping were spotted which can be observed as ring masks on the surface of particles. The size measured through DLS is slightly larger than the size evaluated by TEM analysis because of bio-capping on their surface [51].

The resulted biogenic AgNPs showed effective anti-pathogenic activities against all selected strains of ESKAPE pathogens while especially on MDR Methicillin-resistant *S. aureus* (MRSA- ATCC 33591), *S. aureus* (MTCC 1430) and *P. aeruginosa* (ATCC 27853). Similar studies have been done with MDR strains by using biosynthesized nanoparticles [52]. Therefore, the small amount of AgNPs can be rid off MDR pathogens and may prove an effective besides combating the problematic pathogenic microorganisms [10].

Efficacy of biogenic AgNPs was further validated by measuring the growth kinetics. The exposure of AgNPs on ESKAPE pathogens showing a clear growth pattern of all phase of time kinetics along with the effective inhibition of growth with the time exposure as an antibacterial agent. This inhibition practice showed the diverse efficacy of AgNPs on human pathogenic strains responsible for nosocomial infections [53].

The anti adherence effect of antimicrobial agent on any surface directly affects the accumulation of microbes and biofilms formation which can be reservoirs for pathogenicity. The anti-adherence potential of nano materials may be attributed to their ability to inhibit the attachment of bacteria on a glass surface [54]. The anti-adherence behavior of AgNPs suggested that this could be useful for the development of biologically functionalized silver nanoantibiotics [55].

In live/ dead staining assay, the biofilm was grown upto 72 hrs and stained with LIVE/DEAD BacLight™ Bacterial Viability Kit and treated with increasing concentrations of AgNPs, the SYTO-9 dye specific for live cells to provide substantial resolution between live and dead cells population [24]. The impacts of biogenic AgNPs on MDR pathogen biofilms formed by ESKAPE pathogens showed statistically significant depletion in their population.

Conclusions

The biogenic synthesis of AgNPs presented structural uniqueness with specific binding sites on their surface to interact with the complementary object. In this context, AgNPs were synthesized successfully by using bacterial supernatant with the characteristic size measurement (26.00 nm). Due to their tiny size with the functional group, AgNPs can easily enter across the bacterial cells and causes leakage of their cell wall/membrane or inhibit enzymatic systems in the respiratory chain and thereby alter their DNA synthesis. In the present study, the synthesis and characterizations of nanoparticles were monitored by using UV–Vis, XRD, DLS, FTIR, TGA-DTA, SEM, UHRTEM and optimum conditions were assessed. Eleven strains of ESKAPE pathogens were evaluated against AgNPs at different/ increasing concentrations. The different sets of antimicrobial assays exhibited excellent broad-range anti-pathogenic activities against MDR pathogens. The assessed AgNPs were found extremely suitable for potential use. This study demonstrates the biosynthesis of nanoparticles, a simple and cost effective method, and the possibility of using AgNPs to control the nosocomial infections caused by ESKAPE strains resistant to the majority of antibiotics. Further studies may be conducted to investigate the biocompatibility at molecular level by modification on their binding sites and with impregnation of compatible agents make them a feasible choice in future biomedical as a nano-biomedicine.

Declarations

Disclosure of potential conflicts of interest

Authors have no conflict to report.

Funding

This work was supported by the DST Science and Engineering Research Board (SERB), India [Grant number- SERB/LS-267/2014] for providing fellowship and funds to perform this research.

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Figures

Figure 1

Synthesis of biogenic AgNPs: Panels shows a change in colour due to the excitation of surface plasmon resonance, (A) The changes in colour observation with time indicates formation of AgNPs (B) UV-vis spectra of synthesized AgNPs

Figure 2

X-ray patterns of biosynthesized AgNPs by using AgTB

Figure 3

(A) Dynamic light scattering of biosynthesized AgNPs, (B) TGA-DTA thermograms of biosynthesized AgNPs, (C) FTIR spectra of dried biosynthesized AgNPs.

Figure 4

(A) SEM observations of dried AgNPs: (i) Magnification at $\times 30,000$; insert bar corresponds to 0.5 μm , (ii) Magnification at $\times 15,000$; insert bar corresponds to 1 μm , (B) EDX analysis: The strong spectra of Ag showed the domination of biosynthesized AgNPs, (C) Estimation of the elements: The bar graphs shows the participation of elements present in the sample.

Figure 5

UHR-TEM, (A-F) Images of biogenic AgNPs, and (G) size distribution histogram of particles.

Figure 6

Efficacy of AgNPs at increasing concentrations against ESKAPE pathogens by well diffusion method: (A) 10 µg/ml (B) 50 µg/ml (C) 100 µg/ml (D) 200 µg/ml. Note: [EF = *E. faecium* (MCC 2763), SA-1 = MRSA

(ATCC 33591), SA-2 = *S. aureus* (MTCC 1430), KP-1 = *K. pneumoniae* (ATCC 35657), KP-2 = *K. pneumoniae* (MTCC 432), AB-1 = *A. baumannii* (ATCC 19606), AB-2 = *A. baumannii* (MTCC 1920), PA-1 = *P. aeruginosa* (ATCC 27853), PA-2 = *P. aeruginosa* (MTCC 1688), EA = *E. aerogenes* (MTCC 111), and ES_p = *Enterobacter* sp. (MCC 2296)] The data set represent the mean $\pm$ SD of two independent experiments performed in triplicates. The level of statistically significant differences are indicated by * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ and **** $p < 0.00005$. The significant differences were compared among AgNPs Vs Cefotaxime.

Figure 7

Growth inhibition activity of AgNPs against ESKAPE pathogens: Changes in absorbance are depicted in panels (A-F) at increasing concentrations (7.5, 15, 30 and 60 µg/ml) of AgNPs (A): *E. faecium*- MCC 2763; (B): *S. aureus* (MRSA)- ATCC 33591; (C): *K. pneumoniae*- MTCC 432; (D): *A. baumannii*- ATCC 19606; (E): *P. aeruginosa*- ATCC 27853; (F): *Enterobacter* species- MCC 2296.

Figure 8

Inhibitory effect of biogenic AgNPs on ESKAPE pathogen's adherence: (A) at concentration 5 µg/ml AgNPs, 5 µl/ml H₂O₂ and 5 µl/ml UPH₂O (sterile), (B) 25 µg/ml AgNPs, 25 µl/ml H₂O₂ and 25 µl/ml UPH₂O, (C) 50 µg/ml AgNPs, 50 µl/ml H₂O₂ and 50 µl/ml UPH₂O, (D) 100 µg/ml AgNPs, 100 µl/ml H₂O₂ and 100 µl/ml UPH₂O. The data set represent the mean ± SD of two independent experiments performed

in triplicates. The level of statistically significant differences are indicated by * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ and **** $p < 0.00005$. The significant differences were compared among AgNPs vs. NC (UPH20).

Figure 9

Exposure of AgNPs and Cefotaxime at raising concentrations (10, 50, 100 and 200 µg/ml) to biofilms formed by ESKAPE pathogens: (A): *E. faecium*- MCC 2763; (B): *S. aureus* (MRSA)- ATCC 33591; (C): *K. pneumoniae*- MTCC 432; (D): *A. baumannii*- ATCC 19606; (E): *P. aeruginosa*- ATCC 27853; (F): *Enterobacter* species- MCC 2296. (AgNPs = Silver nanoparticles Cefo = Cefotaxime) The data sets of experiments represent the mean ± SD of two independent experiments performed in triplicates. The level

of statistically significant differences are indicated by * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ and **** $p < 0.00005$. The significant differences were compared with Live vs. Live and Dead vs. Dead among AgNPs and Cefotaxime, respectively

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