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Antimicrobial potential of polyvinyl pyrollidone stabilized silver nanoparticles synthesized by *Sphingobacterium multivorum*

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Biological synthesis of nanoparticles is emerging as a promising solution to tackle issues associated with conventional synthesis methods. Silver nanoparticles (AgNPs), owing to their unique physiochemical and antimicrobial properties, attract more attention. In this study, we have made an attempt to develop ecofriendly and stable AgNPs with antimicrobial potential. AgNPs were synthesized using Gram negative *Sphingobacterium multivorum* supernatant and characterized by UV-Visible spectrophotometric analysis, X-Ray Diffraction analysis, Transmission electron microscopy, Fourier transform infrared and Dynamic Light scattering. Biosynthesized AgNPs exhibited broad spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria. Minimum inhibitory concentration was detected 19.5 µg for *E. coli* and *Staphylococcus aureus*. AgNPs have exhibited significant synergistic effect with all the selected antibiotics. The results demonstrated a rapid, economic and ecofriendly method for the synthesis of stable AgNPs and further evaluation of the antimicrobial activity against Gram-positive and Gram-negative bacteria.

Keywords: AgNPs, Antimicrobial, Dynamic light scattering (DLS), Polydispersity index (PDI)

Nanotechnology, a multidisciplinary field, is emerging exponentially form last few decades owing to its enormous application potential. Advances in nanotechnology have revolutionized many sectors like electronics, defence, cosmetics, agriculture, food, health and medicine[1-6]. Nanobiotechnology, an amalgamation of nanotechnology with biotechnology includes design and synthesis of materials or devices of nanometer range[7]. Ultrafine particles due to their nano scale size possess novel physiochemical and biological properties. The physiochemical properties of metal nanoparticles are based on the size, shape, composition, crystallinity and morphology. Anti-microbial resistance is a global threat growing at an alarming rate. There is a dire need to seek solution for this problem. Inefficacy of conventional antibiotics against drug resistance has pushed search for other novel options. Among metal nanoparticles, silver nanoparticles (AgNPs) gain more attention owing to their unique physiochemical and antimicrobial properties[8]. AgNPs have revolutionized different sectors like electronics, optics, catalysis and Raman scattering, pharmaceuticals and medicine[9,10]. Silver has been used since several years owing to their antimicrobial properties. Silver based products are employed in topical ointments, surgical bandages for wound healing and stents coating to prevent microbial infections[11,12].

A large number of physical and chemical processes have been exploited for synthesis of metal nanoparticles. However, these processes require high temperature/ pressure which is extremely harmful for environment. Selection of environment friendly and cost-effective process for the engenderment of nanoparticles is one of the major challenges in the newly emerging field of nanobiotechnology. Biologically fabricated AgNPs have significantly contributed to different areas like biosensor technology, biomedical, drug delivery, diagnostics, etc.[12-14]. Green synthesis methods are gaining more attention over conventional synthesis methods as they do not require toxic chemicals for their synthesis. Green synthesis processes are based on plant, bacteria and fungi mediated synthesis of nanoparticles. Microbes based synthesis is a rapid, economic, environmentally friendly and easy to scale up as compared to other methods[15-17]. Bacteria mediated synthesis may occur either extracellularly or intracellularly. Extracellular synthesis methods are preferred over intracellular methods due to easy downstream recovery and purification steps. In the

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present study, we have made an attempt to develop a rapid and environment-friendly method for synthesis of stable AgNPs and also evaluated their antimicrobial activity against Gram-positive and Gram-negative bacteria.

Materials and Methods

Isolation and characterization of bacteria from soil

The AgNPs synthesizing bacteria was isolated from metal contaminated soil by serial dilution method[17]. The isolated colonies were further screened for their ability to tolerate silver nitrate. The selected bacterial culture was identified on the basis of 16S rRNA sequencing performed at Institute of Microbial Technology, Chandigarh (India).

Extracellular synthesis of silver nanoparticles

The synthesis of AgNPs was carried out by extracellular method[17] and polyvinyl pyrollidone (0.1% PVP) was used as a stabilizing agent. The isolated bacterial culture DNP 5 was inoculated in basal salt media (BSM) for 48 h at 30°C under shaking conditions of 220 rpm. After incubation, the broth culture was centrifuged at 8000 rpm for 20 min. The supernatant was used for the synthesis of AgNPs. The synthesis of AgNPs was observed at regular intervals visually on the basis of colour change of culture supernatant from a transparent to brown appearance. The brown colour of culture supernatant was considered as a sign of the synthesis of AgNPs.

Characterization of silver nanoparticles

The produced nanoparticles were characterized by UV–Vis spectroscopy and X-Ray Diffraction analysis. The morphological examination of AgNPs was done by Transmission electron microscopy (Hitachi (H-7500) at CIL, Panjab University, Chandigarh. The particle size distribution and zeta potential of synthesized AgNPs was evaluated using dynamic light scattering (DLS) (Microtrac Nanotrac Wave Particle Size and Zeta Potential analyzer. Fourier transform infrared (FTIR) spectrum was recorded using FT-IR spectrophotometer (Horizon ABB) in the range of 4000-400 cm^{-1} (scan speed of 16 cm/s) and elemental composition was confirmed by energy dispersive X-ray spectroscopy (EDX).

Antimicrobial studies

Antimicrobial potential of biofabricated AgNPs was analyzed against 10 different test pathogens (seven bacteria and three fungi). The test microorganisms were *Escherichia coli*, *Bacillus subtilis*, *Psuedomonas aerugenosa*, *P. fluorescens*, *Staphylococcus aureus*, *Streptococcus mutans*, *S. pyrogenes*, *Fusaium graminearum*, *Candida albicans* and *C. glabrata.* The test cultures were procured from MTCC, IMTECH, Chandigarh. Antibacterial activity of synthesized AgNPs was determined by agar well diffusion assay with some modifications[17,18]. Further, the interaction between test pathogen *P. aeruginosa* and synthesised AgNPs was confirmed by TEM analysis.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of AgNPs for different test pathogens (*E. coli*, *P. aerugenosa*, *S. aureus*, *B. subtiliis* and *C. albicans*) was measured by agar well diffusion assay method with some modifications[17,18]. Different concentrations (19.5, 39, 78.1, 156.2, 312.5, 625 and 1250 µg) of synthesized AgNPs suspended in deionized water were used. The smallest concentration of AgNPs that inhibits the test pathogens was recorded as MIC.

Synergistic effect of AgNPs with antibiotic

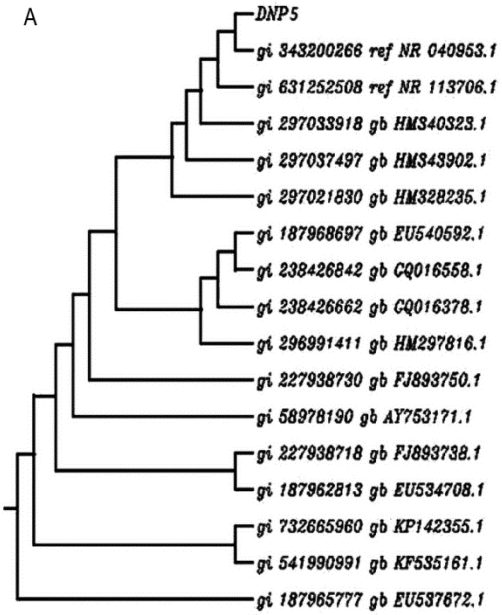
The collective effect of AgNPs and antibiotic was assessed by agar well diffusion assay as discussed earlier. Five different classes of antibiotics i.e. (1) Norfloxacin 10 µg (Quinolone), (2) 15 µg (Macrolide), (3) Rifampicin 5 µg (Rifamycin), (4) Kanamycin 30 µg (Aminoglycoside) and (5) Amoxyclav 30 µg (β Lacatm) were selected to measure synergistic effect of AgNPs. Antibiotic discs were soaked with 30 µL biologically synthesized AgNPs (0.1 mg/mL). The diameter of inhibition zone was measured as mean ± SD of the triplicate experiment[19].

Stability study

The synthesized nanoparticles were stored at 4°C for 40 days. The stability of synthesized nanoparticles was confirmed by UV-Visible Spectrophotometer analysis. Further, these stored nanoparticles were incubated in autoclave at 121°C for 40 min and then analyzed for antimicrobial activity.

Results

In the present study, we carried out bacterial supernatant mediated extracellular biogenesis of silver nanoparticles (AgNPs). The soil sample from metal contaminated site was used for isolation of bacteria capable to synthesize silver nanoparticles. The silver nanoparticles producing bacterial strains were screened on the basis of their growth on nutrient agar containing 1 mM silver nitrate ($AgNO_3$) at 30°C for 48 h. All the bacterial isolates were checked for



B Sequences producing segment alignments:
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Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Sphingobacterium multivorum strain DNP 5 16S ribosomal RNA gene, partial sequence	2503	2503	100%	0.0	100%	KX011877.1
Sphingobacterium multivorum strain IAM 14316 16S ribosomal RNA gene, partial sequence	2503	2503	100%	0.0	100%	NR_040953.1
Uncultured bacterium clone ncd1047h04c1 16S ribosomal RNA gene, partial sequence	2492	2492	99%	0.0	99%	HM340323.1
Uncultured bacterium clone nbt120d01 16S ribosomal RNA gene, partial sequence	2492	2492	100%	0.0	99%	EU540592.1
Bacteroidetes endosymbiont of Metaseiulus occidentalis clone pAJ234 16S ribosomal RNA gene, partial sequence	2488	2488	100%	0.0	99%	AY753171.1
Uncultured bacterium clone ncd1052c09c1 16S ribosomal RNA gene, partial sequence	2486	2486	99%	0.0	99%	HM343902.1
Uncultured bacterium clone nbw774g02c1 16S ribosomal RNA gene, partial sequence	2486	2486	99%	0.0	99%	GQ016558.1
Uncultured bacterium clone C4_9 16S ribosomal RNA gene, partial sequence	2484	2484	99%	0.0	99%	KP142355.1
Sphingobacterium multivorum strain BAB-2422 16S ribosomal RNA gene, partial sequence	2483	2483	100%	0.0	99%	KF535161.1
Sphingobacterium multivorum strain NBRC 14947 16S ribosomal RNA gene, partial sequence	2481	2481	99%	0.0	99%	NR_113706.1
Uncultured bacterium clone ncd490f01c1 16S ribosomal RNA gene, partial sequence	2481	2481	99%	0.0	99%	HM328235.1
Uncultured bacterium clone ncd854c12c1 16S ribosomal RNA gene, partial sequence	2481	2481	99%	0.0	99%	HM297816.1
Uncultured bacterium clone nbw772f12c1 16S ribosomal RNA gene, partial sequence	2481	2481	99%	0.0	99%	GQ016378.1
Uncultured bacterium clone nbt24f07 16S ribosomal RNA gene, partial sequence	2481	2481	99%	0.0	99%	FJ893750.1
Uncultured bacterium clone nbt24d02 16S ribosomal RNA gene, partial sequence	2481	2481	99%	0.0	99%	FJ893738.1
Uncultured bacterium clone nbt75f11 16S ribosomal RNA gene, partial sequence	2481	2481	99%	0.0	99%	EU537672.1
Uncultured bacterium clone nbt182h08 16S ribosomal RNA gene, partial sequence	2481	2481	100%	0.0	99%	EU534708.1
Sphingobacterium sp. B2(2012) 16S ribosomal RNA gene, partial sequence	2477	2477	100%	0.0	99%	JX941542.1
Uncultured bacterium clone ncd490f09c1 16S ribosomal RNA gene, partial sequence	2475	2475	99%	0.0	99%	HM328259.1

Fig. 1 — (A) Graphic summary of BLAST analysis of 16 S rDNA sequence of DNP 5; (B) Phylogenetic tree constructed by UPGMA method; and (C) Description of BLAST analysis

extracellular biosynthesis of AgNPs and out of all bacterial isolates, DNP5 was selected for biosynthesis of AgNPs. On the basis of BLAST analysis, the 16S rDNA sequence of DNP5 exhibited 100 % identity to already reported sequence of *Sphingobacterium multivorum*. 16S rDNA sequence was submitted to NCBI (accession number KX011877) (Fig. 1).

Extracellular synthesis of silver nanoparticles was primarily confirmed by visual monitoring of colour change (transparent to dark brown). The test flask (supernatant, $AgNO_3$ (2 mM) and PVP exhibited light yellow colour within 2 min of incubation. The colour change was completed within 20 min of incubation (Fig. 2). However, control flasks containing only supernatant and silver nitrate remained colourless.

Further, biosynthesized AgNPs were characterized by UV–Visible spectroscopy analysis and spectrum exhibited peak at 418 nm (Fig. 3A). In the present study, one SPR symmetric shape peak was observed in UV-Visible spectral analysis of synthesized AgNPs. Hence, it could be inferred that the synthesized AgNPs are monodispersed and of spherical shape. On the basis of TEM analysis, silver nanoparticles were in the range of 17-30 nm and the average size of biosynthesized AgNPs was 22.28±0.2 nm (Fig. 3 B & C).

The crystal structure of biologically synthesized silver nanoparticles was determined by X-ray

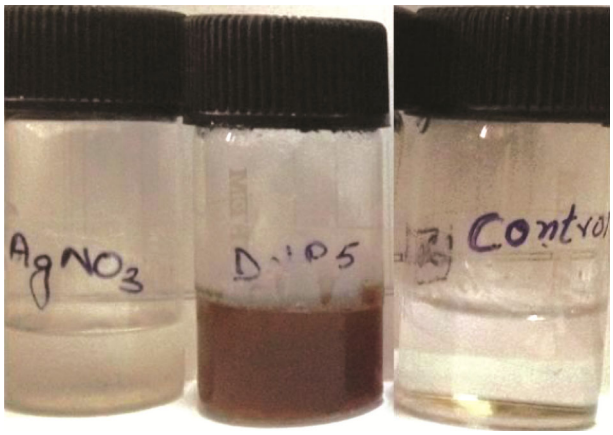

Fig. 2 — Extracellular synthesis of silver nanoparticles

diffraction (XRD) analysis. The XRD pattern of synthesized AgNPs exhibited four peaks at 38.14, 44.35, 64.84 and 77. 53 which belong to 111, 200, 220, and 311 face centered cubic (fcc) planes respectively for metallic silver (Fig. 3D). Average size of biosynthesized silver nanoparticles was determined as 39.38 nm using Debye-Scherer's equation. The calculated size of nanoparticles was same as the size estimated by TEM analysis. The XRD spectrum for synthesized AgNPs was in agreement with JCPDS 04-0783 diffraction standard, which verified the crystalline nature of synthesized Ag-NPs.

The DLS analysis confirmed that silver nanoparticles synthesized using DNP 5 (*Sphingo-*

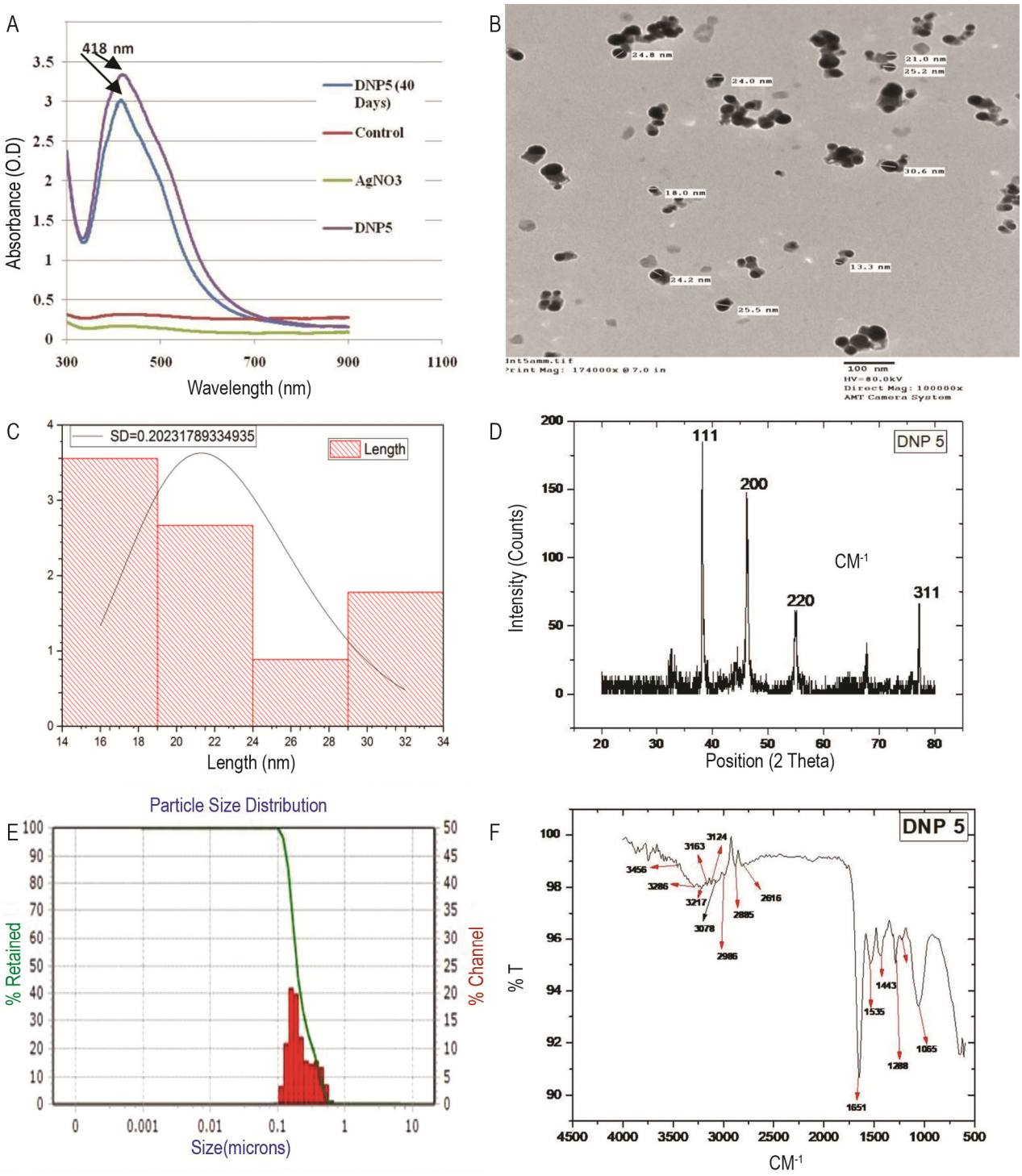

Fig. 3 — (A) UV-Visible spectral analysis; (B) TEM analysis; (C) XRD analysis; (D) DLS analysis; and (E) FTIR analysis of silver nanoparticles synthesized by using culture supernatant of DNP 5

bacterium multivorum) were in the range of 160-230 nm (Fig. 3E). Average size of DNP 5 synthesized silver nanoparticles was approximately 200 nm. The particles bear a charge of -41.55 mV. The polydispersity index (PDI) for biosynthesized AgNPs was less than 0.276.

The silver nanoparticles synthesized using DNP 5 (*Sphingobacterium multivorum*) exhibited peaks at 3456 cm^{-1}, 3124 cm^{-1}, 2986 cm^{-1}, 1774 cm^{-1}, 1651 cm^{-1}, 1535 cm^{-1}, 1288 cm^{-1}, 1227 cm^{-1} and 1065 cm^{-1} in FTIR spectrum (Fig. 3F). The peaks at 1651

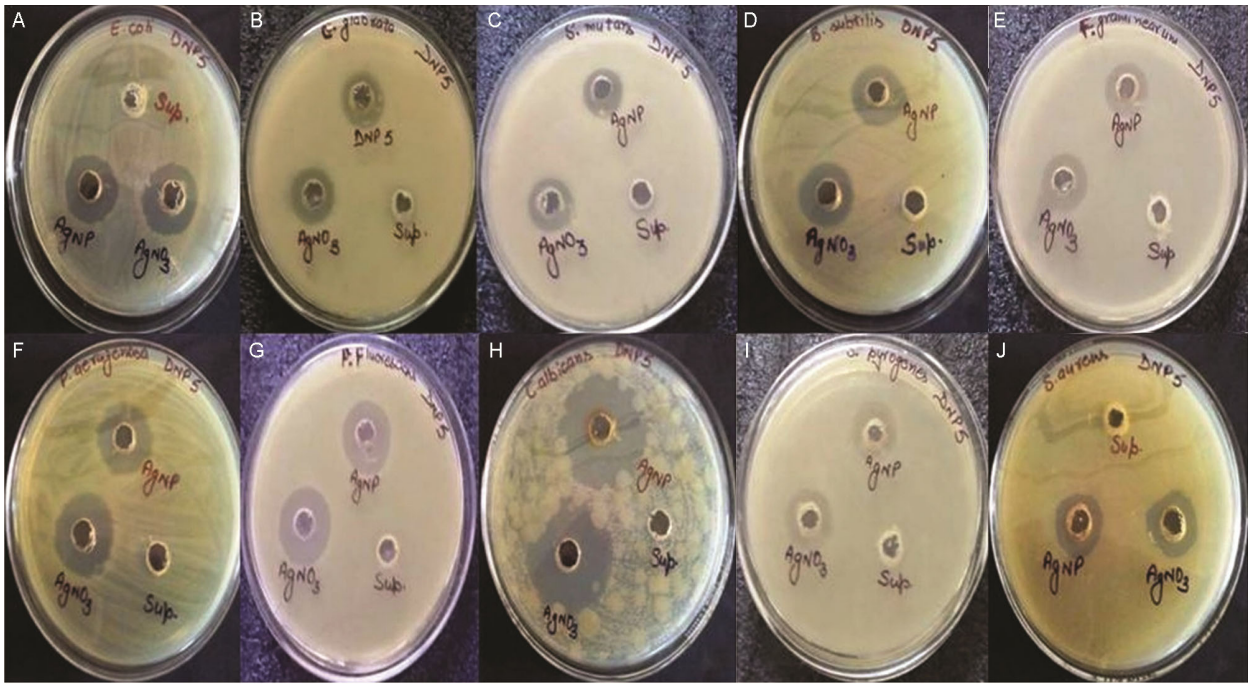

Fig. 4 — Antimicrobial activity of AgNPs synthesized by bacterial DNP 5 against test pathogens (A) *Escherichia coli*; (B) *Candida glabrata*,; (C) *Streptococcus* mutans; (D) *Bacillus subtilis*,; (E) *Fusarium graminearum* (F) *Pseudomonas aerugenosa*; (G) *Pseudomonas fluorescens*; (H) *Candida albicans*; (I) *Streptococcus pyrogenes*; and (J) *Staphylococcus aureus*

cm^{-1} corresponds to stretching of C=O amide I bands of peptide linkage and 1288 cm^{-1} corresponds to bending of NH and CN stretching of peptide. The peak at 1065 cm^{-1} indicated $–CH_3$ bending in amino acid and the peak at 1443 cm^{-1} was related to C=N vibration and 1535 cm^{-1} confirmed change in NO asymmetric stretch due to nitro compound. The peak at 2885 cm^{-1} and 3163 cm^{-1} was observed due to -CH vibration. The peak at 2616 cm^{-1} and higher energy region peaks at 3217, 3456 cm^{-1} correspond to alcohol or phenol O-H stretching. The peak at 3078 cm^{-1} and 3286 cm^{-1} indicated –NH stretching.

The energy dispersive X-ray analysis (EDX) is used to get the qualitative and quantitative information about elements. EDX exhibited strong signal in silver region and substantiated the formation of silver nanoparticles. EDX analysis demonstrated silver (66.2 %), chlorine (18.92%) and oxygen (14.8%) as elemental constituents of fabricated nanoparticles. On the basis of these characterizations, this could be concluded that synthesized particles are of silver and in nano range.

Further, the stability of synthesized AgNPs solutions was determined by UV-Vis spectra at intervals of 1, 15 and 40 days after storage at 4ºC. There was no change in the peak position even after 40 days (Fig. 3A). The constant position of absorbance peak confirmed that nanoparticles did not aggregate. Further, autoclaved nanoparticles also showed the same antimicrobial activity as shown by freshly synthesized silver nanoparticles. This can be inferred that nanoparticles colloidal solution could be stored for 40 days and were stable even at high temperature.

The antimicrobial activity of biosynthesized AgNPs against different test pathogens is shown in Fig. 4. The presence of inhibition zone signified the antimicrobial action of synthesized AgNPs. The inhibitory effect was present against all the test pathogens. The AgNPs synthesized by DNP 5 showed a maximum effect against *C. albicans* with an inhibition zone of 27.16±0.28 mm followed by *S. aureus* with a zone of inhibition of 24.66±0.57 mm. The minimum effect of AgNPs was observed against *S. mutans* (15.66±0.57 mm) and *Fusarium graminearum* (16.66±0.57). The Antimicrobial activity of biofabricated AgNPs was notably higher than $AgNO_3$ against all test pathogens Fig. 5.

In the present study, TEM analysis has also confirmed the attachment and penetration of silver nanoparticles in *Pseudomonas aerugenosa* (Fig. 6). The MIC values of silver nanoparticles for different strains were measured (Fig. 7). The MIC of silver

nanoparticles was measured as 19.5 µg for *E. coli* and *S. aureus*, 39 µg for *P. aerugenosa* and *C. tropicalis* and 78 µg for *B. subtilis*. At concentrations less than MIC, inhibition zone was not observed. The diameter of zone increased with silver concentration

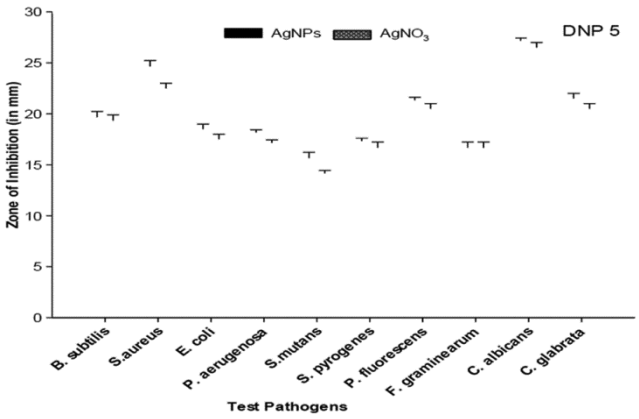

Fig. 5 — Inhibition zone (diameter in mm) of AgNPs synthesized by DNP 5

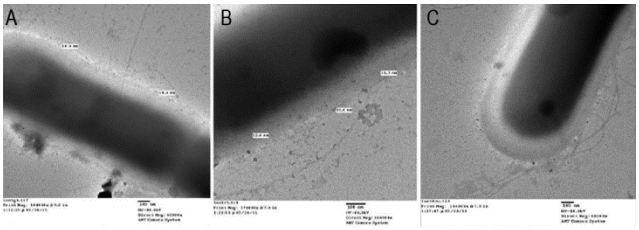

Fig. 6 — TEM analysis of interaction of AgNPs with *P. Aerugenosa*

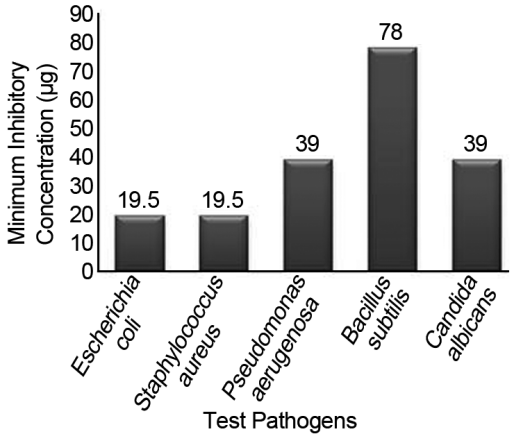

Fig. 7 — Minimum inhibitory concentration (MIC) of biologically synthesized AgNPs against different test pathogens

confirming that the antimicrobial activity is directly proportional to silver nanoparticles concentration.

The synergistic effect of AgNPs with norfloxacin and kanamycin was observed against all selected pathogens except *S. aureus* shown in Table 1. The highest effect was against *P. aerugenosa* for both the antibiotics. *P. aerugenosa* was not sensitive to kanamycin but addition of AgNPs rendered them sensitive. The increase in antimicrobial activity of rifampicin and erythromycin after AgNPs addition was against all pathogens. However, in case of amoxyclav synergistic effect was only against *S. aureus* and *P. aerugenosa*.

The antimicrobial effect of antibiotic combined with AgNPs was statistically different from antibiotic alone. The measured synergistic effect of AgNPs was significant with all the classes of antibiotics.

Discussion

Green synthesis process is rapid, easy to perform, economic and also avoids the use of toxic chemicals and thus has overcome the limitations associated with physical/chemical synthesis methods[19,20]. Biological synthesis methods being ecofriendly are seeking more attention than other methods. Different biological materials like plants, bacteria and fungi have been exploited for the synthesis of nanoparticles[10,11,17,19-24]. Various studies have already exploited different bacterial strains for the synthesis of stable silver nanoparticles (AgNPs)[17,19,22,24].

In the present study, the selected bacterial strain DNP5 identified as *Sphingobacterium multivorum* showed ability to synthesize AgNPs by extracellular mechanisms. Extracellular synthesis method is preferred over intracellular synthesis due to easy scale up and purification which leads to reduced production cost. The screening of microbial isolate for silver nanoparticle synthesis is generally carried out on the basis of colour change[17]. The change in colour was due to the surface plasmon resonance (SPR) of silver nanoparticles formed in the medium[12,24]. The metal type, size and morphology of nanoparticles and

Table 1 — Synergistic effect of silver nanoparticles with antibiotics

Test pathogens	Nx	Nx^+	Rif	Rif^+	E	E^+	K	K^+	Amx	Amx^+
Escherichia coli	27	29	32	33	21	23	24	25	40	40
Staphylococcus aureus	30	30	15	17	25	27	21	21	10	13
Pseudomonas aerugenosa	20	23	13	15	11	13	0	14	0	11
Bacillus subtilis	33	36	21	23	28	30	23	24	30	30
Candida tropicalis	31	33	-	10	12	14	21	23	-	-

dielectric properties of the medium may affect SPR of silver nanoparticles[15,24-27].

Biosynthesized AgNPs were further characterized by UV–Vis spectroscopy, which measures the absorption spectra of AgNPs. The presence of characteristic peak at 418 nm confirmed the synthesis of AgNPs. The UV-Vis absorption peak around 420 nm is due to surface plasmon resonance (SPR) of AgNPs[28]. A single peak represents the spherical shape nanoparticles and two or more peak belongs to the anisotropic molecules[29]. The XRD spectrum of synthesized nanoparticles was in agreement with diffraction standard JCPDS 04-0783, which confirms the presence of elemental silver.

TEM images of biosynthesized AgNPs confirmed the spherical shape and monodispersity of nanoparticles. On the basis of TEM analysis, nanoparticles are in the range of 17-30 nm and average size of synthesized AgNPs is 22 nm.

The dynamic light scattering (DLS) calculate hydrodynamic size of the AgNPs and includes the size of stabilizers absorbed. Therefore, the size measured by DLS is larger than measured by other techniques. Stable AgNPs bear a minimum of ± 30 mV and the polydispersity index (PDI) below 0.3[30]. This inferred the stability of biologically synthesized AgNPs. The result of present study was consistent with the results of already reported studies. The mechanism of synthesis of nanoparticles is not clear; some studies have reported the role of nitrate reductase in reduction of silver nitrate to silver[31]. Different peaks observed in FTIR spectrum indicated the role of biological molecules viz. proteins or enzymes (present in bacterial supernatant) in the synthesis and stability of AgNPs. Stable absorption peak of synthesized AgNPs in UV-Visible spectrophotometric analysis confirmed the stability of nanoparticles. Antimicrobial activity of nanoparticles remained same even after incubating them at high temperature. Previous studies have already reported that PVP coated AgNPs are considered as the most stable nanoparticles in OECD recommended media (chloride present)[32].

Various studies have already documented the antimicrobial activity of AgNPs against micro-organisms[19,33-37]. In the present study, the biosynthesized AgNPs exhibited considerable antimicrobial activity against both bacterial strains and fungi strains. The precise mode of antimicrobial activity is not clear yet, but previous data reported that AgNPs due to small size can easily enter into bacterial cell, harm cell membrane and functions of cell, enhance the free radicals production and finally may leads to cell death[38]. The present study on the basis of TEM analysis also confirmed the attachment and penetration of AgNPs to bacterial membrane.

The Gram-negative bacteria possess external lipopolysaccharides and an inner thin peptidoglycan layer[39]. It is also proposed that the negative charges on lipopolysaccharides may attract positively charged AgNPs[40]. However, in the present study, synthesized nanoparticles are negatively charged. These AgNPs may inhibit the Gram-negative bacteria by metal reduction[41-43]. Different studies have reported different MIC values for the same pathogens[44-47]. Various factors, such as size and shape of nanoparticles, strains of pathogens, pathogen source and stabilization method can affect MIC value[48]. The antimicrobial effect of AgNPs is inversely proportional to their size. Nanoparticles, due to large surface to volume ratio, can easily make contact with pathogens that impart them high biocidal activity. Biosynthesized nanoparticles exhibited synergistic antimicrobial effect in combination with different classes of antibiotic. Various studies have supported that the addition of AgNPs can enhance the antimicrobial effect of antibiotics[49-51]. Different Level of activity increment is reported with different classes of antibioitics[52]. Combination of nanoparticles and antibiotic may decrease the required concentration of antibiotic and may help to fight against resistant microbial infections[53].

Conclusion

In the present study, we developed a rapid, economic and ecofriendly method for the synthesis of stable silver nanoparticles (AgNPs). Size and crystal nature of synthesized particles was in nano range as confirmed by TEM and XRD analysis, respectively. Synthesized AgNPs exhibited broad spectrum antimicrobial activity against different test pathogens and showed synergistic effect with selected classes of antibiotics. Therefore, biosynthesized nanoparticles possess great application potential in the medical field and can be explored to fight against alarming issue of antimicrobial resistance. However, a further mechanistic insight into the antimicrobial action of AgNPs and *in vivo* studies related to toxicity of biofabricated AgNPs need to be conducted before commercial applications.

Conflict of interest

Authors declare no competing interests.

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