

Antimicrobial activity of *Bacillus subtilis* silver nanoparticles

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1. ABSTRACT

We tested the hypothesis that silver nanoparticles (BsAgNPs) made by *Bacillus subtilis* subsp. *inaquosorum* exhibit antibacterial and antifungal activities. The bacteria formed nanoparticles in UV-visible spectra with the peak around 430 nanometer detected by surface plasmon resonance and LambdaMax. The FTIR spectra, SEM and XRD revealed the involvement of interaction of biological moieties in the formation of crystalline cuboidal nanoparticles that were had an

average size of 10-20 nm. The particles exerted dose dependent antibacterial and antifungal activities and high dose equivalent to standard antibiotics. Minimum inhibitory concentration, minimum bactericidal and fungicidal concentrations were in the range from 3.1-25 microgram/ml and 6.3-50 microgram/ml, respectively. BsAgNPs significantly inhibited the growth of multiple strains including multidrug resistant (MDR) bacteria. Moreover, the combination of BsAgNPs and antibiotics showed synergistic inhibition of MDR strains. These data show that BsAgNPs have a great potential in the

treatment of MDR bacteria without or with standard antibiotics.

2. INTRODUCTION

The industrial demand and use of silver (Ag) has steadily increased over the years. Each year, 3125 tons of Ag is used in the production of medicine and 2800 tons is used in food hygiene and water purification (1). Ag has been used by the Romans and Greeks as an antimicrobial agent and disinfectant. Ag has potent antimicrobial effects against bacteria, fungi and viruses (2). These antimicrobial effects can be further increased by creation of nanometer size particles that are less toxic than Ag alone (3). AgNPs are synthesized by numerous physical (irradiation and laser ablation) (4,5) and chemical (aqueous-solution, nonaqueous chemical, electrochemical and photo-induced or photocatalytic reduction) (6) methods, which are expensive or use toxic substances that limits their yield, energy-intensive, difficult to scale up and 'not so favored' methods of synthesis. Employing the biological methods or green synthesis of AgNPs using microbes or plants are an alternate and feasible method for obtaining cheaper, clean, non-toxic, biocompatible, ecofriendly, size-controlled and large-scale synthesis of nanoparticles (7).

Numerous bacteria (8), fungi (9) and plant sources (10) have been utilized in the biological synthesis of AgNPs. Bacteria are able to absorb, accumulate and reduce the metal ions and thus known for the synthesis of nanoparticles (11). Various experiments involving bacteria such as *Pseudomonas proteolytica*, *Pseudomonas meridiana*, *Arthrobacter kerguelensis*, *Bacillus indicus*, etc., were proven to form the nanoparticles effectively (12). Recent studies indicated that prokaryotic bacteria have been most extensively used for the synthesis of metallic nanoparticles (13). The production of AgNPs by *B. subtilis* (14), *B. stearothermophilus* (15) and *B. licheniformis* (16) were also reported. Velmurugan *et al.*, (17) showed the bactericidal action of AgNPs, which were synthesized using *B. subtilis* against gram-positive (*Staphylococcus aureus*) and gram-negative (*Pseudomonas fluorescens*) bacteria.

Globally, the antibacterial resistance becomes a major issue as few bacteria showed resistance even to third generation antibiotics due to their overuse, failure to comply with their treatment regimen and high capability of bacteria to mutate are termed as multidrug resistant (MDR) strains (18). MDR strains causes nosocomial infections that are very difficult to treat and basis for major risk to the common people (19). So the best alternative is the usage of non-conventional therapy to which bacteria are implausibly to develop resistance. AgNPs are considered as alternative antimicrobial agents to antibiotics particularly for the treatment of disease caused by MDR strains of bacteria (20).

In the present experiment, we have made an effort to characterize the AgNPs, which were synthesized using *Bacillus subtilis* subsp. *inaquosorum* (BsAgNPs) isolated from the soil samples of Neyveli mine and investigated its antimicrobial activity against gram positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus* and *Streptococcus mutans*), gram negative bacteria (*Klebsiella Pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*) and fungi (*Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger*, *Candida tropicalis* and *Candida parasilopsis*) to address the broad anti microbial activity. However, beyond this the use of BsAgNPs against multidrug- resistant (MDR) strains of bacteria has not yet been extensively studied. Hence the aim of the current work was to analyse the antimicrobial effect of BsAgNPs against MDR bacterial strains and their synergistic effect with commonly used antibiotics.

3. MATERIALS AND METHODS

3.1. Chemicals

AgNO₃ and all other chemicals used in this investigation were procured from Sigma Aldrich, USA. Freshly prepared double distilled water was used throughout the experimental work.

3.2. Antibiotics

Tetracycline, Fluconazole, Vancomycin, Chloramphenicol, Gentamicin and Ciprofloxacin were purchased from Sigma Chemical Co., ST. Louis, Missouri, USA.

3.3. Microorganisms

Gram positive bacteria such as *S. aureus* (MTCC 39315), *S. pyogenes* (MTCC 1924), *B. cereus* (MTCC 441) and *S. mutans* (MTCC 497), Gram negative bacteria like *K. Pneumoniae* (MTCC 3384), *P. aeruginosa* (MTCC 424), *E. coli* (MTCC 343) and *S. typhi* (MTCC 430) and fungi including *C. albicans* (MTCC 227), *C. neoformans* (MTCC 1353), *A. Niger* (MTCC 1344), *C. tropicalis* (MTCC 1369) and *C. parasilopsis* (MTCC 4448) were collected from the MTCC, Chandigarh. Four clinical MDR bacteria (*E. coli*, *K. Pneumoniae*, *P. aeruginosa* and *S. aureus*) were also collected from the Raja Muthiah Medical College and Research center. The collected isolates were identified using conventional microbiological techniques.

3.4. Biosynthesis of AgNPs

3.4.1. Isolation of the bacteria

Soil samples were collected from Neyveli Lignite Corporation Limited Neyveli, Cuddalore Tamil

Nadu, India. They were serially diluted up to 10^{-5} using sterile distilled water. Then, they were poured into the petri plates and added with sterilized modified Nutrient Agar. The plates were then incubated at 37°C for 48 h on an orbital shaker at 220 rpm. After incubation the cultures were used for nitrate reductase assay (Nitrite/ Nitrate Assay Kit, colorimetric), and cell-free extract was separated by centrifugation at 9,060 g for 10 min and assessed for the synthesis of AgNPs. The potent nitrate reducing isolate was subject to reduction of Ag^{+} ion (AgNO_3). About 1 mM of Ag^{+} ion (40 ml) was mixed with the obtained cell-free extract (10 ml).

3.4.2. Characterization of the isolates

The morphological and physiological characterization of the strictly aerobic isolate was performed according to the methods described in Bergeys manual of determinative bacteriology (21). Genomic DNA of isolate was extracted by the methods described in earlier reports (22). In brief, the 16S ribosomal RNA gene was amplified by using the PCR method with Taq DNA polymerase and with suitable primers. The PCR product obtained was sequenced by an automated sequencer ABI PRISM (Model 3700). The sequences were compared using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

3.4.3. Extracellular biosynthesis of BsAgNPs

For the biosynthesis of BsAgNps, the selected bacterial isolate NMS-1 was inoculated in to 250 ml of conical flask containing 100 ml sterile nutrient broth. The cultured flask was incubated in a rotating shaker at 200 rpm for 48 h at room temperature. The bacterial culture was centrifuged at 12000 rpm for 10 minutes. The supernatant was used for studying extra-cellular production of BsAgNPs by mixing it with filter-sterilized AgNO_3 solution at 1 mM final concentration. The reaction mixture was incubated on rotating shaker (200 rpm) at room temperature for a period of 72 h in bright condition (23).

3.5. Characterization of silver nanoparticles

In this investigation, changes in the colorless solution to brown color with yellow shade showed the bioreduction of Ag^{+} ions. BsAgNPs biosynthesis was confirmed by the firm color development from 20 min of incubation and subsequent enhancement in the intensity of the color till 6 h.

These BsAgNPs were further analysed by using UV –Visible spectrophotometer (SHIMAGU-UV 1800nm) in the wavelengths ranging from 200-800nm to check the maximum absorbance (λ_{max}) (24).

3.5.1. Fourier Transform Infrared (FTIR)

Dried powder of BsAgNPs was scanned in the FTIR range of $400\text{--}4000\text{ cm}^{-1}$ to analyze the interaction between protein and AgNPs.

3.5.2. Scanning Electron Microscope (SEM)

The sample was kept on copper grid stained with uranyl acetate and lead citrate and then observed under SEM (JEOL-JSM-5610LV) to determine the surface morphology of the BsAgNPs.

3.5.3. X-Ray diffraction

The X-ray diffraction (XRD) measurement of BsAgNPs was carried out using $\text{Cu-K}\alpha$ radiation source in a wide range of Bragg angles 2θ at a scanning rate of $0.388^{\circ}/\text{min}$ in powder diffractometer (Phillips X'Pert Pro X-Ray diffractometer), under 50 kilo Volt and 30 milli Ampere current.

3.6. Antimicrobial activity of silver nanoparticles

The BsAgNPs were centrifuged ($21,000\times g$; 30 min) and the Ag found in the supernatant was discarded. Ultra-sonicator was used to dissolve the pellet and which was lyophilized to get the AgNPs powder and used after suspended in deionized water at various concentrations.

The antimicrobial activities of BsAgNPs were demonstrated by disc diffusion method. After the solidification of culture medium, various pathogenic gram positive bacteria such as *S. aureus*, *S. pyogens*, *B. cereus* and *S. mutans*, gram negative bacteria like *K. pneumoniae*, *P. aeruginosa*, *E. coli*, *S. typhi* and fungi such as *C. albicans*, *C. neoformans*, *A. niger*, *C. tropicalis* and *C. parasilopsis* were swabbed on these plates. The sterile disc was dipped in BsAgNPs solution (5, 10, 15 and $20\mu\text{g}/\text{ml}$ concentration) and placed on the agar plate and incubated at 37°C for 24 h along with standard antibiotics (Tetracycline and Fluconazole). The zone of inhibition was measured in mm (25).

3.7. Minimal Inhibitory Concentration (MIC)

Bacterial strains were grown overnight on MHA plates at 37°C before being used. The antimicrobial activity of BsAgNPs was examined using the standard broth dilution method (CLSI M07-A8). The MIC was determined in nutrient broth Hi-Media (Mumbai, India) using serial two-fold dilutions of Ag-NPs in concentrations ranging from 200 to $1.5625\mu\text{g}/\text{ml}$, initial bacterial inoculums of $2\times 10^8\text{ CFU}/\text{ml}$ and the time and temperature of incubation being 24 h at 37°C , respectively. The MIC is the lowest concentration of

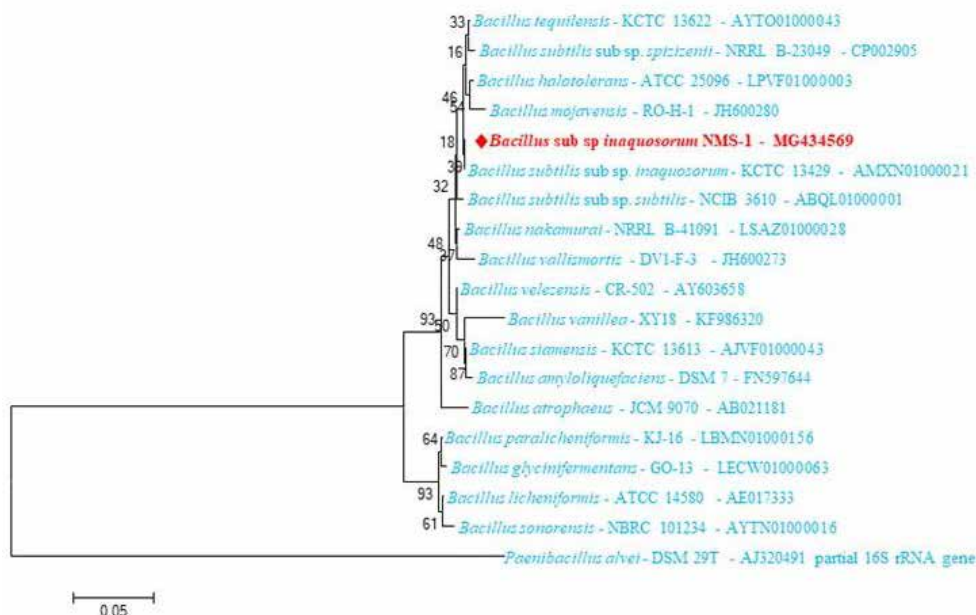


Figure 1. The phylogram showing the position of *Bacillus subtilis* sub sp. *inaquosorum* MG434569 with other *Bacillus* based on 16S rRNA partial gene sequence based on phylogenetic tree analysis.

antimicrobial agents that completely visually inhibits 99% growth of the microorganisms. The MIC measurement was done in triplicate to confirm the value of MIC for each tested bacteria (26).

3.8. Minimal Bactericidal Concentration (MBC)

After MIC determination of the BsAgNPs tested, aliquots of 50 µl from all the tubes in which no visible bacterial growth was observed were seeded in MHA plates not supplemented with BsAgNPs and incubated for 24 hrs at 37° C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills 100% of the initial bacterial population (26).

3.9. Minimal Fungicidal concentration (MFC)

The MFC of the AgNPs were determined by plating loopful of culture onto SDA plates and incubated under aseptic condition for 72 h at 28° C. The lowest concentration of the BsAgNPs that showed no visible growth on solid media was recorded as the MFC.

3.10. Antibacterial activity of AgNPs against Multiple Drug Resistant Pathogens

The antibacterial efficacy was assayed by the standard Kirby–Bauer disc diffusion method and antibacterial activity of synthesized BsAgNPs were observed against multi drug resistant clinical isolates such as *E. coli*, *K. Pneumoniae*, *P. aeruginosa* and *S. aureus*. *E. coli* strain were isolated from urine sample that were resistant to antibiotics such as Cefepime,

Ciprofloxacin, Aztreonam, Ceftazidme, Ampicillin, Gentamicin, Meropenam, Cefoxitin, Amikacin, Amoxycylan, Piperacillin–Tazobactam and sensitive to Chloramphenicol. *K. Pneumoniae* strain were isolated from pleural fluid that were resistant to Imipenem, Cefepime, Ciprofloxacin, Cefetazidme, Aztreonam, Ceftazidme, Ampicillin, Gentamicin, Meropenam, Cefoxitin, Amikacin, Amoxycylan, Piperacillin – Tazobactam and sensitive to Chloramphenicol. *P. aeruginosa* strain was isolated from pus sample that were resistant to Cefepime and sensitive to Ciprofloxacin. *S. aureus* was isolated from blood and that were resistant to Cefepime, Ciprofloxacin, Linezolid, Clindamycin and Gentamicin. The bacterial suspension was swabbed on the Muller Hinton Agar (MHA) plates using sterile cotton swab. The sterile disc at 6 mm dimension was impregnated with BsAgNPs (20 µg/ml) and standard antibiotics such as vancomycin, chloramphenicol, gentamicin and ciprofloxacin (5 µg/ml). The disc with standard antibiotics (5 µg/disc) located on the plates was maintained as control. These discs were gently pressed in Muller Hinton Agar plates and incubated in inverted position for 24 hrs at 37 °C. However, the susceptibility of the test organisms was determined by measuring the diameter of the zone of inhibition using Hi-Media zone scale.

4. RESULTS

4.1. Screening of BsAgNPs

16S rRNA partial gene sequencing analysis and phylogenetic tree construction (Figure 1) showed maximum identity with *Bacillus subtilis* (NMS-1).



Figure 2. Visual observation of the biosynthesis of AgNPs by *Bacillus* sp. MG434569 M1 isolate selected for the study after 72 h incubation: a) Culture supernatant (no colour change), b) AgNO₃ solution (no colour change) and c) Silver nanoparticles (bacterial biomass with AgNO₃ solution - colour change from pale yellow to brown).

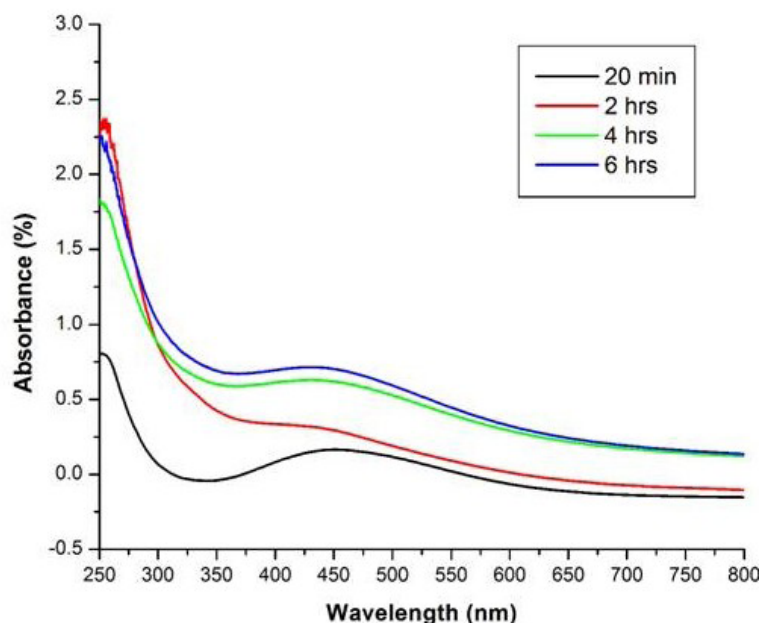


Figure 3. The UV-Vis absorption spectrum of AgNPs synthesized by supernatant of *Bacillus subtilis* subsp. inaquosorum NMS1 isolate. The absorption spectrum of AgNPs exhibited a strong broad peak at 430 nm and observation of such a band is assigned to surface Plasmon resonance of the particles.

Screening of BsAgNPs was done primarily by observing the color change of the culture supernatant from pale yellow to dark brown, after mixing it with silver nitrate. In the present study, after AgNO₃ addition to the culture, the colorless mixture changed into dark brown. No color change in the control, which contained only the medium and AgNO₃ solution, indicated the absence of BsAgNPs (Figure 2). As to authenticate AgNPs formation, the medium were observed by a UV-

Vis absorption spectrum (250–800 nm). It showed an absorption peak at 250 nm and 430 nm, which are the characteristics of un-reacted Ag and formed AgNPs respectively (Figure 3).

4.2. FTIR

The FTIR spectra of BsAgNPs confirmed the presence of NH stretching (3,287 cm⁻¹), C=O group

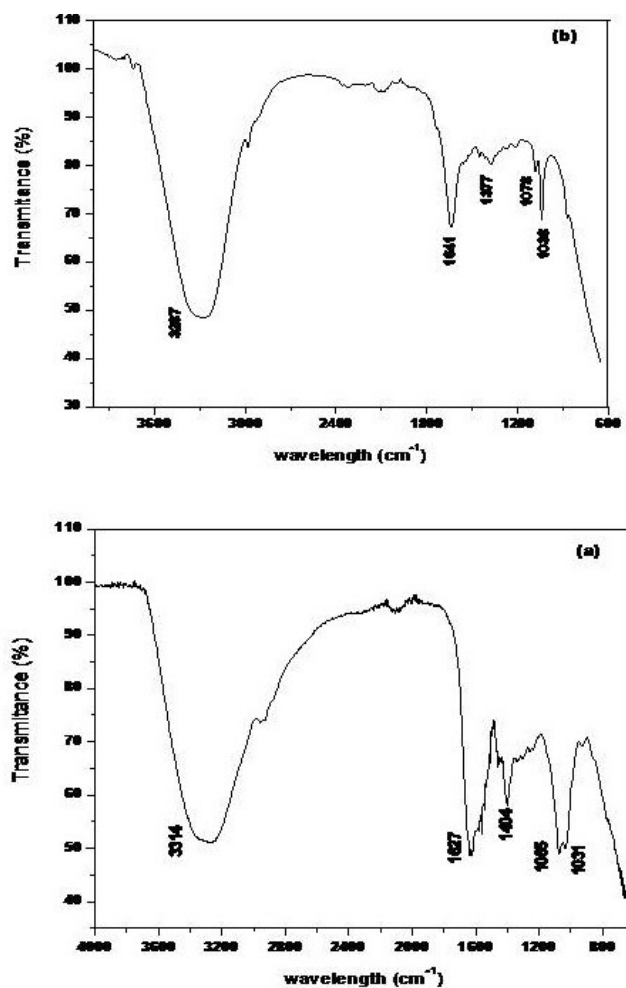


Figure 4. Fourier Transform Infra-Red spectroscopy of (a) Culture supernatant alone; (b) BsNPs. Absorption peaks located at 3287, 1641, 1377, 1078, 1038 cm⁻¹ were observed upon culture supernatant, whereas absorption peaks located at 3314, 1627, 1404, 1065 and 1031, cm⁻¹ were observed for the BsAgNPs.

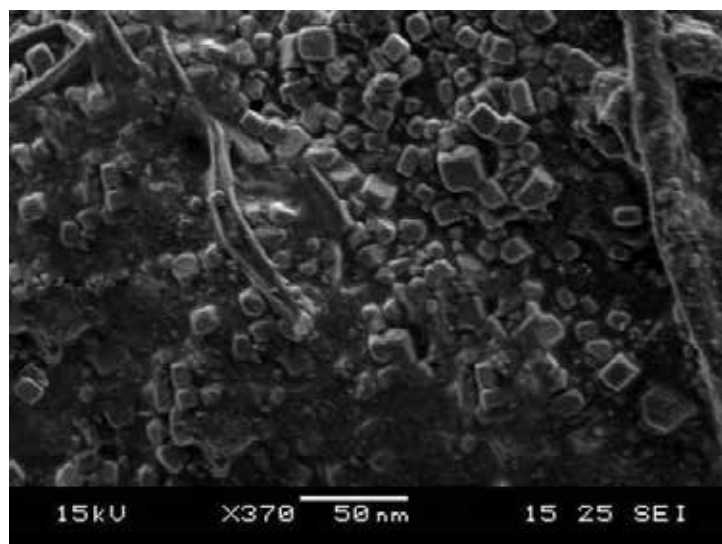


Figure 5. TEM image indicating the shape, size and form of AgNPs synthesized from *Bacillus subtilis* subsp. inaquosorum strain

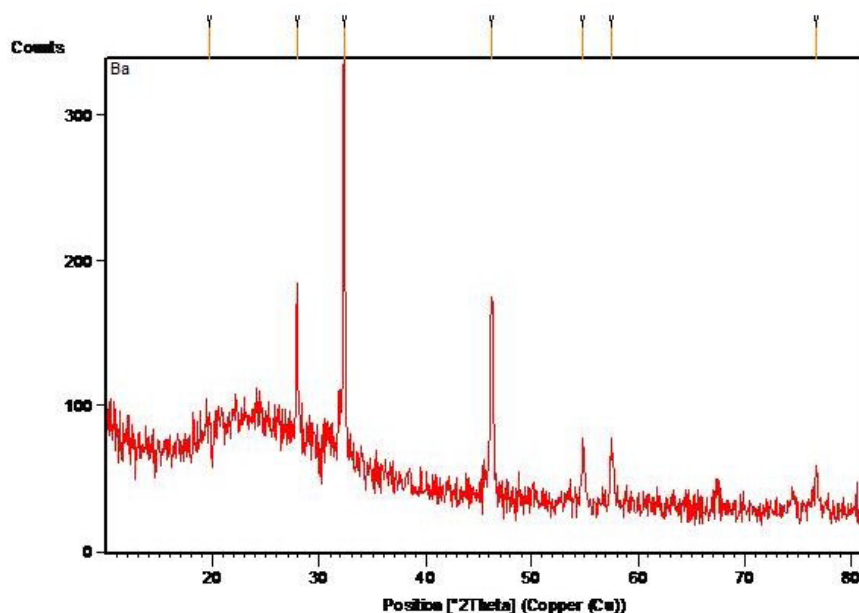


Figure 6. XRD patterns of AgNPs synthesized from *Bacillus subtilis* subsp. inaquosorum strain.

(1,641 cm^{-1}), strong absorption peaks (1377 cm^{-1}), C-O stretching (1,078 cm^{-1}) and C-N stretching vibrations of the aliphatic amines (1038.63 cm^{-1}) respectively. These observations indicated the presence and binding of proteins with AgNPs which could lead to their possible stabilization (Figure 4).

4.3. Scanning Electron Microscopy studies

The SEM micrograph depicts the size of BsAgNPs in the range of 10-20 nm. The nanoparticles were not in aggregates indicating the stabilization of the nanoparticles (Figure 5).

4.4. XRD analysis

XRD analysis confirmed the crystalline character of AgNPs, that depicts peaks at 2θ values of four distinct diffraction peaks at 38.2.8°, 44.3.8°, 64.5.4° and 77.6.4° corresponding to XRD planes of (111), (200), (220), (311) Bragg's reflection based on the fcc structure of AgNPs (Figure 6).

4.5. Biological activities of BsAgNPs

The increasing concentration of AgNPs (5-20 microg/ml) displayed dose dependent increase in antibacterial and antifungal activity, as indicated by diameter of inhibition zones of 8–20 mm. The standard antibiotic tetracycline, and antifungal Fluconazole (5 microg/ml) disk showed enhanced inhibitory activity against all the studied pathogens. However, BsAgNPs showed antibacterial effect closer to tetracycline against *S. aureus* and *E. coli*.

4.6. MIC and MBC of BsAgNPs

Bacteriostatic effect of BsAgNPs towards *S. mutans* were recorded at very low concentration (3.1. $\mu\text{g/ml}$), whereas for *S. pyogenes* and *K. Pneumoniae* at high concentration (6.3. $\mu\text{g/ml}$), however for *S. aureus*, *S. typhi* and *E. coli* at higher concentration (12.5. $\mu\text{g/ml}$) and for *P. aeruginosa* at highest concentration (25 $\mu\text{g/ml}$). Bactericidal activity of BsAgNPs for abovementioned organisms were found at almost double the bacteriostatic concentration (from 6.3. to 50 $\mu\text{g/ml}$).

MIC test showed the highest fungistatic effect of BsAgNPs towards *A. niger* and *C. parasilopsis* (6.3. $\mu\text{g/ml}$), followed by *C. albicans* and *C. tropicalis* (12.5. $\mu\text{g/ml}$) and *C. neoformans* (25 $\mu\text{g/ml}$). Fungicidal activity of BsAgNPs for abovesaid organisms were found at almost double the fungistatic concentration (from 6.3. to 50 $\mu\text{g/ml}$).

4.7. Synergistic effect of BsAgNPs with standard antibiotics against MDR pathogens

Antimicrobial potential of BsAgNPs were evaluated against MDR pathogens, *S. aureus*, *K. Pneumoniae*, *E. coli* and *P. aeruginosa* and in combination of different antibiotics such as vancomycin, chloramphenicol, Gentamicin and ciprofloxacin. Among the different antibiotics, Gentamicin showed antibacterial activity against all the studied bacteria. Chloramphenicol yielded maximum zone of inhibition against *K. Pneumoniae* and *E. coli*. The *S. aureus* and *E. coli* had acquired resistance against ciprofloxacin. *E. coli* had showed resistance against vancomycin,

Table 1. Comparison of antibacterial and antifungal activities of AgNPs with standard antibiotics such as tetracycline and fluconazole

Pathogen	Zone of inhibition (mm) Concentrations (µg/ml)				Standard Tetracycline (5 µg/disc)
	5	10	15	20	
Gram Positive bacteria					
<i>Staphylococcus aureus</i>	10	12	14	21	22
<i>Streptococcus pyogenes</i>	9	10	11	17	22
<i>Bacillus cereus</i>	8	10	11	15	23
<i>Streptococcus mutans</i>	8	10	12	19	24
Gram Negative bacteria					
<i>Klebsiella Pneumoniae</i>	10	12	13	16	23
<i>Pseudomonas aeruginosa</i>	7	9	11	18	24
<i>Salmonella typhi</i>	10	11	13	17	21
<i>E.coli</i>	10	13	15	21	22
Pathogen	Zone of inhibition (mm) Concentrations (µg/ml)				Standard Fluconazole (5 µg/disc)
Fungi					
<i>Candida albicans</i>	9	10	12	16	21
<i>Cryptococcus neoformans</i>	9	10	11	14	20
<i>Aspergillus niger</i>	7	9	10	12	20
<i>Candida tropicalis</i>	11	12	14	16	22
<i>Candida parasilopsis</i>	10	13	15	17	23

Table 2. MIC and MBC and MFC of AgNPs synthesized by NMS-1 (*Bacillus subtilis subsp. inaquosorum*)

Pathogen	MIC µg/ml	MBC µg/ml
Bacteria		
<i>Staphylococcus aureus</i>	12.5	25
<i>Streptococcus pyogenes</i>	6.3	12.5
<i>Bacillus cereus</i>	25	50
<i>Streptococcus mutans</i>	3.1	6.3
<i>Klebsiella pneumoniae</i>	6.3	12.5
<i>Pseudomonas aeruginosa</i>	25	50
<i>Salmonella typhi</i>	12.5	25
<i>E. coli</i>	12.5	25
	MIC µg/ml	MFC µg/ml
Fungi		
<i>Candida albicans</i>	12.5	25
<i>Cryptococcus neoformans</i>	25	50
<i>Aspergillus niger</i>	6.3	12.5
<i>Candida tropicalis</i>	12.5	25
<i>Candida parasilopsis</i>	6.3	12.5

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, MFC: Minimum Fungicidal Concentration

whereas *Pseudomonas aeruginosa* had developed resistance against chloramphenicol. But when BsAgNPs were added simultaneously with antibiotics, a clear zone of inhibition was observed.

5. DISCUSSION

In the total of 25 isolates, *B. subtilis* isolate NMS 1 was identified as a potent nitrate reducer and was used for further study. It was named according to our convenience for further processing of isolates from NMS 1–25. The formation of AgNPs was indicated

by the changing of the colourless solution into brown color, which occurs due to excitation of surface plasmon vibration (27). *Bacillus* sp. are reported to secrete the NADH, a cofactor for NADH-dependent enzymes particularly nitrate reductase that are accountable for the bioreduction of Ag⁺ to Ag⁰ and subsequently forms AgNPs (28). Reports indicated that 2 to 100 nm sized metal nanoparticles exhibited a peak near to 430 nm due to the surface plasmon resonance (27). The UV-Visible spectra of the synthesized AgNPs were noted at various time intervals (20 min, 2 h, 4 h and 6 h) at room temperature. The absorbance peaks indexed as

Table 3. Synergistic action of silver nanoparticles synthesized by the isolate NMS-1 (*Bacillus subtilis* subsp. *inaquosorum*) against multi drug resistant Pathogens

Multi Drug Resistant Pathogens	Zone of inhibition (mm)							
	Antibiotics (5 µg/disc)				Antibiotics (5µg) +AgNPs (20µg)			
	Vancomycin	Chloramphenical	Gentamicin	Ciprofloxacin	Vancomycin	Chloramphenical	Gentamicin	Ciprofloxacin
<i>Staphylococcus aureus</i>	10	19	28	-	13	22	33	12
<i>Klebsiella pneumoniae</i>	16	30	18	21	19	33	22	25
<i>E.coli</i>	-	31	10	-	10	34	13	10
<i>Pseudomonas aeruginosa</i>	10	-	12	18	12	10	14	23

different colors indicated the reduction of AgNO_3 . The reaction was completed within 6 h as the intensity of the color did not exaggerate after 6h in our study. The peak surface plasmon resonance of AgNPs was found at 430 nm in UV-Visible spectroscopy throughout 6h reaction period indicating the distribution of nanoparticles in the aqueous solution with no proof for aggregation even after the end of the reaction.

The topographical imaging of nanoparticles in SEM is typically used for investigating the size, shape, impurities and stabilization of particles. The SEM studies indicated that the particles are nearly cubic identical in shape, mono-dispersive and crystalline nature. Generally green synthesis results in the formation of various sized and shaped nanoparticles (29). The size of the BsAgNPs was calculated by the scale represented in each micrograph and the calculated range was found within 10 - 20 nm in this study. The presence of the organic part in the AgNPs was confirmed by FT-IR as the amino groups of the protein forms bond with BsAgNPs (30). The stretching vibration of carbonyl amino groups appeared due to amide linkages with a protein, which is commonly responsible for the reduction process. The presence of carbonyl and hydroxyl groups on the AgNPs indicated the mechanisms associated with Ag reduction. The FT-IR results indicated that the secondary structures of proteins were not affected as a consequence of reaction with Ag^+ ions or binding with AgNPs.

The XRD-spectrum illustrated four intense peaks, which is corroborated with the Bragg's reflection of AgNPs (31). It was also in concurrence with the standard values reported for face centered cubic (fcc) published by the Joint Committee on Powder Diffraction Standards (File No. 04-0783). In the present study, XRD analysis illustrated the crystalline nature of synthesized BsAgNps.

The antibacterial and antifungal activities of BsAgNPs were found to be concentration dependent, which was enhanced with the increased AgNPs concentration. However as compared to standard antibiotics, zone of inhibition of higher concentration of AgNPs (20 µg/ml) were almost equal to certain pathogens including *S. aureus*, *E. coli*, *S. typhi* and *C. albicans*. AgNPs exhibited higher bactericidal activity among both the Gram positive and Gram negative bacterial species indicating its broad spectrum antibacterial action.

The MIC values of BsAgNPs were found to be within 3.1. to 25µg/ml, whereas MBC values were found within 6.3. to 50 µg/ml. The inhibitory effect of AgNps on microbes is probably due to (i) Ag induced free radicals formation and breakdown of membrane lipids (32), (ii) interaction of Ag with phosphorus groups of DNA and thereby inhibiting replication or transcription processes (33) and (iii) binding of Ag to sulfur-contain-

ing amino acids, thereby blocking respiration in association with oxygen and sulfhydryl (–S–H) groups in the cell membrane and or cell wall (34). AgNPs showed efficient antifungal property due to their large surface area, which offer good contact with microorganisms and leads to the breakdown of membrane lipid bilayer, inducing pores and reducing the electrical potential of the membrane (35). The antibacterial and antifungal activities of standard drugs were increased in the presence of AgNPs against test strains.

The mechanism of antimicrobial action of AgNPs were reported due to the (i) induction of damage to bacterial cell membranes resulting in enhanced permeability due to structural changes (36), (ii) presence of distinctive electrical, optical and thermal properties with more surface area to volume ratio leading to improved interaction with bacterial surfaces (37), (iii) sustained release and penetration of cationic silver (38) followed by elevated production of free radicals (28, 39), (iv) binding to the cellular membranes, nucleic acids and proteins causing structural changes (38) and (v) deactivation of several key enzymes by interacting with thiol groups (40) and are implicated in the synthesis of reactive oxygen species (41). This synergistic effect may be raised by the interaction between nanosilver and active groups like hydroxyl and amido groups of the antibiotic molecules by chelation (42). Previous studies by Hwang *et al.*, (43) indicated that a synergistic action of AgNPs with antibiotics such as chloramphenicol, ampicillin and kanamycin against gram-positive and -negative pathogenic bacteria, which is inconsistent with our study.

The *S. aureus* and *E. coli* had acquired resistance against ciprofloxacin, *E. coli* had showed resistance against vancomycin, whereas *P. aeruginosa* had developed resistance against chloramphenicol, but when BsAgNPS were added simultaneously with antibiotics, it yielded a clear zone of inhibition, which was due to the synergistic activity of BsAgNPs. With the antibiotics to which the pathogen was resistant, there was a synergistic increase in zone of inhibition when both antibiotics and silver nanoparticles were applied simultaneously.

Multidrug therapies in the form of combination or sequential is a probable way to nullify the evolution of multidrug resistance (44). MDR reversion to ineffective antibiotics by combination with AgNPs could be a novel strategy to battle infections caused by MDR pathogens. Biogenic silver nanoparticles are reported to have synergistic effects with various antibiotics that can oppose the MDR acquired by the pathogenic microbes (45). Smekalova *et al.* (46) demonstrated the synergistic effect of AgNPs with penicillin G, Gentamicin and colistin against MDR bacteria. The therapeutic response of AgNPs with ineffective antibiotics was increased probably due to the formation of complex

or conjugate, which enhance the antibiotics concentration at the site of bacterium–antibiotic interaction and improve the binding of antibiotics to bacteria (47). Moreover Naqvi *et al.*, (48) demonstrated that the combination of AgNPs with ciprofloxacin and imipenem were most effective in inhibiting bacteria because even if bacteria develops resistance to one of them, the other bactericidal agent would kill the bacteria. However, more studies are desirable to find out the exact mechanism of action to develop a novel antimicrobial drug against multidrug-resistant bacteria.

6. REFERENCES

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Abbreviations: AgNPs: Silver nanoparticles, BsAgNPs: *Bacillus subtilis* synthesized silver nanoparticles, FTIR: Fourier Transform Infrared, MBC: Minimal Bactericidal Concentration, MFC: Minimal Fungicidal concentration, MIC: Minimal Inhibitory Concentration, SEM: Scanning Electron Microscope

Key Words: *Bacillus subtilis*, subsp. *inaquosorum*, Silver nanoparticles, Biosynthesis, Antibacterial activity, Antifungal activity, Multidrug resistant

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