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Biogenic Synthesis, Characterization and Antibacterial Properties of Silver Nanoparticles against Human Pathogens

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Abstract: Biogenic synthesis of silver nanoparticles (AgNPs) is more eco-friendly and cost-effective approach as compared to the conventional chemical synthesis. Biologically synthesized AgNPs have been proved as therapeutically effective and valuable compounds. In this study, the four bacterial strains *Escherichia coli* (MT448673), *Pseudomonas aeruginosa* (MN900691), *Bacillus subtilis* (MN900684) and *Bacillus licheniformis* (MN900686) were used for the biogenic synthesis of AgNPs. Agar well diffusion assay revealed to determine the antibacterial activity of all biogenically synthesized AGNPs showed that *P. aeruginosa* AgNPs possessed significantly high ($p < 0.05$) antibacterial potential against all tested isolates. The one-way ANOVA test showed that that *P. aeruginosa* AgNPs showed significantly ($p < 0.05$) larger zones of inhibition (ZOI: 19 to 22 mm) compared to the positive control (rifampicin: 50 µg/mL) while no ZOI was observed against negative control (Dimethyl sulfoxide: DMSO). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) concentration against four test strains also showed that among all biogenically synthesized NPs, *P. aeruginosa* AgNPs showed effective MIC (3.3-3.6 µg/mL) and MBC (4.3-4.6 µg/mL). Hence, *P. aeruginosa* AGNPs were characterized using visual UV vis-spectroscopy, X-ray diffractometer (XRD), fourier transform infrared (FTIR) and scanning electron microscopy (SEM). The formation of peak around 430 nm indicated the formation of AgNPs while the FTIR confirmed the involvement of biological molecules in the formation of nanoparticles (NPs). SEM revealed that the NPs were of approximately 40 nm. Overall, this study suggested that the biogenically synthesized nanoparticles could be utilized as effective antimicrobial agents for effective disease control.

Key words: biogenic silver nanoparticles, tube dilution method, agar well diffusion method, antibacterial activity, MIC and MBC determination

1 Introduction

Nanotechnology is the emerging field of science with lots of benefits to humans, animals and nature. Out of many excellent findings of nanotechnology, the nanoparticles (NPs) are very much beneficial[1]. With the advancement in nanotechnology, humans have gained the ability to synthesize nanoparticles in the labs[2]. The particles having nanometers size are known as NPs. There are many kinds of metallic and non-metallic NPs synthesized using metals such as Au, Ag, Ce, Pt, Pd and Zn[3], but most important are silver nanoparticles (AgNPs). The AgNPs have extensively been studied for their activities particularly antimicrobial activi-

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ties[4, 5] and emerging as promising nano antibiotics now a days[6]. Because of their broad-spectrum activities, AgNPs grab great attention in the field of biomedical, medicine, agriculture, insect control and several other industries[7]. By using an ecofriendly approach, AgNPs applications have been increased in preparation of large number of products such as pests, electronic devices and in controlling microbe's growth and infection[8, 9]. Silver nanoparticles (AgNPs) are most commercialized among inorganic nanoparticles. They are nontoxic, safe inorganic antibacterial agent used for centuries and has great potential to kill disease causing microorganisms.

The use of silver as suspension and in nano-particulate form has a dramatic revival in nanotechnology. It has eminence antibacterial potency against human pathogens. The main task in nanoparticles synthesis is the control of their physical properties like uniform particle size, similar shape, chemical composition, morphology and crystal structure. The reduced effectiveness of drugs, antibiotics against the pathogenic bacteria, particularly against multi-drug resistance (MDR) bacteria could be the major threat to the life of the human being[1, 10]. AgNPs have very strong antibacterial activity against the human pathogens, because of their effectiveness against most of the microbial pathogens, microorganisms have been probed as potential bio-factories for metallic nanoparticles synthesis such as silver, copper, zinc and gold (biogenic synthesis)[11].

Moreover, the antibacterial activity of biogenic nanoparticles in combination with antibiotics signifies their importance in combating multi-drug resistant (MDR) pathogenic bacteria in planktonic[12] and biofilm mode (microorganisms embedded in elf produced polymeric matrix)[13]. It was also observed that drug-loaded silver nanoparticles (Ciprofloxacin + 10 mM) showed a stronger antibacterial potential than the synthesized silver nanoparticles and ciprofloxacin alone to restrict the development of *E. coli* and *E. aerogenes*[14]. Biogenically synthesized nanoparticles are easy to produce biocompatible, economic, environmental friendly and offer different catalytic abilities compared to chemically synthesized ones. They have anticancer and antioxidant properties. Furthermore, they are naturally stabilized, as the natural organic material (citrate, sodium dodecyl sulfate) of bacteria work as natural capping layers surrounding the biogenic nanoparticles, make these active, stable and reusable[1, 12, 15, 16]. These act as promising antimicrobial agent due to their long term stability and biocompatibility[17].

The application of biogenic AgNPs has been demonstrated since it arrests the growth and multiplication of many bacteria such as *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Citrobacter koseri*, *Salmonella typhii*, *P. aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, and *Candida albicans* by binding Ag/Ag^+ with the biomolecules present in the microbial cells. It has been suggested that biogenic AgNPs produce reactive oxygen species and free radicals which cause cell death through apoptosis and prevent their replication. Since AgNPs are smaller than the microorganisms, they diffuse into cell and rupture the cell wall which has been shown from SEM. It has also been observed that smaller nanoparticles are more effective than the bigger ones[1, 18].

In this study, microbial synthesis of AgNPs was investigated by using culture supernatant of the bacterial strains *E. coli* (MT448673), *P. aeruginosa* (MN900691), *B. subtilis* (MN900684) and *B. licheniformis* (MN900686), as a reducing agent. The biogenically synthesized AgNPs was further characterized for the antibacterial potency against four human pathogenic bacteria (both Gram negative and Gram positive) using agar well diffusion method. The *P. aeruginosa* AgNPs showed significant antibacterial potential, hence were further characterized by UV-vis spectroscopy, X ray diffraction (XRD), scanning electron microscopy (SEM) and fourier transform infrared spectroscopy (FTIR).

2 Materials and Methods

2.1 Test microorganisms

Four human pathogenic bacteria (both Gram negative and Gram positive) such as *E. coli* (MT448673), *P. aeruginosa* (MN900691), *B. subtilis* (MN900684) and *B. licheniformis* (MN900686) were obtained from Microbiology lab, Government College University, Lahore and used in the current study.

2.2 Chemicals and reagents

The chemicals, media, reagents and $AgNO_3$ used during experiments were highly pure and up to the analytical grade. The chemicals including silver nitrate (Merck, Germany), Luria bertani (LB) agar and LB broth were purchased from Sigma-Aldrich, USA.

2.3 Preparation of supernatant

Luria bertani (LB) broth was prepared by dissolving 5.0 g Sodium chloride, 5.0 g peptone; 3.0 g yeast extract in 500 mL distilled water, which after adjusting pH was made up to 1 liter. The prepared LB was sterilized by autoclaving at 121℃ for 15 minutes at 15 Ib and inoculated with 100 μL (OD: 1 ± 0.2) fresh cultures of *E. coli*, *P. aeruginosa*, *B. subtilis* and *B. licheniformis*, separately. The culture flasks were incubated for 24 h in incubator at 37℃ at a rotatory shaker (2g-force). Following incubation, the bacterial cultures were centrifuged at 10000 rpm for 10 min twice. The supernatants were saved for further study.

2.4 Biosynthesis of silver nanoparticles

For synthesis of AgNPs, each supernatant was mixed with 10 mM solution of $AgNO_3$, in 2:1 (supernatant: $AgNO_3$

solution) while two control flasks, each with $AgNO_3$ (10 mM) and bacterial culture separately were run in parallel. The prepared solutions were incubated for 72 h on rotatory shaker at 2g-force at 37℃. All the solutions were kept in dark to prevent photochemical reaction during the experiment. After three days, flasks having supernatants and $AgNO_3$ turned showed color change from yellow solution to brown confirming synthesis of AgNPs. No colour change was observed in control flask. The cultures were centrifuged at 3g-force for 10 minutes, the pellet was discarded and the supernatant was saved for further characterization.

2.5 Antibacterial activity of AgNPs

The antibacterial activity of AgNPs was analyzed through agar well diffusion method against the test pathogens i.e., *E. coli*, *P. aeruginosa*, *B. subtilis* and *B. licheniformis*[19−22]. The Muller Hinton agar plates were prepared and well of 6 mm diameter were made using sterile cork borer. The test pathogens culture was adjusted to 0.5 McFarland turbidity standard and was spread on the media plate uniformly. The 100 μL of AgNPs (1 mg/mL) was added into each well. DMSO, rifampicin (50 μg/mL) and $AgNO_3$ were used as controls. The plates were kept at room temperature for one hour. Afterwards, plates were incubated at 37℃ for 24 h. Following incubation period, the clear zones around the wells were taken as zone of inhibition (ZOI) and were recorded in millimeter (mm).

2.6 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of AgNPs were measured using tube dilution method following Liaqat *et al.*[23] with slight modifications. In brief, 3 mL of freshly prepared nutrient broth was added into the test tubes. About 10 μL of bacterial culture which (adjusted to 0.5 McFarland turbidity standard) was added to the tubes containing broth. The test tubes were supplied with various concentrations (3-12 μg/mL) of AgNPs and incubated at 37℃ for 24 h. Controls having $AgNO_3$ and LB medium were run in parallel. Following incubation, MIC was measured by considering the lowest possible concentration that inhibited the bacterial growth visually, while the MBC was determined by spreading the lowest MIC on media plates which killed 99.9% of bacteria.

2.7 Characterization of *P. aeruginosa* AgNPs

P. aeruginosa synthesized AgNPs showed excellent antibacterial potential hence subjected to the further characterization via UV-Visible spectroscopy, FTIR, SEM, and XRD in order to confirm the formation and specificity (size, shape and peak) of AgNPs.

2.7.1 UV-Visible spectroscopy

The *P. aeruginosa* AgNPs supernatant was qualitatively analyzed by UV-visible spectroscopy using AE-S70-1U UV-visible spectrophotometer and $AgNO_3$ solution was used as control. UV-vis spectrophotometer from 300 to 770 nm operated at a resolution of 1 nm was used as a function of wavelength for spectral analysis of AgNPs. Occurrence of peak between 400-470 nm showed formation of AgNPs and reduction of silver nitrate.

2.7.2 Fourier transform-infrared (FTIR) spectroscopy

The FTIR spectroscopy of *P. aeruginosa* AgNPs was performed in order to check the influence of bio-molecules which were responsible for the reduction, stabilization and capping of AgNPs as well as to determine the functional groups of the AgNPs. The completely dried samples of AgNPs were used in order to perform FTIR. The spectrum was recorded on FTIR (IR Prestige-21 (P/N 206-72010. SHIMADZU)) in the transmission range of 4000-500 cm^{-1} [24].

2.7.3 Scanning Electron Microscopy (SEM)

The size and morphology of the *P. aeruginosa* AgNPs was analyzed by coating the air dried AgNPs and observing under scanning electron microscope (EM6200).

2.7.4 X-ray diffraction (XRD)

Formation of *P. aeruginosa* AgNPs was further checked by XRD technique using an X-ray diffractometer (Phillips PW 1729/40) operated at 40 kV, 40 mA, step size of 0.2, over the 2θ range of 20-80°. Glass slides coated with AgNPs were tested following manufacturer guidelines.

2.8 Statistical analysis

All experiments were performed in triplicates. Microsoft Excel 2019 was used to draw graphs while SPSS version 10 was used to calculate means, standard error and one way ANOVA test followed by Tureky's test was used to determine the significance level at $p \leq 0.05$[23]. The figure of FTIR data was made using origin 2019A (8.5.1). The pictures of SEM and XRD were derived from one replicate.

3 Results

3.1 Synthesis of AgNPs

Biogenic synthesis of AgNPs by using four bacterial isolates (*E. coli*, *P. aeruginosa*, *B. subtilis* and *B. licheniformis*) was confirmed by the change of color from pale yellow to brown after 72 hours incubation compared to the two controls. The formation of AgNPs indicated that certain reducing agent released by the tested bacteria are actually involved in the reduction of Ag^+ ions to AgNPs. In control group, the reduction of Ag^+ ions did not occur due to the absence of reducing agent produced by bacteria, hence no color change was observed (Fig. S1).

3.2 Antibacterial activity of AgNPs

Biogenically synthesized AgNPs showed significant antibacterial activity against human pathogenic strains such as *E. coli*, *P. aeruginosa*, *B. subtilis* and *B. licheniformis*. *E. coli* synthesized AgNPs showed 17.6 mm ZOI against *E.*

coli, 18.3 mm against *P. aeruginosa*, 19.6 mm against *B. subtilis* and 16 mm against *B. licheniformis*. *P. aeruginosa* synthesized AgNPs showed 19 mm ZOI against *E. coli*, 20.3 mm against *P. aeruginosa*, 21.6 mm against *B. subtilis* and 22.5 mm against *B. licheniformis*. *B. subtilis* synthesized AgNPs showed 16.6 mm ZOI against *E. coli*, 15.6 mm against *P. aeruginosa*, 17.3 mm against *B. subtilis* and 15 mm against *B. licheniformis*. *B. licheniformis* synthesized AgNPs showed 17.6 mm ZOI against *E. coli*, 20 mm against *P. aeruginosa*, 19 mm against *B. subtilis* and 17 mm against *B. licheniformis*. On the other hand, positive control (rifampicin 50 μg/mL) showed 5.0 mm ZOI against *E. coli*, 6.0 mm against *P. aeruginosa*, 7.0 mm against *B. subtilis* and 6.3 mm ZOI against *B. licheniformis*. *P. aeruginosa* AgNPs showed highest ZOI (19-22 mm) against all tested human strains. The negative control (DMSO) was unable to inhibit the growth of any test strain while the positive control (rifampicin) showed 5.0 to 7.0 mm ZOI (Fig. 5 and Table 1). The one-way ANOVA showed that *P. aeruginosa* AgNPs showed significant antibacterial activity ($p<0.05$) as compared other biogenically synthesized AgNPs and positive control.

3.3 MIC and MBC determination

MIC was measured to determine the lowest concentration which is effective to inhibit bacterial growth. It was observed that MIC values of *E. coli* AgNPs ranged from 6.0 to 8.6 μg/mL, *P. aeruginosa* AgNPs from 2.6 to 3.3 μg/mL,

Table 1 Antibacterial Activity of Biogenically Synthesized AgNPs.

Sr No.	Strains	*E. coli*	*P. aeruginosa*	*B. subtilis*	*B. licheniformis*	Rifampicin (PC) (50 μg/mL)	DMSO (NC)	$AgNO_3$ (1 mg/mL)
		Zone of inhibition in mm (Mean ± S.E)						
1	*E. coli* AgNPs (100 μg/mL)	17.6 ± 0.33^{c}	18.3 ± 0.33^{cd}	19.6 ± 0.33^{d}	16 ± 0.57^{b}	5.0 ± 0^{a}	0.0 ± 0^{a}	2.0 ± 0^{a}
2	*P. aeruginosa* AgNPs (100 μg/mL)	19.0 ± 0.57^{c}	20.3 ± 0.66^{d}	21.6 ± 0.33^{d}	22.5 ± 0.28^{c}	6.0 ± 0.57^{b}	0 ± 0^{a}	2.0 ± 0.32^{b}
3	*B. subtilis* AgNPs (100 μg/mL)	16.6 ± 0.88^{de}	15.6 ± 0.33^{cd}	17.3 ± 0.88^{e}	15.0 ± 0.57^{c}	7.0 ± 0.57^{b}	0 ± 0^{a}	2.0 ± 0.24^{b}
4	*B. licheniformis* AgNPs (100 μg/mL)	17.6 ± 0.33^{c}	20.0 ± 0^{c}	19.0 ± 0.57^{c}	17.0 ± 0.57^{c}	6.3 ± 0.33^{b}	0 ± 0^{a}	2.3 ± 0.25^{b}

PC, Positive control; NC, negative control.

Table 2 MIC and MBC of biogenically synthesized AgNPs against test pathogens.

Compound	Test Pathogens	MIC (μg/mL)	MBC (μg/mL)
E. coli AgNPs	*E. coli*	8.6^{b}	11.6^{d}
	P. aeruginosa	6.0^{a}	7.0^{a}
	B. subtilis	6.0^{a}	10.3^{c}
	B. licheniformis	6.7^{ab}	8.3^{b}
P. aeruginosa AgNPs	*E. coli*	3.3^{a}	4.6^{a}
	P. aeruginosa	3.6^{a}	4.3^{a}
	B. subtilis	3.3^{a}	4.6^{a}
	B. licheniformis	2.6^{a}	4.6^{a}
B. subtilis AgNPs	*E. coli*	5.6^{ab}	6.3^{a}
	P. aeruginosa	6.3^{b}	6.6^{a}
	B. subtilis	4.6^{a}	5.6^{a}
	B. licheniformis	5.6^{ab}	6.6^{a}
B. licheniformis AgNPs	*E. coli*	6.0^{b}	7.3^{bc}
	P. aeruginosa	7.7^{c}	8.7^{c}
	B. subtilis	5.4^{ab}	6.5^{ab}
	B. licheniformis	4.7^{a}	5.6^{a}

MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration.

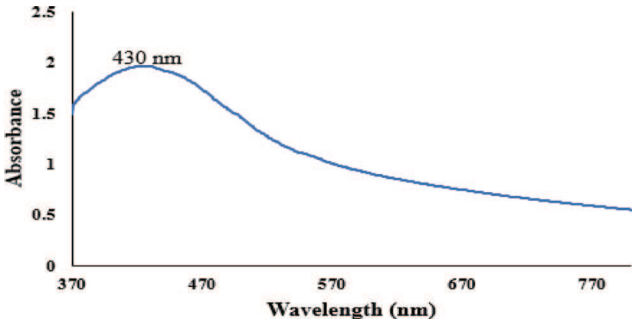

Fig. 1 UV-vis spectra of *Pseudomonas aeruginosa* synthesized AgNPs was showed. The formation of absorbance peek at 430 nm indicates the reduction of silver nitrate and the formation of AgNPs.

B. subtilis AgNPs from 4.6-6.3 μg/mL and *B. licheniformis* AgNPs from 4.7-7.7 μg/mL. Regarding MBC, *E. coli* AgNPs showed values ranging from 7.0-11.6 μg/mL, *P. aeruginosa* AgNPs from 4.3-4.6 μg/mL, *B. subtilis* AgNPs from 5.6-6.6 μg/mL and *B. licheniformis* AgNPs from 5.6-8.7 μg/mL. MIC values were calculated on the basis of visual observation. The maximum MIC value (8.6 μg/mL) was noted against *E. coli* strain by *E. coli* AgNPs while lowest value was 4.3 μg/mL by *P. aeruginosa* AgNPs against *B. licheniformis*. Overall, *P. aeruginosa* AgNPs showed the lowest MIC and MBC values against all tested strains, hence subjected to characterization study (Table 2).

3.4 Characterization study

3.4.1 UV-visible spectroscopy

The *P. aeruginosa* AgNPs were analysed using UV visible spectrophotometer. The absorption spectrum for AgNPs was measured from 370 nm-770 nm which showed peaks from 400 nm-430 nm. The absorption peak was observed around 430 nm (Fig. 1). The *P. aeruginosa* AgNPs showed broad peaks due to variation in the size of the NPs. Increased peak intensity is the clear indication of increased NPs in the solution.

3.4.2 FTIR spectroscopy

The FTIR result of *P. aeruginosa* AgNPs showed number of bands in the region 4000-500 cm^{-1}. The spectrum analysis of AgNPs showed absorption bands at different peaks (Fig. 2). The bands present at 3261, 3061, 2947, 2358, 1338 and 1394 cm^{-1} corresponded to the stretching vibrations of alcohol (O-H), primary amines (N-H), alkane (C-H), amine (C-N) and alcohol (C-O) groups, respectively. Amides containing carbonyl groups (C = O) were observed at 1633 and 1575 cm^{-1}. The other peaks at 1456, 1398 and 1072 cm^{-1} can be assumed to the C-O stretching vibrations aromatic and aliphatic amines, respectively (Fig. 2). The above information confirmed the presence of stabilizing agents, responsible for AgNPs stabilization.

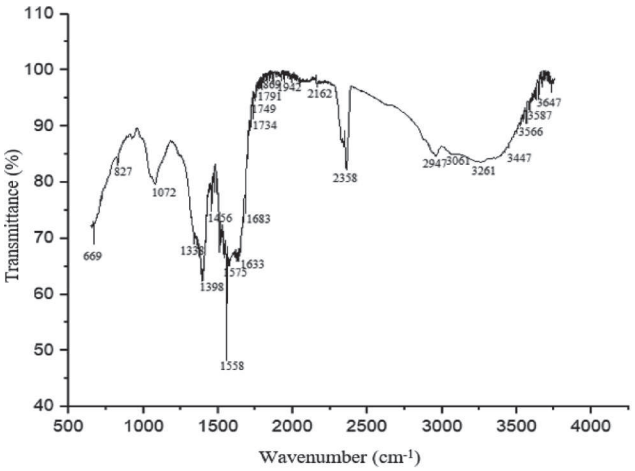

Fig. 2 FTIR spectra of *Pseudomonas aeruginosa* synthesized AgNPs. The different bands indicate various functional groups.

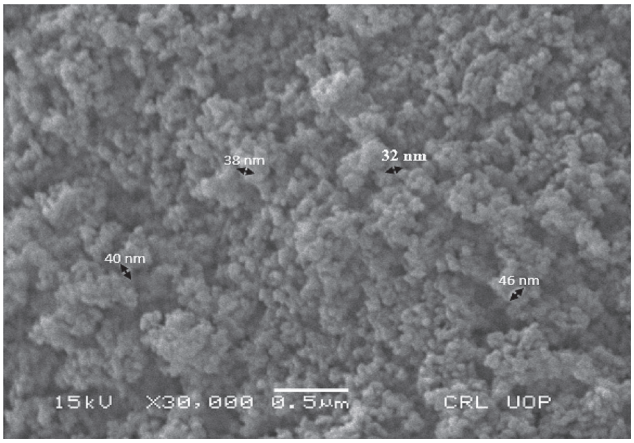

Fig. 3 SEM micrograph of *Pseudomonas aeruginosa* synthesized AgNPs taken at scale of 0.5 μm. AgNPs of spherical shape can be seen.

3.4.3 Scanning Electron Microscopy (SEM)

The size and morphology of *P. aeruginosa* AgNPs was examined by SEM analysis. The *P. aeruginosa* AgNPs were mostly spherical in shape with size ranging from 32-46 nm (scale bar of 0.5 μm). There were few NPs aggregation suggesting that the protein molecules may play crucial role as capping agent for NPs thus preventing agglomeration and providing stability to the synthesized NPs (Fig. 3).

3.4.4 X-ray Diffraction (XRD)

The XRD pattern of the *P. aeruginosa* AgNPs showed unique diffraction peaks at about 34.4°, 46.8°, 62.0°, and 79.1° which indicated the presence of 111, 200, 220 and 311 orientations, respectively (Fig. 4). The sharp peaks of AgNPs resulted from capping agents, which act as stabilizers for NPs. The diffraction peak present at 34.4° was ascribed to the 111 lattice plane of face centred cubic (FCC),

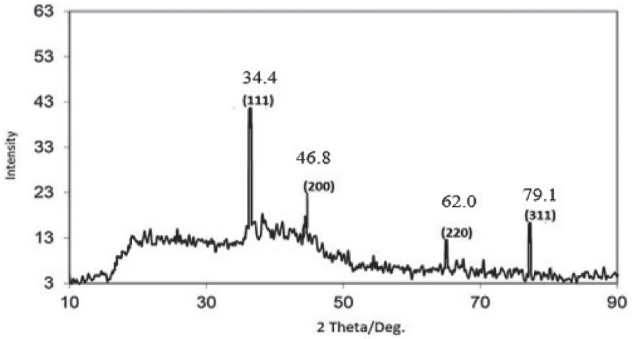

Fig. 4 XRD analysis of *Pseudomonas aeruginosa* synthesized AgNPs. Sharp peeks indicate the various capping agents which stabilize the AgNPs.

clearly indicated that the NPs were made up of silver (Fig. 4). The XRD pattern confirmed the crystalline nature of biogenically synthesized AgNPs.

4 Discussion

Biologically synthesized AgNPs have been proved as therapeutically effective and valuable compounds. They also have excellent antimicrobial and antiviral activity[1, 25, 26]. Although, there are a lot of techniques available which can be used for the synthesis of AgNPs. Much of these techniques were focused on use of chemicals. However, green synthesis or biogenic synthesis of AgNPs is more eco-friendly approach[26, 27]. In the present study, four bacterial strains i.e., *E. coli*, *P. aeruginosa*, *B. subtilis* and *B. licheniformis* were used for the biogenic synthesis of AgNPs. Synthesis of AgNPs was confirmed by color change from pale yellow to brown. Previously, Gurunathan *et al.*[16] described color change to brown as confirmed biogenic synthesis of AgNPs. Likewise, Masum *et al.*[28] reported that the formation of AgNPs get confirmed by brown coloration and more saturated brown color indicates the more AgNPs in the solution. The change in color is due to the excitation of the surface plasmon vibrations in metal NPs[11]. After visual observation the solution was further analyzed by UV visible spectrophotometer.

Following formation, all four biogenically synthesized AgNPs were tested for antibacterial potential against four human isolates using agar well diffusion assay. It was observed that *E. coli* AgNPs showed ZOI from 16.0-19.6 mm against four isolates. *P. aeruginosa* AgNPs showed 19.0 to 22.5 mm ZOI, *B. subtilis* AgNPs showed 7.0 to 17.3 mm ZOI and *B. licheniformis* AgNPs showed 17.0 to 20.0 mm ZOI against all tested isolates. Among all biogenic AgNPs, *P. aeruginosa* AgNPs showed significant antibacterial potential even higher than positive control (rifampicin 50 μg/mL), because the size of *P. aeruginosa* synthesized AgNPs were 10-100 nm and showed strong antimicrobial effect against both Gram-positive and negative bacteria. The small particle size enables AgNPs to adhere to the cell wall and penetrate into the bacteria cell easily, which in turn improves their antimicrobial activity against bacteria (Table 1). The findings of this study corroborate with Aziz *et al.*[25] who observed the significant antibacterial potential AgNPs

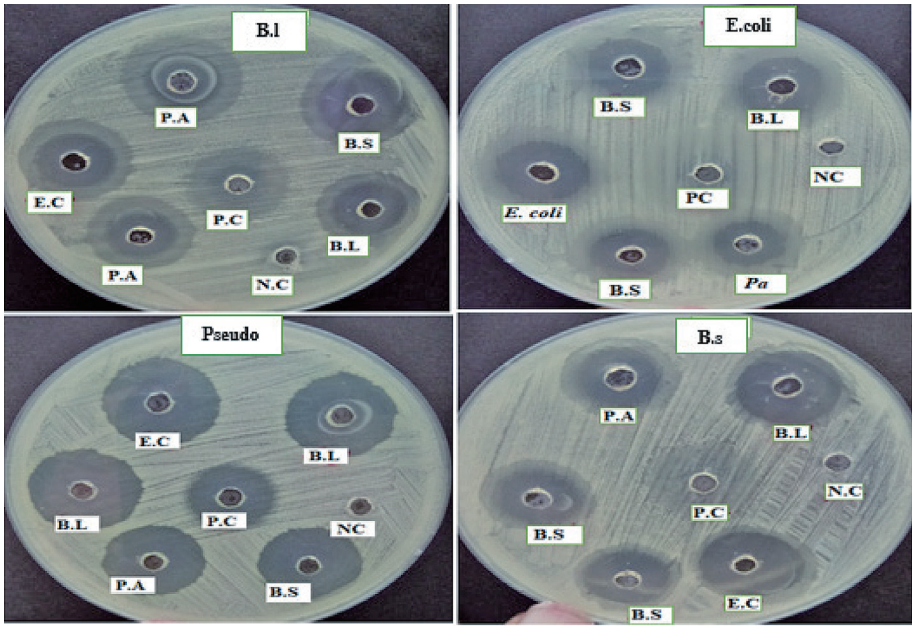

Fig. 5 The antibacterial activity of biogenically synthesized AgNPs against *B. licheniformis*, *E. coli*, *P. aeruginosa* and *B. subtilis*. The E.C; *E. coli*, B.S; *B. subtilis*, P.A; *P. aeruginosa*, B.L; *B. licheniformis*, P.C; Positive control and N.C; Negative control.

even at lower concentration. Previously, Hamouda *et al.*[29] reported that AgNPs release Ag^+ ions from the surface and these ions are responsible for the bactericidal efficacy of AgNPs. The more Ag^+ ions release will kill more bacteria which results in bigger ZOI. The observed ZOI by biogenically synthesized NPs were obtained after subtracting the ZOI by $AgNO_3$, hence indicating the actual antibacterial potential of biogenic NPs.

Silver nitrate has shown different effects against bacteria at high concentrations, killing bacteria by different mechanisms, which are binding to the thiol groups of protein and denaturing them, programmed cell death (apoptosis) and causing the DNA to be in the condensed form, which inhibits cell replication. While low concentrations (5-10 mM) of $AgNO_3$ leads to the formation of silver NPs[30]. This study used low concentration 10 mM $AgNO_3$ for forming AgNPs. However, it's already stated that in depth antibacterial mechanism of NPs is still unclear. In particular, various bacterial isolates, their mechanism of action, resistance profile, and NP potential have been reported previously, which make it difficult to compare bacteriostatic vs bactericidal activity. In addition, no single method/criteria justify the exact through information about antibacterial mechanisms of tested NPs[31].

In this study, tube dilution method was used for the determination of MIC and MBC. The maximum MIC value (8.6 μg/mL) was noted against *E. coli* by *E. coli* AgNPs while lowest value was 4.3 μg/mL by *P. aeruginosa* AgNPs against *B. licheniformis*. Overall, *P. aeruginosa* AgNPs showed the lowest MIC and MBC values against all tested strains, hence subjected to characterization study. The MIC values were 4.3 to 8.6 μg/mL against *E. coli*, 6 to 7.6 μg/mL against *P. aeruginosa*, 4.3 to 6 μg/mL against *B. subtilis* and 4.6 to 6.6 μg/mL against *B. licheniformis*. These results were significant ($p<0.05$). The above results showed that AgNPs have lowest minimum inhibitory concentration against pathogenic bacteria suggesting the broad spectrum nature of their antimicrobial activity[32]. The MBC values were 5.6 to 11.6 μg/mL against *E. coli*, 6.3 to 8.8 μg/mL against *P. aeruginosa*, 5.6 to 10.3 μg/mL against *B. subtilis* and the MIC value against *B. licheniformis* was 5.5 to 8.3 μg/mL (Table 2). The findings of this study are consistent with the results by[33].

The *P. aeruginosa* synthesized AgNPs show strong peek at 430 nm[28]. The occurrence of peek around 430 nm indicates the presence of AgNPs. Aziz *et al.*[25] reported that the reduction of silver nitrate causes the occurrence of peek around 430. Biosynthetic mechanism of AgNPs has been assumed that the Ag^+ ions required NADPH-dependent nitrate reductase enzymes for their reduction, which were released by tested bacteria in their extracellular environment[34].

FTIR of *P. aeruginosa* AgNPs was performed to check the involvement of biological molecules. The bands present at 3261, 3061, 2947, 2358, 1338 and 1394 cm^{-1} corresponded to the stretching vibrations of alcohol (O-H), primary amines (N-H), alkane (C-H), amine (C-N) and alcohol (C-O) groups respectively, as reported previously by Liaqat[35] and Kalyanasundaram *et al.*[36]. The SEM data showed that silver nanoparticles are in spherical shape with average size of 40 nm. The formation of various sharp peeks in XRD, indicated the presence of different molecules, involved in the stabilization of the NPs. Similar result was reported by Kumar *et al.*[37].

5 Conclusion

This study showed the effective antibacterial potential of biogenically synthesized AgNPs and suggested these as alternative source of antimicrobial agents against human pathogenic bacteria. AgNPs showed strong bactericidal effect even at lower concentration against the test human pathogenic strains. Ongoing *in vivo* study will prove their toxicity analysis and safety in pharmaceutical and therapeutic biomedical applications.

Conflict of Interests

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Author Contributions

I. Liaqat designed the project and supervised the complete study. S. Tufail did experimental work on characterization and wrote the first draft. I. Liaqat and S. Tufail did the statistical analysis. I. Liaqat wrote the final draft. S. Andleeb, S. Naseem, U. Zafar, and A. Sadiqa proof read the manuscript. I. Liaqat, N. M. Ali, A. Bibi, N. Arshad and G. Saleem helped in experimental work. All authors approved the final version of manuscript.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess21291

References

1) Tufail, M.S.; Liaqat, I. Silver nanoparticles and their applications-A comprehensive review. *Pure Appl. Biol.* **11**, 315-330 (2021).
2) Cha, K.E.; Myung, H.J. Cytotoxic effects of nanoparti-

cles assessed *in vitro* and *in vivo*. *J. Microbiol. Biotechnol.* **17**, 1573-1578 (2007).
3) Ahmed, S.; Ahmad, M.; Swami, B.L.; Ikram, S. A review on plants extract mediated synthesis of silver nanoparticles for antimicrobial applications: a green expertise. *J. Adv. Res.* **7**, 17-28 (2016).
4) Kaliamurthi, S.; Selvaraj, G.; Çakmak, Z.E.; Çakmak, T. Production and characterization of spherical thermostable silver nanoparticles from *Spirulina platensis* (Cyanophyceae). *Phycologia* **55**, 568-576 (2016).
5) Satyavani, K.; Gurudeeban, S.; Ramanathan, T. Influence of leaf broth concentration of *Excoecaria agallocha* as a process variable in silver nanoparticles synthesis. *J. Nanomed. Res.* **1**, 11 (2014).
6) Borrego, B.; Lorenzo, G.; Mota-Morales, J.D.; Almanza-Reyes, H.; Mateos, F.; López-Gil, E.; Bogdanchikova, N. Potential application of silver nanoparticles to control the infectivity of Rift Valley fever virus *in vitro* and *in vivo*. *Nanomed- Nanotechnol. Biol. Med.* **12**, 1185-1192 (2016).
7) Benelli, G. Plant-mediated biosynthesis of nanoparticles as an emerging tool against mosquitoes of medical and veterinary importance: A review. *Parasitol. Res.* **115**, 23-34 (2016).
8) Chari, N.; Felix, L.; Davoodbasha, M.; Ali, A.S.; Nooruddin, T. *In vitro* and *in vivo* antibiofilm effect of copper nanoparticles against aquaculture pathogens. *Biocatal. Agric. Biotechnol.* **10**, 336-341 (2017).
9) LewisOscar, F.; MubarakAli, D.; Nithya, C.; Priyanka, R.; Gopinath, V.; Alharbi, N.S.; Thajuddin, N. One pot synthesis and anti-biofilm potential of copper nanoparticles (CuNPs) against clinical strains of *Pseudomonas aeruginosa*. *Biofouling* **31**, 379-391 (2015).
10) Morris, S.; Cerceo, E. Trends, epidemiology, and management of multi-drug resistant gram-negative bacterial infections in the hospitalized setting. *Antibiotics* **9**, 196 (2020).
11) Sastry, M.; Ahmad, A.; Khan, M.I.; Kumar, R. Biosynthesis of metal nanoparticles using fungi and actinomycete. *Curr. Sci.* **85**, 162-170 (2003).
12) Singh, R.; Wagh, P.; Wadhwani, S.; Gaidhani, S.; Kumbhar, A.; Bellare, J.; Chopade, B.A. Synthesis, optimization, and characterization of silver nanoparticles from Acinetobacter calcoaceticus and their enhanced antibacterial activity when combined with antibiotics. *Int. J. Nanomedicine* **8**, 4277-4290 (2013).
13) Liaqat, I.; Sabri, A.N. Effect of biocides on biofilm bacteria from dental unit water lines. *Curr. Microbiol.* **56**, 619-624 (2008).
14) Sadiqa, A.; Gilani, S.R.; Anwar, A.; Mehboob, A.; Saleem, A.; Rubab, S. Biogenic fabrication, characterization and drug loaded antimicrobial assay of silver nanoparticles using *Centratherum anthalminticum* (L.) Kuntze. *J. Pharm. Sci.* **110**, 1969-1978 (2021).
15) Deepak, V.; Umamaheshwaran, P.S.; Guhan, K.; Nanthini, R.A.; Krithiga, B.; Jaithoon, N.M.H.; Gurunathan, S. Synthesis of gold and silver nanoparticles using purified URAK. *Colloids Surf. B* **86**, 353-358 (2011).
16) Gurunathan, S.; Kalishwaralal, K.; Vaidyanathan, R.; Venkataraman, D.; Pandian, S.R.K.; Muniyandi, J.; Hariharan, N.; Eom, S.H. Biosynthesis, purification and characterization of silver nanoparticles using *Escherichia coli*. *Colloids Surf. B* **74**, 328-335 (2009).
17) Singh, R.; Shedbalkar, U.U.; Wadhwani, S.A.; Chopade, B.A. Bacteriagenic silver nanoparticles: synthesis, mechanism, and applications. *Appl. Microbiol. Biotechnol.* **99**, 4579-4593 (2015).
18) Siddiqi, K.S.; Husen, A.; Rao, R.A. A review on biosynthesis of silver nanoparticles and their biocidal properties. *J. Nanobiotechnology* **16**, 1-28 (2018).
19) Liaqat, I.; Bachmann, R.T.; Sabri, A.N.; Edyvean, R.G.; Biggs, C.A. Investigating the effect of patulin, penicillic acid and EDTA on biofilm formation of isolates from dental unit water lines. *Appl. Microbiol. Biotechnol.* **81**, 349-358 (2008).
20) Liaqat, I.; Arshad, N.; Arshad, M.; Mirza, S.A.; Ali, N.M.; Shoukat, A. Antimicrobial activity of some medicinal plants extracts against food industry isolates. *Pak. J. Zool.* **49**, 565-572 (2017).
21) Liaqat, I.; Sumbal, F.; Sabri, A.N. Tetracycline and chloramphenicol efficiency against selected biofilm forming bacteria. *Curr. Microbial.* **59**, 212-220 (2009).
22) Liaqat, I.; Liaqat, M.; Tahir, H.M.; Ali, N.M.; Arshad, M.; Arshad, N. Motility effects biofilm formation in *Pseudomonas aeruginosa* and *Enterobacter cloacae*. *Pak. J. Pharm. Sci.* **32**, 927-932 (2019).
23) Liaqat, I.; Arshad, N.; Arshad, M.; Mirza, S.A.; Ali, N.M.; Shoukat, A. Antimicrobial activity of some medicinal plants extracts against food industry isolates. *Pak. J. Zool.* **49**, 565-672 (2017).
24) Liaqat, I. Fourier transform infrared spectroscopy of dental unit water line biofilm bacteria. *Spectroscopy* **23**, 175-189 (2009).
25) Aziz, N.; Fatma, T.; Varma, A.; Prasad, R. Biogenic synthesis of silver nanoparticles using *Scenedesmus abundans* and evaluation of their antibacterial activity. *J. Nanopart. Res.* **2014**, 689419 (2014).
26) Zhang, X.F.; Liu, Z.G.; Shen, W.; Gurunathan, S. Silver nanoparticles: Synthesis, characterization, properties, applications, and therapeutic approaches. *Int. J. Mol. Sci.* **17**, 1534 (2016).
27) Iravani, S.; Korbekandi, H.; Mirmohammadi, S.V.; Zolfaghari, B. Synthesis of silver nanoparticles: Chemical, physical and biological methods. *Res. Pharm. Sci.* **9**, 385-406 (2014).
28) Masum, M.; Islam, M.; Siddiqa, M.; Ali, K.A.; Zhang, Y.;

Abdallah, Y.; Li, B. Biogenic synthesis of silver nanoparticles using *Phyllanthus emblica* fruit extract and its inhibitory action against the pathogen *Acidovorax oryzae* strain RS-2 of rice bacterial brown stripe. *Front. Microbiol.* **10**, 820(2019).

29) Hamouda, R.A.; Hussein, M.H.; Abo-Elmagd, R.A.; Bawazir, S.S. Synthesis and biological characterization of silver nanoparticles derived from the *Cyanobacterium oscillatoria* limnetica. *Sci. Rep.* **9**, 1-17(2019).

30) Pandian, S.R.K.; Deepak, V.; Kalishwaralal, K.; Viswanathan, P.; Gurunathan, S. Mechanism of bactericidal activity of silver nitrate-A concentration dependent bi-functional molecule. *Braz. J. Bio.* **41**, 805-809(2010).

31) Wang, L.; Hu, C.; Shao, L. The antimicrobial activity of nanoparticles: present situation and prospects for the future. *Int. J. Nanomedicine* **12**, 1227-1249(2017).

32) Ramalingam, V.; Rajaram, R.; PremKumar, C.; Santhanam, P.; Dhinesh, P.; Vinothkumar, S.; Kaleshkumar, K. Biosynthesis of silver nanoparticles from deep sea bacterium *Pseudomonas aeruginosa* JQ989348 for antimicrobial, antibiofilm, and cytotoxic activity. *J. Basic Microbiol.* **54**, 928-936(2014).

33) Liao, S.; Zhang, Y.; Pan, X.; Zhu, F.; Jiang, C.; Liu, Q.; Chen, L. Antibacterial activity and mechanism of silver nanoparticles against multidrug-resistant *Pseudomonas aeruginosa*. *Int. J. Nanomedicine* **14**, 1469-1487(2019).

34) Kalishwaralal, K.; Deepak, V.; Ramkumarpandian, S.; Nellaiah, H.; Sangiliyandi, G. Extracellular biosynthesis of silver nanoparticles by the culture supernatant of *Bacillus licheniformis*. *Mat. Lett.* **62**, 4411-4413 (2008).

35) Liaqat, I. Fourier transform infrared spectroscopy of dental unit water line biofilm bacteria. *Spectroscopy* **23**, 175-189(2009).

36) Kalyanasundaram, G.T.; Doble, M.; Gummadi, S.N. Production and downstream processing of(1→3)-β-D-glucan from mutant strain of *Agrobacterium* sp. ATCC 31750. *AMB Express* **2**, 1-10(2012).

37) Kumar, V.; Yadav, S.K. Plant mediated synthesis of silver and gold nanoparticles and their applications. *J. Chem. Technol. Biotechnol.* **84**, 151-157(2009).