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Bacterial nanobiotic potential

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Abstract: Antibiotics are the chemicals responsible for killing pathogenic bacteria but inappropriate and extensive use of antibiotics is hazardous causing adverse impact on human health. Excessive use of antibiotics has led to the development of multiple-drug resistant bacteria posing health hazards to mankind. The study of nanoparticles has revolutionized the problem solving concerns regarding fields of agriculture, chemistry and medicine. Nanoparticles are smaller than atomic nuclei offering more surface area and greater reactivity. Bacterial silver nanoparticles (AgNPs) were studied for their antibacterial potential. AgNPs from *Bacillus subtilus* show the highest antibacterial activity. Nanoparticles exhibiting antibacterial activity can be helpful to reduce the toxic impact of synthetic antibiotics. Present work deals with the green production of silver nanoparticles by exploiting indigenous bacteria. These AgNPs were characterized through Fourier transform infrared spectroscopic (FTIR) analysis, transmission electron microscopy (TEM) and UV spectroscopic analysis and were also evaluated for their antibacterial and antifungal potential. The data suggested the extracellular biosynthesis method to be very effective for the biosynthesis of AgNPs in some bacterial strains. Keeping in view the antibacterial potential of studied AgNPs, the present work suggests green production of nanoparticles which can be effectively utilized as environment friendly antibacterial and antifungal agents.

Keywords: AgNPs, antibiotics; antifungal; antibacterial; *Bacillus*

List of abbreviations

AgNPs	silver nanoparticles
FTIR	Fourier transform infrared spectroscopy
TEM	transmission electron microscopy
UV	ultraviolet
NPs	nanoparticles

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1 Introduction

Nanotechnology is one of the fast growing fields especially in the area of biotechnology and medicine [1]. Nanoparticles are nanoscale particles (1-100 nm) which have unique chemical and physical properties. Due to their smaller size and extraordinary properties, they are commonly used in various fields of science and technology. Various methods are being used now-a-days for the synthesis of nanoparticles like physical, chemical and biological methods. Physical methods are highly exothermic reactions with lesser yields. Chemical methods exert toxic effects due to the production of large amount of hazardous by-products. On the other hand, biological nanoparticle synthesis is comparatively economical, ecofriendly and appreciable. In biological process of metal nanoparticles synthesis, principles of green chemistry are applied. Microorganisms (bacteria, fungi and actinomycetes) and plants are used as a reducing agent for conversion of silver metal to stabilized silver nanoparticles of various sizes [2]. Microbial infections have now become a global health concern due to the antibiotic resistance developed by pathogenic microbes and hence, confronting proper medical treatments. This increasing antibiotic resistance is responsible for elevated risk of health disorders, rate of mortality, expenditure and low life probability. We need an alternative method as a permanent solution for this emerging problem [3]. Silver has excellent potential to be used as an antibacterial and antiseptic agent since remote past. Silver nanoparticles are important metal particles which are commonly used in photocatalysis, photonics, micro-electronics and antimicrobial activities. Silver nanoparticles are unique inorganic nanoparticles because of their unique size and properties like catalysis, surface plasmon resonance (SPR), biomedicine, nanoelectronics and sensing. The antimicrobial activity of bacterial AgNPs can offer a unique option to reduce the use of antibiotics thereby minimizing the development of antibiotic resistance [4]. The current study deals with the bacterial AgNPs having antimicrobial potential and characterization of these biosynthesized AgNPs through spectroscopic and microscopic techniques.

2 Materials and methods

2.1 Nitrate reductase assay

Four already isolated bacterial strains i.e., *Serratia marcescens* (S4c1), *Brevundimonas diminuta* (S5a), *Bacillus cereus* (So3II), and *B. subtilis* (Mt3b) by Wagi and Ahmed [5] were screened for nitrate reductase activity following Thamilselvi and Radha [6].

2.2 Bacterial silver nanoparticles synthesis

Bacterial biosynthesis of silver nanoparticles was carried out using extracellular and intracellular method following Wagi and Ahmed [5].

2.2.1 Extracellular synthesis of bacterial silver nanoparticles

Extracellular synthesis of bacterial silver nanoparticles (AgNPs) was carried out following Wagi and Ahmed [5]. Bacterial cell supernatant of all the bacterial strains (S4c1, S5a, So3II and Mt3b) was treated with $AgNO_3$ solution in the dark for extracellular synthesis of AgNPs and was incubated for 24 h at 37°C.

2.2.2 Intracellular biosynthesis of bacterial silver nanoparticles

Intracellular synthesis of bacterial silver nanoparticles (AgNPs) was carried out following Wagi and Ahmed [5] using bacterial cell pellet. Bacterial cell pellet of all the bacterial strains (S4c1, S5a, So3II and Mt3b) were treated with $AgNO_3$ solution in the dark for intracellular synthesis of AgNPs and was incubated for 24 h at 37°C.

2.3 Factors affecting bacterial silver nanoparticles synthesis

Factors affecting the bacterial synthesis of silver nanoparticles were observed.

2.3.1 Cell supernatant and residue

Extracellular and intracellular synthesis of bacterial silver nanoparticles (AgNPs) was carried out using bacterial cell supernatant and bacterial cell pellet, respectively.

2.3.2 $AgNO_3$ concentration

The impact of $AgNO_3$ concentration on the synthesis of AgNPs was evaluated by varying the concentration of $AgNO_3$ used for AgNPs synthesis. Three different concentrations of $AgNO_3$ i.e. 1, 2 and 3 mM were used in the current study to evaluate the effect of $AgNO_3$ on silver nanoparticles synthesis.

2.3.3 Reaction time

Silver nanoparticles synthesis was also evaluated at varying reaction times i.e., 4, 6, 24, 48 and 72 h of reaction time.

2.3.4 Reaction temperature

Varying temperatures (37°C and 60°C) were used for the synthesis of AgNPs and its effect on nanoparticles synthesis was also recorded.

2.4 Characterization of bacterial silver nanoparticles

2.4.1 Fourier transform infrared spectroscopic (FTIR) analysis

The bacterial AgNPs were examined through FTIR analysis (400-4000 cm^{-1}) following Thamilselvi and Radha [6].

2.4.2 Transmission electron microscopic (TEM) analysis

Transmission electron microscopic (TEM) analysis of bacterial silver nanoparticles was carried out following Mishra et al. [7].

2.4.3 UV visible spectroscopic analysis

UV-visible spectroscopic analysis of synthesized silver nanoparticles was carried out using UV visible spectrophotometer at 400 nm following Arun et al. [1].

2.5 Antimicrobial activity of bacterial silver nanoparticles

The antibacterial activity of bacterial AgNPs was studied by using bacterial strains i.e., *Enterobacter* sp., *Aeromonas* sp., *Butyricum* sp., *Proteus* sp., *Pasturella* sp.

and *E. coli* obtained from a Tertiary Care Hospital, Lahore. Disc diffusion method was used to check the antimicrobial activity of the bacterial AgNPs. After 24 h of incubation, inhibition zones produced around the disc on the plates were recorded following Mishra et al. [7]. The experiment was performed in triplicate.

2.6 Antifungal activity of bacterial silver nanoparticles

The bacterial silver nanoparticles were evaluated for their antifungal potential to inhibit the growth of *Phytophthora palmivora* a fungus obtained from Shenk Lab, Australia, causing several plant diseases following Thamilselvi and Radha [6]. The experiment was performed in triplicate and the percentage radial inhibition of fungal growth in the treated and control samples were recorded.

3 Results

3.1 Nitrate reductase assay

Four already isolated and identified bacterial strains i.e., *Serratia marcescens* (S4c1), *Brevundimonas diminuta* (S5a), *Bacillus subtilis* (Mt3b) and *Bacillus cereus* (So3II) from indigenous environment by Wagi and Ahmed [5] were used in the current study. These bacterial strains were screened for their nitrate reductase potential. All the bacterial strains have shown nitrate reductase production potential which was confirmed through color change. The isolate *Serratia marcescens* (S4c1) has shown maximum nitrate reductase (NR) potential (Table 1).

3.2 Bacterial silver nanoparticles synthesis

3.2.1 Extracellular synthesis of bacterial silver nanoparticles

Change in color of reaction mixture manifested the synthesis of AgNPs. Maximum absorbance was recoded at 400 nm. Optimum AgNPs production potential was exhibited by *Serratia marcescens* (S4c1) (Figure 1).

Table 1: Nitrate reductase potential of bacterial strains.

Sr. No.	Strains	Nitrate reductase activity
1.	S4c1	++
2.	S5a	+
3.	So3II	+
4.	Mt3b	+

3.2.2 Intracellular synthesis of bacterial silver nanoparticles

Among the four bacterial strains used, the bacterial strain *Serratia marcescens* (S4c1) has shown maximum production of AgNPs by intracellular method (Figure 1).

3.3 Factor affecting bacterial silver nanoparticles synthesis

3.3.1 Cell supernatant and residue

Cell supernatant was observed to be more effective in the synthesis of AgNPs by some bacterial strains while on the other hand some bacterial strains utilized intracellular method in which cell residue was responsible for the reduction of $AgNO_3$ (Figure 1).

3.3.2 $AgNO_3$ concentration

Increase in $AgNO_3$ concentration caused improvement in the amount of AgNPs synthesized (Figure 2). Bacterial strains *Serratia marcescens* (S4c1), *Brevundimonas diminuta* (S5a) and *Bacillus subtilis* (Mt3b) have shown optimum production of AgNPs upto a certain threshold concentration of $AgNO_3$ which was recorded to be upto 2 mM and further increase resulted in gradual reduction in AgNPs synthesis. On the other hand, bacterial strain *Bacillus cereus* (So3II) has shown quite different behavior and AgNPs production was quite low at 1 and 2 mM concentration of $AgNO_3$ which gradually increased at 3 mM $AgNO_3$.

3.3.3 Reaction time

After 72 h of incubation time, the production of AgNPs was increased hundred times with the bacterial strains *Serratia marcescens* (S4c1), *Brevundimonas diminuta* (S5a) and *Bacillus subtilis* (Mt3b) compared to 4 h incubation while *Bacillus cereus* (So3II) has shown quite different behavior and reduction in AgNPs production was recorded after 72 h of reaction time as compared to 4 h of reaction time (Figure 3).

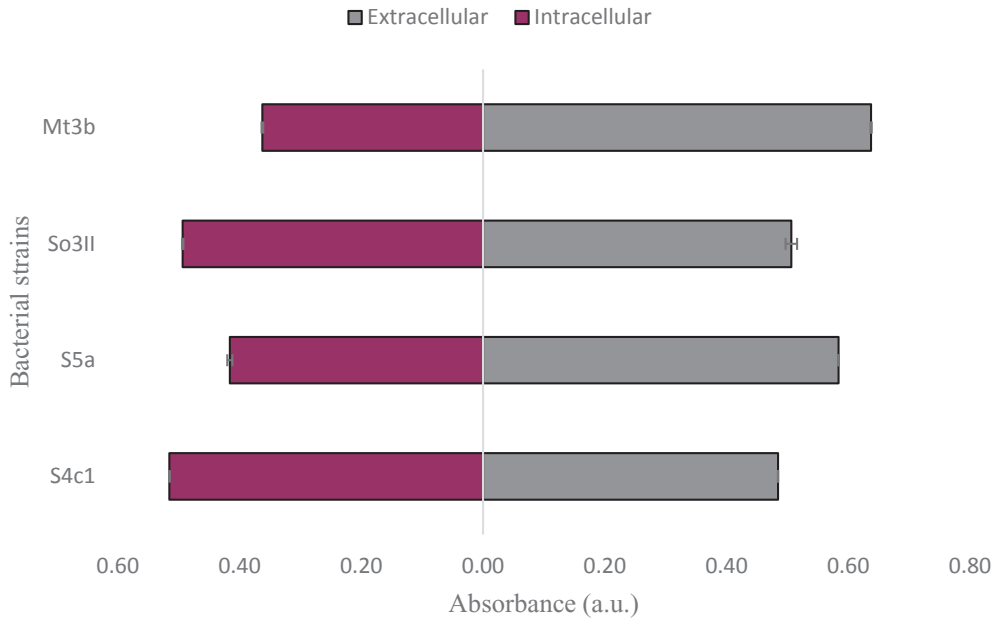

Figure 1: Biosynthesis of bacterial AgNPs.

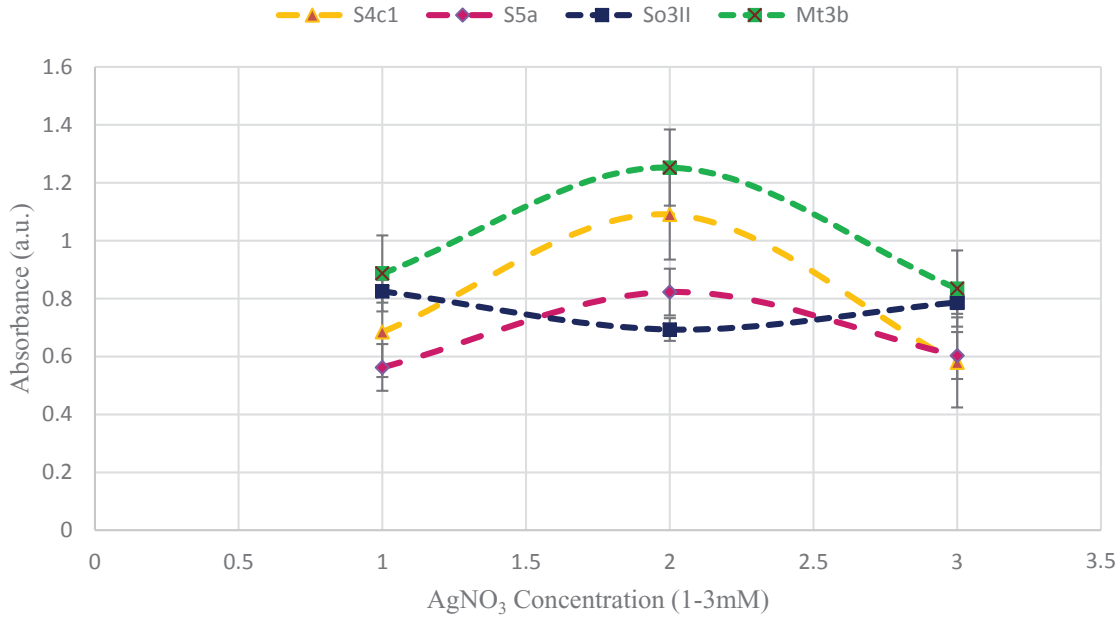

Figure 2: Effect of $AgNO_3$ concentration on AgNPs synthesis.

3.3.4 Reaction temperature

The ideal temperature for the production of AgNPs was found to be 60°C. All the bacterial strains i.e., *Serratia marcescens* (S4c1), *Brevundimonas diminuta* (S5a), *Bacillus cereus* (So3II) and *Bacillus subtilis* (Mt3b) exhibited optimum potential for the production of bacterial AgNPs at 60°C.

3.4 Characterization of bacterial silver nanoparticles

3.4.1 Fourier transform infrared spectroscopic (FTIR) analysis

IR spectrum of bacterial AgNPs was examined for the possible presence of compounds responsible for biological

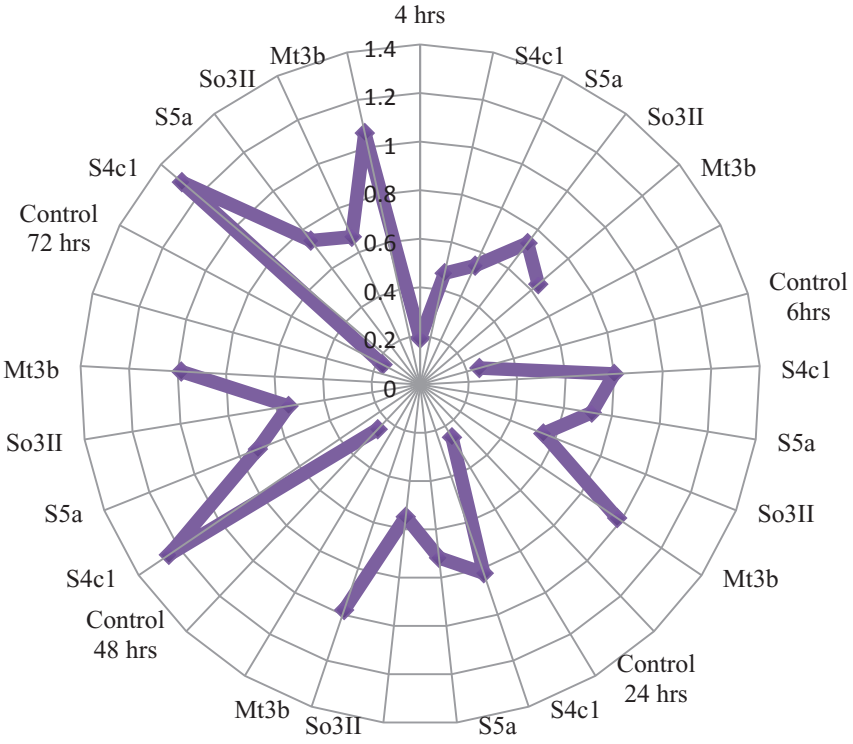

Figure 3: Effect of reaction time on AgNPs synthesis.

reduction of silver ions and stability of silver nanoparticles. The IR spectrum of bacterial AgNPs was scanned in the range of 450-4000 cm^{-1} which showed intense bands at 3466.77 cm^{-1} representing the stretching vibrations of amines while band at 1637.88 cm^{-1} represented phenyl ring substitution and its banding. Also band at 1637 cm^{-1} has shown carbonyl stretch in the amide linkages of protein. NO_2 group exhibited vibrations at 1390 cm^{-1} (Figure 4). For the reduction of Ag^+ to AgNPs, bacterial proteins might have reacted and bound with metals particles and caused its reduction to silver nanoparticles. Bacterial proteins developed a coating on AgNPs resulting in its stability (Figure 4).

3.4.2 Transmission electron microscopic (TEM) analysis

Transmission electron microscopic analysis showed that bacterial AgNPs are poly dispersed, spherical and ranges from 1-100 µm in size (Figure 5).

3.4.3 UV-vis spectroscopic analysis

Serratia marcescens (S4c1) has shown maximum absorbance at 400 nm for both extracellular and intracellular nanoparticles i.e., 1.344 and 1.4293, respectively (Figure 1).

3.5 Antimicrobial activity of bacterial silver nanoparticles

Bacterial silver nanoparticles have shown excellent antimicrobial activity against all bacterial strains used. Strong antibacterial activity was recorded with AgNPs synthesized using *Bacillus subtilis* (Mt3b) against *Butyricum* exhibiting an inhibition zone of 20 mm. AgNPs obtained from *Brevundimonas diminuta* (S5a) proved effective against *Pasturella* with an inhibition zone of 10 mm. Nanoparticles obtained from *Bacillus cereus* (So3II) have shown strong antimicrobial potential against *Aeromonas* with an inhibition zone of 10 mm. AgNPs from *Serratia marcescens* (S4c1) have also shown antibacterial potential against *Aeromonas* with an inhibition zone of 8 mm (Figure 6).

3.6 Antifungal activity of bacterial silver nanoparticles

Bacterial silver nanoparticles showed the potential to inhibit the growth of *Phytophthora palmivora*, a fungus causing several plant diseases. Extracellular nanoparticles obtained from *Bacillus subtilis* (Mt3b) have shown strong antifungal potential against *Phytophthora palmivora*. Extracellularly synthesized

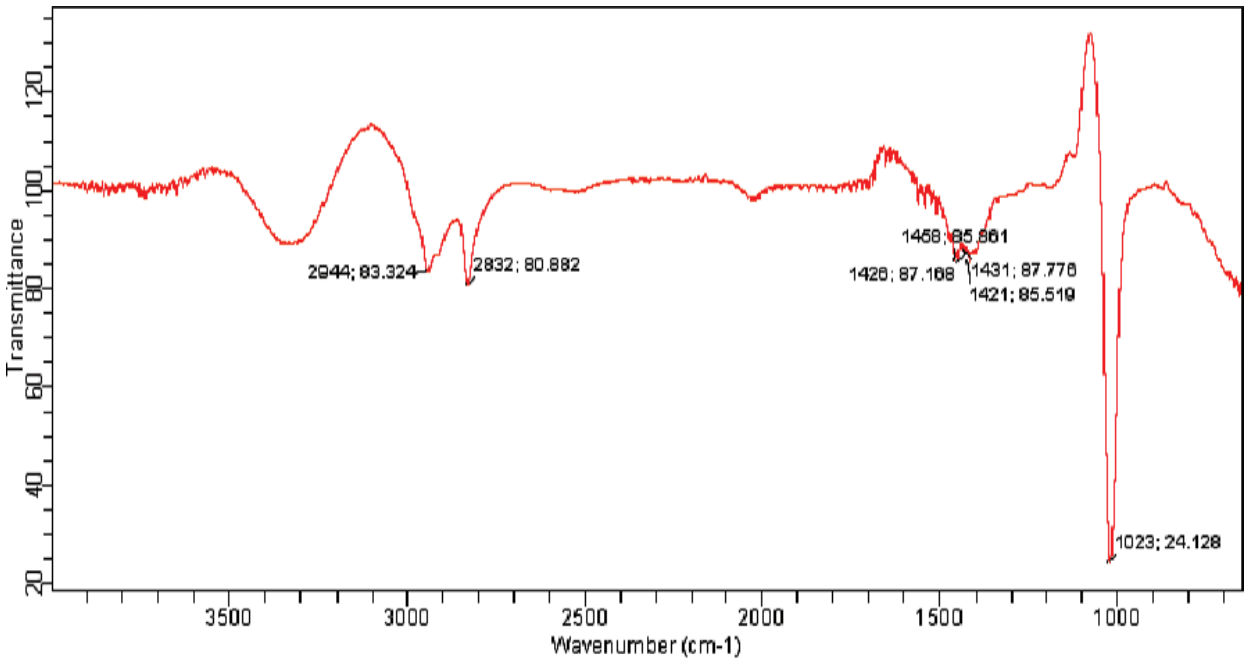

Figure 4: IR spectra of bacterial AgNPs.

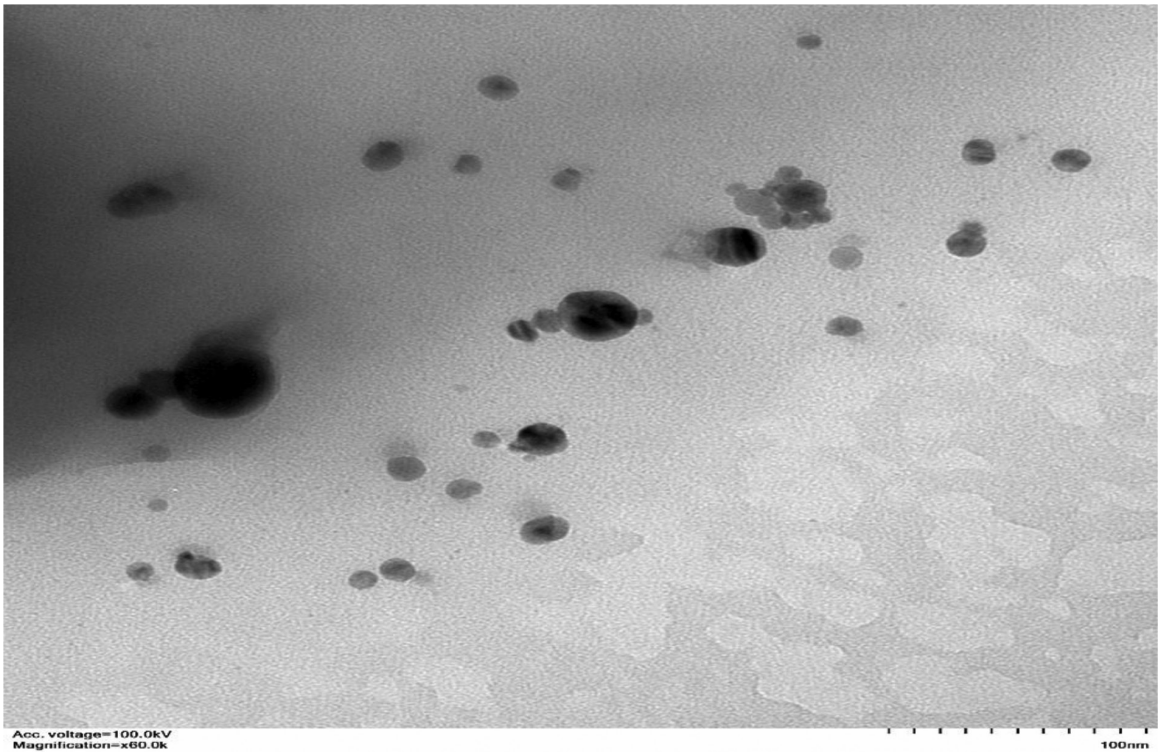

Figure 5: Transmission electron microscopic (TEM) analysis of bacterial AgNPs.

AgNPs obtained from *Bacillus subtilis* (Mt3b) exhibited a percentage radial inhibition of 81.81%. The AgNPs obtained from *Serratia marcescens* (S4c1), *Brevundimonas diminuta* (S5a) and *Bacillus cereus* (So3II) have shown percentage radial inhibition up to 77.27, 63.63 and 45.45%, respectively. Intracellular nanoparticles were not effective as an antifungal agent against *Phytophthora palmivora* (Figure 7).

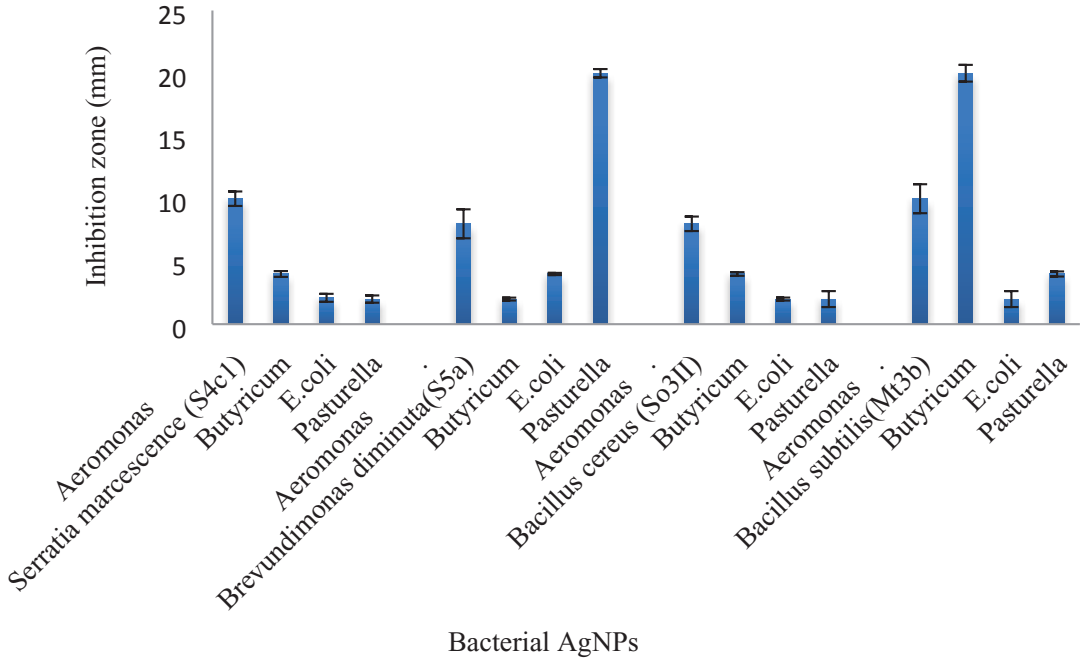

Figure 6: Antimicrobial potential of bacterial AgNPs.

Figure 7: Antifungal potential of bacterial AgNPs.

4 Discussion

Bacterial production of silver nanoparticles is a defensive mechanism against metal toxicity. This defensive mechanism of bacteria is employed in the synthesis of silver nanoparticles and is advantageous over conventional chemical method for being ecofriendly. Bacterial cell wall play a pivotal role in the synthesis of silver nanoparticles because bacterial cell wall is negatively charged and it interacts electrochemically with positively charged metal

ion (Ag^+) and hence, caused bioreduction of metal ions to metal nanoparticles AgNPs [8]. All the four bacterial isolates [*Serratia marcescens* (S4c1), *Brevundimonas diminuta* (S5a), *Bacillus cereus* (So3II) and *Bacillus subtilis* (Mt3b)] used in the current study showed nitrate reductase potential (Table 1). Nicotinamide adenine dinucleotide (NADH) dependent nitrate reductase enzyme is a powerful tool for converting silver ions to silver nanoparticles extracellularly in some bacteria and fungi [9]. All the bacterial strains utilized in the current study have the potential to produce AgNPs by utilizing both extracellular and intracellular method of AgNPs production. The bacterial strain *Serratia marcescens* (S4c1) was most effective in production of bacterial AgNPs (Figure 1). Different factors such as temperature, incubation time and concentration of silver nitrate influence production of silver nanoparticles. Bacterial cell pellet was more effective as compared to cell supernatant for the synthesis of silver nanoparticles in some bacterial strains (Figure 1). The amount of silver nitrate is also an important factor for the synthesis of AgNPs. The production of AgNPs was recorded to be increased exponentially with increase in concentration of $AgNO_3$ but it is not the case for every strain and varies from species to species (Figure 2). Extended reaction time was responsible for the greater production of silver nanoparticles (Figure 3). FTIR analysis confirmed the stabilization of nanoparticles with amines interaction (Figure 4). Surface bound proteins of bacteria play an important role in the production and stabilization of silver nanoparticles. The proteins were observed to cap the nanoparticles using amino acids and amines, hence, caused reduction of Ag^+ to AgNPs [8]. Silver ions (Ag^+) bind with silver nanoparticles which prevent disruption of secondary structure of proteins [9]. Thamilselvi and Radha [6] also reported extended incubation method for the synthesis of silver nanoparticles. Bacterial silver nanoparticles act as an effective biocide agent to inhibit the growth of both gram positive and gram negative bacteria as recorded using disc diffusion method. Six different gram-positive and gram-negative bacterial strains were used in the current study to evaluate the antibacterial potential of bacterial silver nanoparticles. Silver nanoparticles synthesized by *Bacillus subtilis* (Mt3b) were applied to inhibit the growth of bacterial strain *Butyricum* (Figure 6). Bacterial nanoparticles inhibit the growth of pathogenic bacteria which might be due to the production of free radicles from silver which cause lipid degradation of the membrane and hence, change membrane structure and function. Free radicles are also responsible for changing the structure of bacterial cell wall [10]. The mechanism involved in the antibacterial activity of AgNPs is that pathogenic bacterial strains uptake silver ions easily from the surroundings which results in blocking the ATP and DNA synthesis pathways and produce reactive oxygen species (ROS) that results in direct damage of bacterial cell membrane resulting in cell lysis [11]. *Phtyophthora palmivora* is an Oomycetic fungus infecting multiple hosts worldwide like coconut, papaya, mango and many other plants of economic importance. This pathogen is a causative agent of many diseases like kole roga, fruit rot and bud rot. In the current study extracellular AgNPs of *Bacillus subtilis* (Mt3b) was most effective bio-control agent of this fungus (Figure 7). Silver nanoparticles inhibited production of protective substances like spores in fungi. Silver nanoparticles are responsible for the inactivation of sulfhydral group in the fungal cell membrane and produce various toxic compounds hence, disrupt and change the structure of membrane attached proteins and lipids ultimately resulting in cell death of fungus thus exhibiting antifungal potential.

5 Conclusion

In conclusion, the current study has emphasized on the optimization and impact of various factors affecting the synthesis of bacterial AgNPs. In the present wok, bacterial AgNPs synthesized by the bacterial strains exhibited prominent antimicrobial and antifungal potential. In the current era of increasing antibiotic resistance, the antimicrobial activity of bacterial AgNPs can be effectively exploited for human benefit. In vivo analysis is among the future prospects of our current work, which if positive, will allow us to exploit these bacterial AgNPs with nanobiotic potential to help minimize the antibiotic resistance which is causing great harm not only to mankind but to the whole ecosystem.

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