ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES
Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

Biogenic synthesis and characterization of silver nanoparticles from bacteria isolated from garden soil and its antibacterial activity against *Enterococcus faecalis*

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Article History:

Received on: 21.03.2018
Revised on: 11.10.2018
Accepted on: 15.10.2018

Keywords:

Antimicrobial activity,
Green synthesis,
MDR *Enterococcus faecalis,*
Silver nanoparticles

ABSTRACT

Silver is a nontoxic, safe inorganic antimicrobial agent used from decades, which has a diverse perspective in the number of biological applications, predominantly in form of nanoparticles. The present study includes synthesis of silver nanoparticles (AgNps) from the bacterium isolated from garden soil samples. The AgNPs were synthesized by reduction of silver nitrate ($AgNO_3$) solution by the bacterium after incubation for 2 days at room temperature. The synthesis of AgNps was initially indicated by the colour change from pale yellow to brown. Further characterization of AgNps was done using UV-visible spectroscopy, Fourier transforms infrared spectroscopy (FTIR) and transmission electron microscopy (TEM). The synthesized AgNPs were found to be spherical in shape and size in the range of 20 nm as demonstrated by TEM. FTIR spectra confirmed the presence of proteins bound to AgNPs act as reducing and stabilizing agent. Antibacterial assay of the synthesized silver nanoparticles was done against MDR (Multidrug resistant) bacteria *Enterococcus faecalis* by standard NCCLS disc diffusion test. AgNPs were found to have antibacterial activity against *Enterococcus faecalis* at 100µg/ml concentration of AgNps. Therefore, the antimicrobial activity of the synthesized silver nanoparticles proves the application potential of green synthesis in the area of nano-medicine.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v10i1.1774

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INTRODUCTION

Nowadays, the application of nanoparticles in almost various fields has developed considerably which have distinctive physicochemical features, such as high reactivity, a high ratio of surface area to mass and sizes in the range of nanometers. In nano-chemistry, nanoparticles have been used to increase the activity and immobilization of catalysts. In pharmaceutics, it is used for the distribution of therapeutic agents (Zhang *et al.,* 2008; De Azeredo, 2009; Rai *et al.,* 2009; Neouze 2013; Dixit *et al.,* 2017). Over the past decades, metallic silver is widely applied in surgical prosthesis and splints and as fungicides (Forough *et al.,* 2010). Contradictorily, for treating various health illness and disorders, such as epilepsy, gonorrhoea and gastroenteritis, silver metal ions are being used for long. Silver is relatively non-carcinogenic and non-toxic to human nervous, reproductive, immune and cardiovascular systems. Thus, it has been considered as a safe and effective anti-bactericidal metal, as it is highly toxic to bacteria (Chen *et al.,* 2008; El-Kheshen *et al.,* 2012). Hence, silver-based compounds have received much attention as an antibacterial agent for burn care applications (Pasupuleti *et al.,* 2013). In recent years, due to the increasing threat of

antibiotic resistance by various MDR bacterial species which is caused by the misuse of antibiotics, there is an urgent need to find an alternative for the treatment of such MDR bacteria. In this regard the proposed possible antibacterial mechanisms of silver nanoparticles is thought to be effective for inhibition of bacterial growth and proliferation by the adhesion of ultra-small sized silver nanoparticles onto the cell wall of bacteria, resulting in changes in the cell wall which in turn is unable to protect the interior of the cell thus leads to penetration of the silver nanoparticles into the bacterial cell, which damages the DNA, thus leading to cell death (Pal., 2007; Li *et al.,* 2008; Kumar *et al.,* 2016). Due to the antimicrobial nature of AgNps it becomes an alternative for treatment of infections caused by MDR bacteria. In recent years, there has been increasing interest in the biosynthesis and study of silver nanoparticles (AgNPs), because of its antimicrobial properties and nontoxic, safe and eco-friendly nature (Rai., 2009; Eckhardt *et al.,* 2013; Rajora *et al.,* 2016; Sharma *et al.,* 2016; Kumar *et al.,* 2016; Dixit *et al.,* 2017). Moreover, extracellular biogenic synthesis of nanoparticles, especially by bacteria, has been increased due to its easy synthesis, less downstream processing, least toxicity and better optimization control (Kowshik *et al.,* 2002; Gurunathan *et al.,* 2009; Thakkar *et al.,* 2010). A number of bacterial species including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus thuringiensis*, *Bacillus cereus*, etc. (Zhang *et al.,* 2005; Nanda *et al.,* 2009; Jain *et al.,* 2010) have been explored to synthesize AgNPs.

AgNPs were shown to have antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermis* (MRSE) (Nanda *et al.,* 2009; Balaji *et al.,* 2009). This study deals with the biosynthesis of AgNPs using garden soil microflora. Characterization of AgNPs was carried out using FTIR, TEM and spectrophotometric analysis. Furthermore, biochemical characterisation has been done to identify the bacteria involved in the biosynthesis of the nanoparticles and antimicrobial studies have also been done using standard NCCLS disk diffusion method against the MDR bacteria *Enterococcus faecalis* (*Ef*).

MATERIAL AND METHODS

Collection and Isolation of Bacteria

Soil samples were collected from 10 cm deep in a garden area with a sterile spatula. Collected soil samples were taken to the lab within 4 hrs in sterile zip-lock bags. 1gm of soil was serially diluted in PBS and plated onto nutrient agar followed by incubation at 37°C for 24 hours. One most prominent colony was picked and further sub-cultured with Nutrient Broth (NB) and Nutrient Agar (NA) media.

Identification of bacteria

Bacteria were identified by colony morphology, Gram's staining, IMViC test, catalase test and oxidase test (Kumar *et al.,* 2016).

Synthesis of Silver Nanoparticles (AgNPs) from Bacteria

Before final synthesis of AgNP, optimisation study has been done in order to find the best concentration of silver nitrate ($AgNO_3$) for the biogenic synthesis of AgNPs (Table 1). It was found that 2mM $AgNO_3$ gives the best result. Thus, 2mM $AgNO_3$ was used as a standard concentration in this study. 20ml overnight culture (NB) was harvested by centrifuging at 6000 rpm for 10min and the supernatant was transferred in a sterile culture tube. To this, 2mM $AgNO_3$ was added and incubated for 72 hours at 37°C in dark condition. AgNP synthesis was confirmed by a change of broth colour from pale yellow to reddish brown.

Characterization of Silver Nanoparticles

Analysis by UV-Vis spectroscopy

The power of retention or intensity (A) or optical density (OD) as a function of wavelength, is the basis of spectrophotometric analysis. The absorbance is directly related to the path length (L), and the concentration (C) of the absorbing

Table 1: Optimisation of AgNO3 concentration

S. No.	Volume of Sample	Concentration of $AgNO_3$	Volume of Stock Solution
Control	5ml	-	-
1	5ml	0.25mM	6.25µl
2	5ml	0.5mM	12.5µl
3	5ml	1.0mM	25.0µl
4	5ml	1.5mM	37.5µl
5	5ml	2.0mM	50.0µl

Table 2: Colony morphology

Form	Elevation	Margin	Surface	Opacity	Pigmentation
Round	Raised	Entire	Smooth	Opaque	Whitish

particles. Absorption measurements were carried out on Thermo Scientific Biomate-3S Double Beam UV-Visible Spectrophotometer.

FTIR spectroscopy analysis

FTIR measurements were done to detect the possible biomolecules, responsible for the reduction of silver ions to AgNPs and stabilization of AgNPs in colloidal solution. For FTIR analysis, we freeze-dried AgNP samples in Nisco Lyophilizer. The recurrence extent is measured as wave numbers normally over the reach 4000-600 cm^{-1}.

TEM analysis

TEM analysis was done to understand the morphology of AgNPs synthesized. For TEM analysis, carbon-coated copper grids were impregnated with a drop of solution containing synthesized AgNPs and kept in infrared light to dry it up before loading them onto the specimen holder. TEM micrographs were taken and size and shapes were analyzed.

Anti-bacterial sensitivity test

The antibacterial assays were performed by standard NCCLS disc diffusion method against *Ef*.

Antibacterial assay in broth media

Antibacterial assay was performed in suspension culture of *Ef.* The organism was inoculated in nutrient broth in two different culture tubes. One tube was kept as control (no AgNP added) and to the other 50 µg/ml AgNP solution was added. At regular time intervals from 6 hours to 24 hours, optical density (OD) at 600 nm was taken.

Percent kill assay

10 µg, 50 µg, 100 µg AgNP samples were dissolved respectively in 1ml NB media inoculated with the *Ef* culture for antimicrobial testing. 1ml *Ef* inoculated NB media without any AgNP addition was taken as control. Then, all the samples were incubated with shaking at 37^0C for 1h. The samples were then diluted 10-fold and spread plated on NA plates, followed by incubation at 37^0C overnight. The number of the viable cells was determined by colony counting method. Bacterial killing percentage (% kill) is defined as $(N_{control}-N_{sample})/N_{control} \times 100$, where $N_{control}$ and N_{sample} represents the number of bacteria in control and the samples respectively.

RESULTS

Isolation of Bacteria

The most prominent colony was selected and cultured in NB and plated on NA for further analysis. Prominent round whitish opaque colonies were visible throughout the plate (Figure 1).

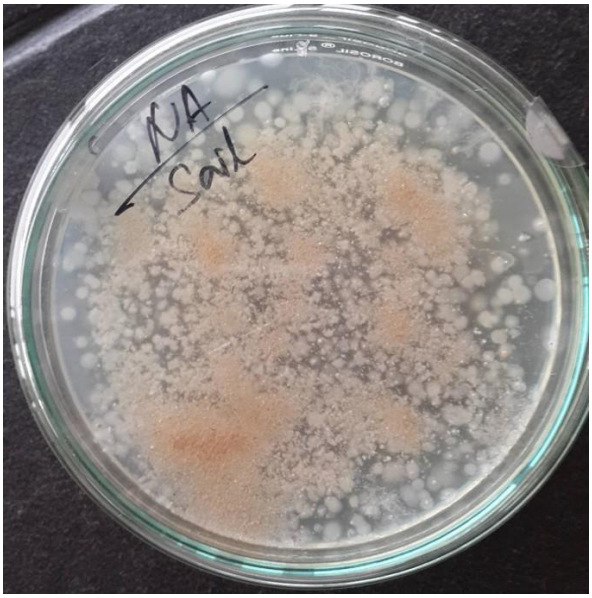

Figure 1: Isolation of bacterial culture

Colony morphology

As shown in Table 2.

Gram's staining

Gram's staining showed that the bacterium is Gram-positive (G+ve) bacilli (Figure 2). Gram-positive cells have a thick peptidoglycan cell wall that is able to retain the crystal violet-iodine complex that occurs during staining. Morphologically, it is, i.e. bacilli. With reference to the results obtained, it may be stated that the colony isolated from the soil sample is *Bacillus sp*.

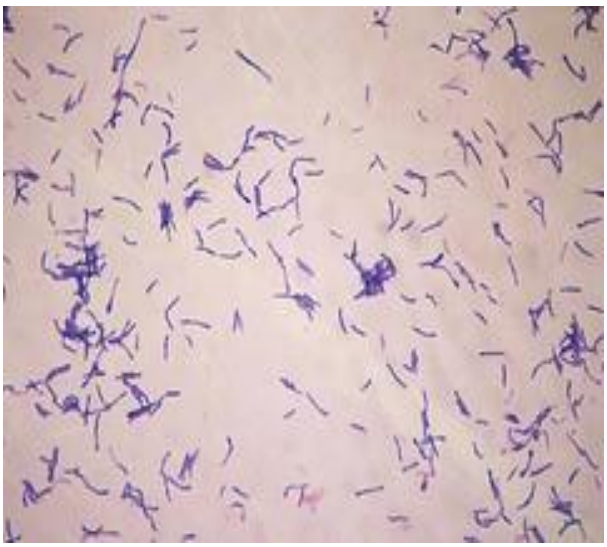

Figure 2: Gram staining showing positive rods

Synthesis of AgNps

After 72 hours, the pale yellow coloured supernatant containing $AgNO_3$ changed to reddish brown because of reduction of Ag^+ to Ag^0 which is due to the excitation of surface plasmon vibrations

in the particles and thus gave a convenient means to visually determine the presence of AgNPs. Uninoculated control showed no colour change. Thus, indicating successful biosynthesis of AgNPs (Figure 3).

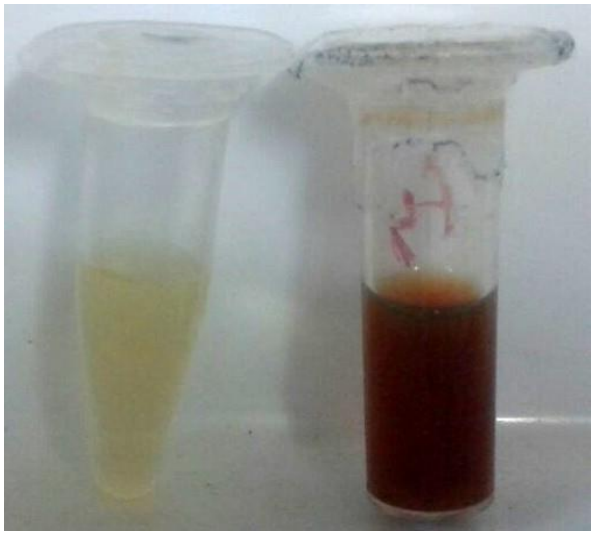

Figure 3: First vial is control and the second vial showing biosynthesized AgNPs

Characterization of AgNps

UV-VIS spectrophotometric analysis confirmed the biosynthesis of AgNPs. The UV-VIS absorption spectra of the synthesised AgNPs were observed in the range of 300-800 nm. A strong peak was obtained at 430 nm, which is specific for AgNP (Figure 4). TEM analysis was done to study the morphological characteristics of synthesized AgNPs which was found to be spherical in shape with 20 ± 5 nm size and well dispersed in the aqueous medium (Figure 5). FTIR analysis was also done to check the presence of proteins around AgNP. FTIR analysis of silver nanoparticle powder is shown in figure (Figure 6). Absorbance bands are shown in the regions 2958.24, 1581.99, 1402.99, and 1060.64cm^{-1}.

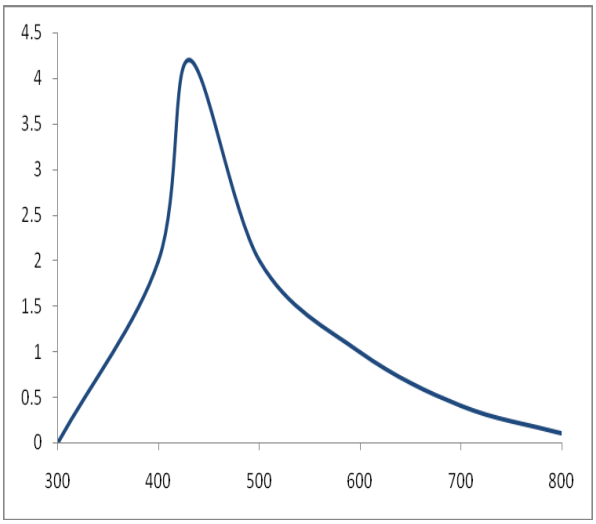

Figure 4: OD scan at UV visible spectra

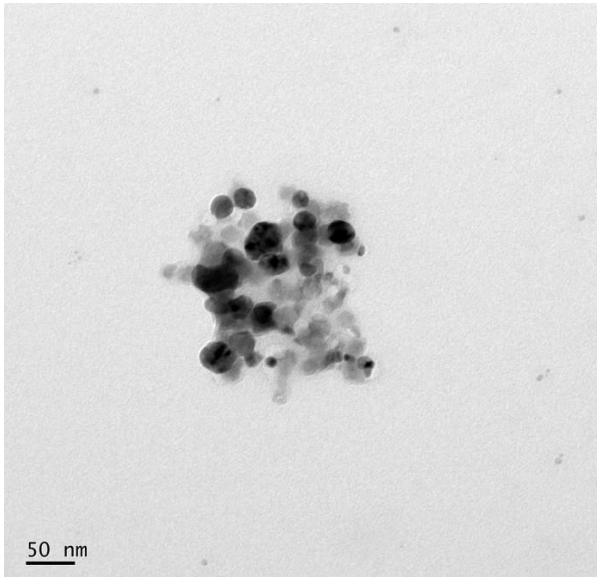

Figure 5: TEM analysis of AgNPs

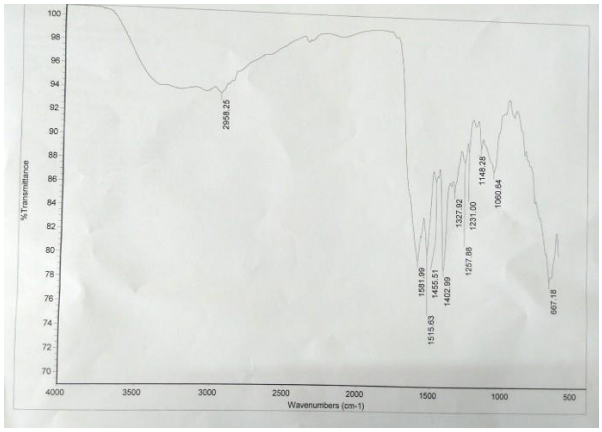

Figure 6: FTIR analysis of AgNPs

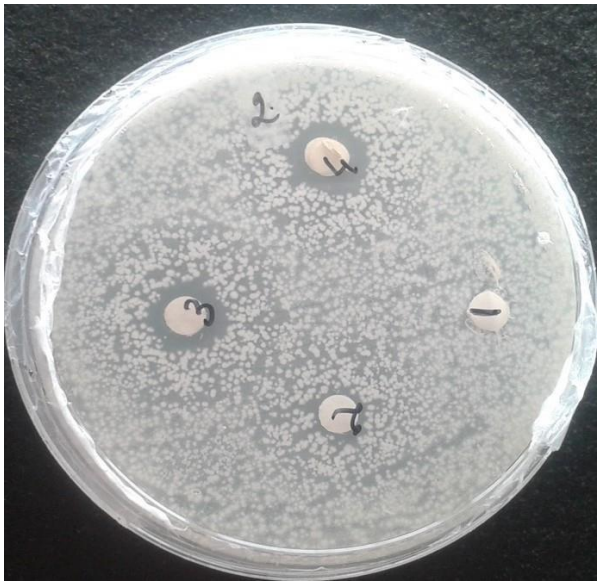

Figure 7a: Disk diffusion test

Antibacterial activity of silver nanoparticles

The antibacterial activity of the synthesized AgNPs was tested against MDR *Enterococcus faecalis* which were determined by NCCLS disc diffusion method (Figure 7a and 7b), antibacterial broth

assay (Figure 8) and percent kill assay method (Figure 9). As indicated from the observations, the zone of inhibition increased with increase in the concentration of AgNps in NCCLS disc diffusion method. Antibacterial broth assay showed negative-to-slight growth of the sample with AgNP while confluent growth is observed for control. Percent kill assay indicated an increase in killing percentage of *Ef* cells with an increase in the concentration of AgNP. These studies showed that 100µg/ml AgNP is the effective concentration to arrest the bacterial growth. Therefore, these studies of antimicrobial activity of the synthesized AgNp prove the potential application of green synthesis of AgNP.

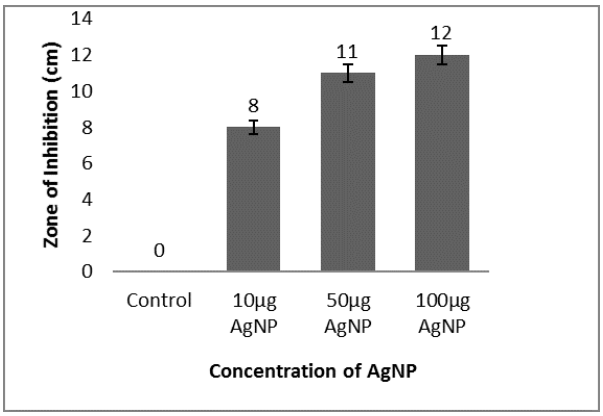

Figure 7b: Graph showing zone of inhibition

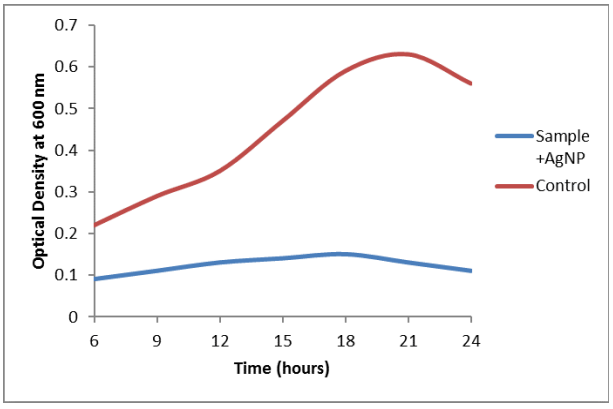

Figure 8: Antibacterial broth assay

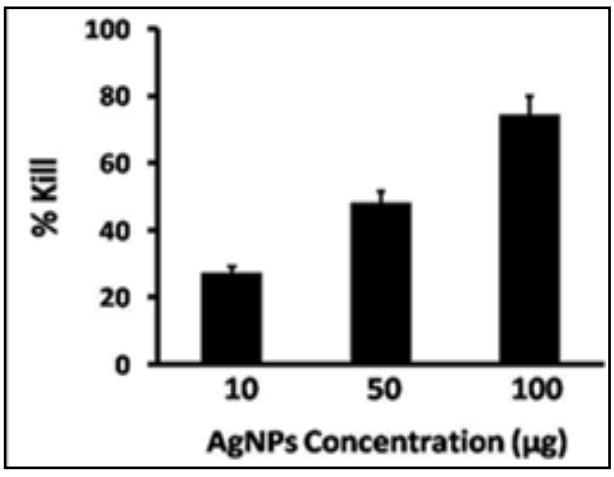

Figure 9: Percentage kill assay

DISCUSSION AND CONCLUSION

Garden soil is an abode of various microorganisms ranging from Actinomycetes to spore-bearing *Clostridium tetani*. But, in this study effort has been made towards employing these microbes in biogenic AgNP synthesis, which is the immense need of the current antibiotic resistance genre. Due to the growing antibiotic resistance, silver has gained interest as an antibacterial agent (Deshpande *et al.,* 1994). Previous studies have reported that the antimicrobial activity of AgNPs is more than the antimicrobial activity of silver metal alone (Kim *et al.,* 2007; Balaji *et al.,* 2009). In the present study, we have studied the synthesis of AgNPs using soil samples and identified the bacterium responsible for it to the genus level by biochemical characterisation. The synthesized AgNPs have been characterised by spectrometry, FTIR and TEM analysis. Further, the antibacterial assay has been done against MDR *Enterococcus faecalis* using the standard NCCLS disc-diffusion, antibacterial broth assay and percent kill assay methods, which had shown that 100µg/ml AgNP is the effective concentration to arrest the bacterial growth and proliferation. Thus, this study supports the effective biogenic AgNP synthesis, its sanctity and its effective antibacterial dose against MDR *Enterococcus faecalis*.

Conflict of Interest

The Authors declare no competing interests.

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