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Biosynthesis, characterization and antibacterial activity of silver nanoparticles by soil fungi *Pencillium sps.*

G Swathi[1], A Sridevi[1], A Sandya[2], B Praveen[1], G Narasimha[1*]

***Corresponding author:**

G Swathi

[1]Applied Microbiology Laboratory, Department of Virology, Sri Venkateswara University,Tirupati-517502Andhra Pradesh India
[2].Department of Microbiology and Biotechnology Sri Padmavati Mahila Visvavidyalayam,Tirupati-517502 India

Abstract

In the present days microbial synthesis of nanoparticles is an eco-friendly green chemistry approach that correlates with nanotechnology and microbial biotechnology. In this study exposure of fungal biomass to aqueous 1Mm $AgNO_3$ solution resulted in the reduction of the metal ions by the nitrate reductase enzyme present in the fungal cell wall membranes and formation of silver nanoparticles. Synthesized silver nanoparticles were characterized using UV-visible spectroscopy, SEM, TEM and FTIR analysis and size and shapes were determined. The synthesed silver nanoparticles were exhibited an excellent antibacterial activities against both gram negative and gram positive pathogenic bacterial strains which causes the diseases in human beings.

Keywords: Silver nanoparticles. Biological synthesis, Characterization, Antibacterial activity

Introduction

Due to increase of microbial resistance to antibiotics, a great care has been taken in the preparation of the nanoparticles, which have applications in a rapidly changing medical field. As new research and experience broaden our knowledge synthesis of nanoparticles by using unicellular or multicellular microorganisms [1,2] like yeast, bacteria, fungi which are eco-friendly in nature [3,4] apart from chemical and physical synthesis. Biological synthesized nanoparticles behave like are new generation antibiotics in the field of medicine because of their good conductivity, chemical stability, catalytic and antibacterial activity [5,6]. The procedures using for the synthesis of nanoparticles should be clean, non-toxic and eco-friendly. Nanoparticles are relatively new in modern medicine.

Silver has a long and intriguing history as an antibiotic in human health care. The antimicrobial properties of silver have been known to cultures all around the world for many centuries. The silver ion is biologically active and readily interacts with proteins, amino acid residues, free anions and receptors on mammalian and eukaryotic cell membranes.

In 1928, Sir Alexander Fleming noticed that the fungi *penicillium* prevented the *Staphylococcus* bacteria from growing and reasoned that the fungus releasing a chemical that was toxic to the bacteria. From this concern in the present study we studied on fungi *penicillium* spp. Which is isolated from soil used as a source for the production of silver nanoparticles (AgNps) It is fact that Microbes secretes more amount of proteins which directly translate to higher productivity of nanoparticle formation [7], and by controlling parameters such as pH, temperature, time, size and shape of the nanoparticle can be strictly controlled [8]. The possible mechanism of silver nanoparticle formation through nitrate reductase, characterization and the antibacterial activity of the silver nanoparticles are reported in the present investigation.

Materials and Methods

In this study, Fungal culture *pencillium sps* (isolated from agricultural soil) obtained from Applied Microbiology laboratory, Sri Venkateswara University, Tirupati and cultured in a liquid medium with the following chemical composition (g/L) $NaNO_3$–2.0; KH_2PO_4–0.1; $MgSO_4\,7H_2O$–0.5; KCl–0.5; sucrose-3.0; streptomycin-0.03. 100 ml medium was inoculated with fungal spores at a rate of 2.0 10^6 and incubated at 28±4°C and speed of 100 rpm in an orbital shaker (ORBITEK LJE). After 72 h of incubation, the fungal biomass was filtered through Whatmann No.1 filter paper. The fungal biomass was washed thrice with sterile double distilled water to prevent the contamination of medium components like sucrose in the formation of silver nanoparticles. Approximately, 10g of fungal biomass was transferred into 250mL Erlenmeyer flask containing 100mL sterile double distilled water and incubated for 72 h in an orbital shaker at speed of 120 rpm and 28±4°C. After incubation, the fungal filtrate was obtained by passing through Whatmann No.1 filter paper. $AgNO_3$ was added to 100 mL of fungal filtrate at a concentration of 1 mM and incubated at 28±4°C and speed of 100 rpm in an orbital shaker. This reaction was carried out for a period of 72h or until reddish brown color formation occurs which indicates formation of AgNps.

Characterization of silver nanoparticles

To verify reduction of silver ions the solution was scanned in the range of 200–600 nm in a UV- Vis spectrophotometer (GENESYS 10 UV- thermo scientific) using a quartz cuvette with water as the reference. After freeze drying of the purified silver metallic nanoparticles, the structure and composition were analyzed by scanning electron microscopy. Samples of the silver nanoparticles were prepared by dispersing the products on a copper flake and then evaporating gold onto them. Scanning electron microscope images were obtained with a JEOL-JSM-6700F instrument operated at an accelerating voltage 5kV. The size and morphology of the nanoparticles were analyzed with a Transmission electron microscope (JEOL model 1200EX). The sample was prepared by placing a drop of silver nanoparticles on carbon-coated copper grids and subsequently the solvent to evaporate, before transferring it to the microscope operated at an accelerated voltage of 80 kV. Fourier transform infrared (FTIR) spectroscopy measurements of the reaction solution, deposited on KBr pellets and dried in vacuum at room temperature were performed on a Niolet Avatar 660 (Nicolet, USA) Fourier transform infrared spectrophotometer between 4000 and 400 cm^{1} with a resolution of 4 cm^{1}.

Nitrate reductase assay

Nitrate reductase is the enzyme that converts nitrate to nitrite. The enzyme activity was measured according to the procedure described by Harley [9] where the activity was measured by putting in the substrate for the enzyme (nitrate) and then measuring the amount of nitrite after 1 h. The net increase in nitrite at 1 h is the amount of nitrate reductase activity. The reagents were: assay medium: 30 mM KNO_3 and 5% propanol in 0.1 M phosphate buffer pH 7.5; nitrite solution: 25µM $NaNO_2$ (Nitrite) solution; nitrite assay reagents: sulphanilamide solution: 1% (w/v) in 25% (v/v) HCl and N-(1-napthy) ethelene diamine dihydrochloride solution (NEED): 0.02% (w/v) in distilled water. The amount of nitrite produced is measured by comparing the tubes between A, B (duplicates) and C, D, E (triplicates) based on the incubation time of tubes in water bath after addition of assay medium. Five tubes (A,B,C,D,E) each containing 5 mL of the fungal culture supernatant, taken from the 5-day incubation culture were prepared. Duplicates (A, B) were placed in boiling water bath for 5 minutes immediately after adding assay medium without any pre-incubation. Whereas triplicates (C, D, E) were mixed with assay buffer and incubated in the dark for 60 minutes before placing in boiling water bath for 5 minutes. After cooling tubes were mixed with 2.5ml of sulphanilamide solution and NEED solution and incubated for 20 minutes at room temperature. After incubation absorbance readings of the tubes were measured at 540nm and amount of nitrite produced is calculated by substracing duplicates from triplicates and amount of nitrite produced is expressed as nmol/hour/ml.

Antibacterial activity of silver nanoparticles

The antimicrobial activity of the synthesized silver nanoparticles was tested using the standard micro dilution method, which determines the minimum inhibitory concentration (MIC) leading to the inhibition of bacterial growth (*E. coli, Pseudomonas, Bacillus and S. aureus*) with different concentrations of silver nanoparticles (25µl, 50µl, 75µl, 100µl). The MIC was read after 24 h of incubation at 37 ^{0}C.

Results and discussions

UV-vis absorption spectroscopy is a proven technique to analyse AgNps [10] and it is well known that the size, shape, stabilization of the silver nanoparticles reflects the absorbance peak [11, 12]. The generation of dark brown color is due to the surface plasmon resonance (SPR) exhibited by the nanoparticles. The UV–vis spectrum in Figure. 1 showed an SPR peak of silver nanoparticles at 420 nm. The SPR peak shifts to longer wavelengths with increase in particle size [13]. The size of silver nanoparticles has a linear correlation with the peak intensity. The mechanism of silver nanoparticle production by fungi is trapping of Ag+ ions at the surface of the fungal cells and the subsequent reduction of the silver ions by the enzymes present in the fungal system [14]. The extracellular enzymes like naphthoquinones and anthraquinones are said to facilitate the reduction [15] postulated that a NADH-dependent nitrate reductase is involved in Ag NPs synthesis by *Fusarium oxysporum.*

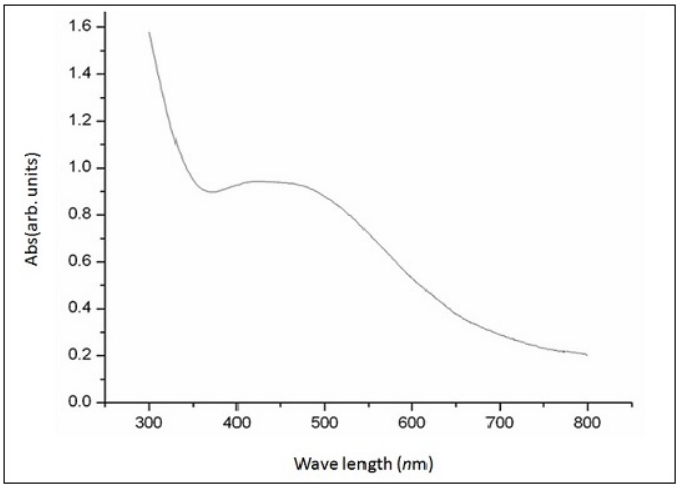

Figure 1. UV-Vis spectrum of Ag nanoparticles synthesized by reduction of Ag ion solution with the pencillium mycelia. The inset shows a digital image of the as-prepared Ag colloidal solution.

Scanning Electron Microscopy (SEM) analysis finally confirmed the synthesis of spherical silver nanoparticles in the reaction mixture (figure.2). The synthesized silver nanoparticles were found to be in the range of 20-60 nm, the larger size of the nanoparticles might be due to the capping of nanoparticles by proteins as confirmed from FTIR analysis.

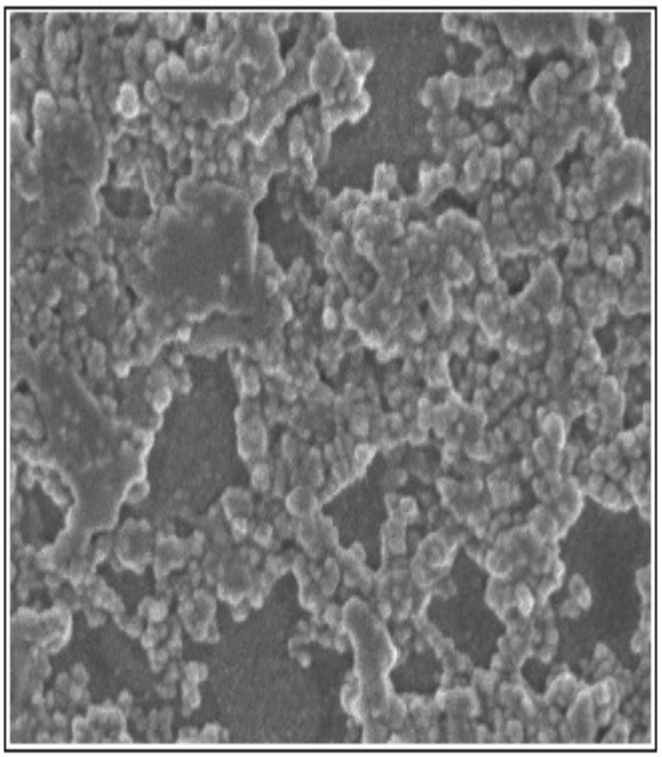

Figure 2. SEM image of synthesized silver nanoparticles

Transmission Electron Microscopy (TEM) Measurements recorded from the microbial synthesized silver nanoparticles at the end of the reaction with mycelia of *pencillium.* The TEM image shows (figure.3) that the silver nanoparticles are polydispersed with a roughly spherical in morphology. The particle size histogram of silver particles Figure. 4 show that the particles range in size from 20 to 60 nm.

Fourier Transform Infrared (FTIR) Spectroscopy Measurements were carried out to identify the potential biomolecules in the mycelia *pencillium* responsible for the reduction of the chloroaurate ions and also the capping reagent responsible for the stability of the bio reduced silver nanoparticles. Figure 5. represents the FTIR spectrum of absorption bands at 1724 cm^{-1} and 1618 cm^{-1}. The shoulder at 1724 cm^{-1} is characteristic of carbonyl stretch vibrations in ketones, aldehydes and carboxylic acids. The 1618 cm^{-1} band is assigned to aromatic C–C skeletal vibrations/N–H deformations, most likely from indoleacetic acid. Among them, the absorption peak at around 1032 cm-1 can be assigned as absorption peaks of -C-O-C- or –C-O-[16]. The absorption at about 1383 cm-1 is notably enhanced indicating residual amount of NO_3- in the solution [17]. Bands at 1513 and 1283 cm-1 were corresponded to the amide II and III bands of proteins respectively [18, 19]. Above all bands confirmed the presence of proteins as capping agent in the reaction solution.

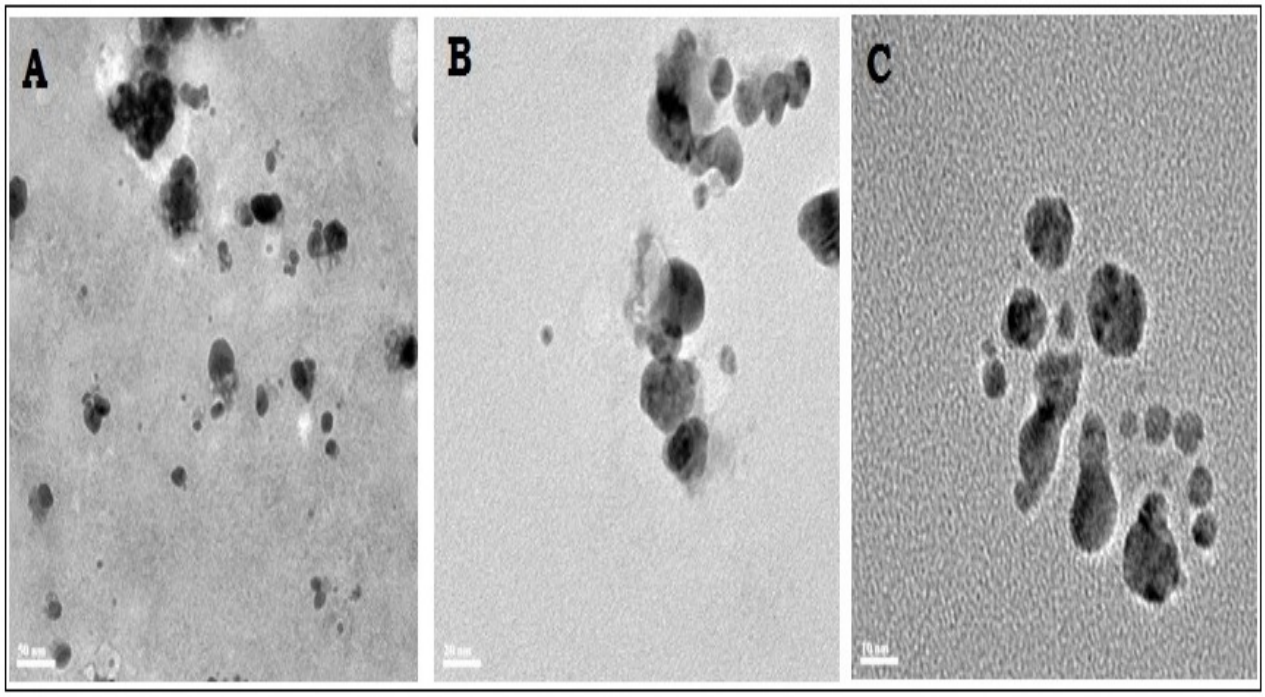

Figure 3.TEM image A, B, C (particle size is measured in different nm (50, 20, 10 nm) of synthesized silver Nanoparticles.

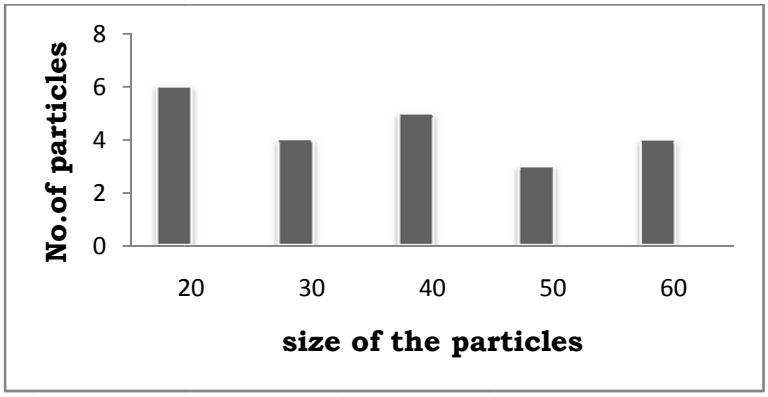

Figure 4. A particle size distribution histogram of as synthesized silver nanoparticles determined from Transmission Electron Microscopy (TEM) images.

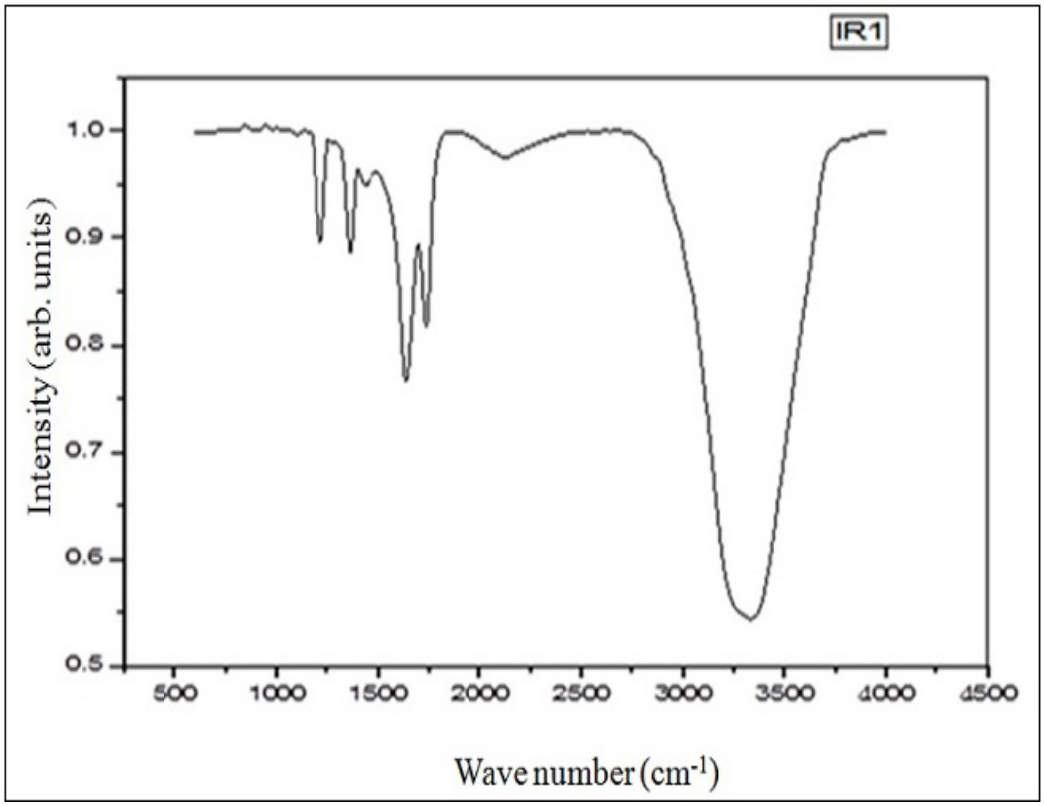

Figure 5. Fourier Transform Infrared Spectroscopy (FTIR) spectrum of silver nanoparticles synthesized by reduction of silver ions by pencillium.

Antibacterial activity

The antibacterial activityof silver nanoparticles were tested against both gram positive and gram negative bacterial strains and shown in figure 6, table 1. The Ag+ ions released from the surface of Ag nanoparticles are responsible for their antibacterial activity [20, 21, 22, 23, 25] for aqueous systems, the results found by Lok et al. 2006 [24] show that the antibacterial activity of Ag+ ions is low at the concentrations levels reached by releasing, and the presence of nanoparticles is vital, which reinforces the idea that the greatest the surface area the greatest the antibacterial activity [23, 26]. The Minimum inhibitory concentration (MIC) of sample is tested against both gram positive (*B. subtilis* and *Pseudomonas*) & gram negative bacteria (*E. coli* and *S. aureus*). The clinical pathogens showed the zone of inhibition (ZOI) i.e Minimum inhibitory concentration (MIC) of Gram negative bacteria is more than that of the Gram positive bacteria as shown in figure 6. As we know specific concentration of metals are toxic to microorganisms which reacts with proteins [27] after penetration into the bacteria causes protein denaturation results in inhibition of complete cellular metabolism and also

capable of inactivating the bacterial enzymes, and in turn releases hydrogen peroxide, leading to bacterial cell death [28] . The formation of clear zone around the cavity is an indication of antibacterial activity. The zone of inhibition of diameters was determined at different concentrations, respectively table 1.

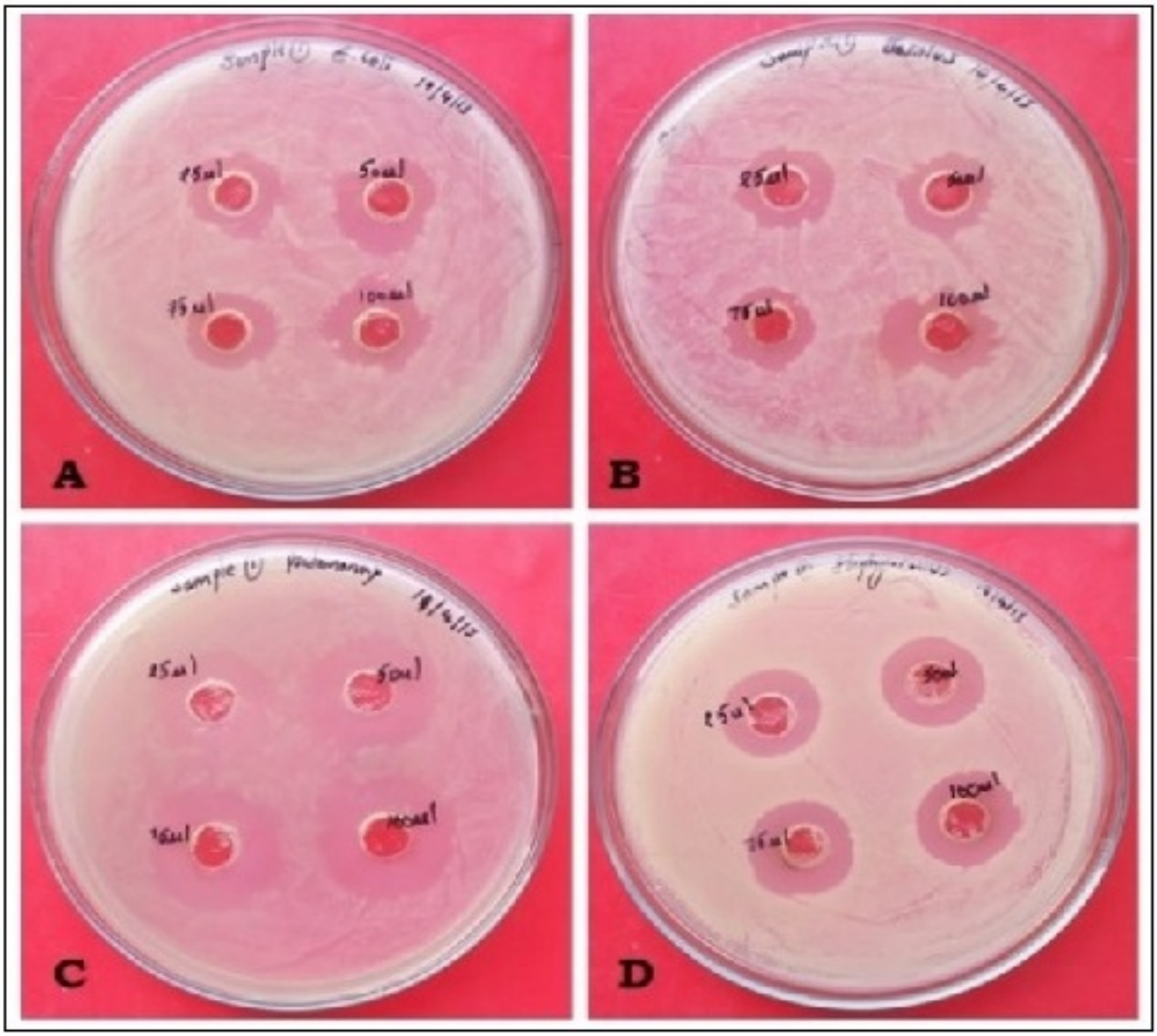

Figure 6. Appearance of inhibitory zones on agar plates at different concentrations of silver nanoparticles. In each figure the concentrations of silver nanoparticles are as 25µl, 50 µl, 75 µl, 100 µl. (*E.coli, bacillus, pseudomonas and staphylococcus*).

Table 1.Inhibitiory activity of silver nanoparticles on bacterial strains.

S.NO	Organism	Zone of Inhibition (mm) at different concentrations(µl)			
		25µL	50 µL	75 µL	100 µL
1	*E.coli*	19	22.75	16.25	24.75
2	*Bacillus*	29.25	27	25.5	39
3	*Pseudomonas*	19.75	27	26.75	27.25
4	*Staphylococcus*	12	12.5	13	13.25

*All values represented the in the table are average zone of inhibition of conducted experiment.

Conclusion

In the present study silver nanoparticles were synthesized from ecofriendly or biological process by fungal strain *pencillium* isolated from soil. The nanoparticles were characterized by standard metods, UV, SEM, TEM, and FTIR analysis. The antibacterial efficacy of silver nanoparticles was tested against gram positive and gram negative clinical pathogenic bacteria strains. Synthesized AgNPs showed an excellent antibacterial efficacies against Gram negative bacteria compared to Gram positive bacteria. Though the Gram Negative bacteria are resistant to many antibiotics and having two layered cell wall our AgNPs have more inhibition on the growth of *Pseudomonas* when compared to *Ecoli*, and inhibition on growth of *Staphylococcus* is more when compared to *Bacillus* .Finally we conclude that the silver nanopatciels synthesised use of physical and chemical synthesis of AgNPs are found to be expensive and there may be effect to AgNPs by various toxic chemicals, whereas biological synthesis is the more preferred option for nanoparticles with high efficacy of antibacterial activity.

Conflict of Interest

All authors declare having no conflict of interest.

Author's contribution

All authors contributed extensively to the work presented in this paper.

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