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Biosynthesis of Silver Nanoparticles using *Bacillus sp.* and Evaluation of its Antibacterial Activity

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ABSTRACT

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Nanotechnology has recently emerged as an elementary discipline of science that explores the interaction of synthetic and biological materials. Nanotechnology is currently employed as a tool to exploit the darkest avenues of medical sciences to combat dreadful diseases caused by drug resistant microbes. Silver nanoparticles (Ag NPs) have been well known for its inhibitory and bactericidal effects. Silver Nanoparticles was synthesized by ecofriendly biogenic approach mediated by using the culture supernatant of *Bacillus sp.* DRI-6. The biogenic silver nanoparticles were characterized by UV-visible spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM) and Transmission electron microscopy (TEM). Ag NPs exhibited maximum antibacterial activity against *E.coli* and *Pseudomonas sp.*

Introduction

Nanotechnology has recently emerged as an elementary division of science that explores the interaction at cellular level between synthetic and biological entities with the help of nanoparticles. ‘Nano’ is a Greek word synonymous to *dwarf* meaning extremely small (Kushwaha *et al.*, 2015). The word “nano” is used to indicate one billionth of a meter or 10^{-9} . Nanoparticles are clusters of atoms in the size range of 1–100 nm. A wide range of nanophasic and nanostructured particles are being fabricated globally with the aim of developing clean, nontoxic and eco-friendly technologies. Use of ambient

biological resources in nanotechnology is rapidly acquiring significant importance owing to its alarming success and simplicity (Sinha *et al.*, 2009). Nanobiotechnology, the combination of biotechnology and nanotechnology greatly focuses on the development of the environmental benign biogenic approach and technology for synthesis of nanomaterials (Sahayaraj and Rajesh, 2011).

Nanobiotechnology combines biological principles with physical and chemical approaches to produce nano-sized particles

with specific functions, representing an economic substitute for chemical and physical methods of nanoparticles formation. Biosynthesis of NP'S can be divided into intracellular and extracellular (Ahmad *et al.*, 2005). Among them, the metallic nanoparticles are considered to be the most promising ones, as they contain significant antibacterial and antifungal properties due to their large surface area to volume ratio, which is of great interest to researchers due to the growing microbial resistance against metal ions, antibiotics and the development of resistant strains (Gong *et al.*, 2007).

Silver nanoparticles (Ag NPs) have several important applications in the field of biolabelling, sensors, antimicrobial agents and filters. They are capable of purifying drinking water, degrading pesticides and killing human pathogenic bacteria (Bhainsa and D'Souza, 2006). Recently, biological synthesis of silver nanoparticles has received a special attention due to environmental friendly green synthesis and easy to scale-up. Many researchers demonstrated that the green synthesis of silver nanoparticles including bacteria, actinomycetes, fungi and plants (Lavanya *et al.*, 2013). The recent advances in researches on metal nanoparticles appear to revive the use of silver nanoparticles (Ag NPs) for antimicrobial applications. Ag NPs have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities for bacteria, fungi, and virus since ancient times (Lok *et al.*, 2006). The mechanism of inhibition by silver ions on microorganisms is partially known. It is believed that DNA loses its replication ability and cellular proteins become inactivated upon silver ion treatment (Gupta *et al.*, 2008). Furthermore, higher concentrations of Ag⁺ ions have been shown to interact with cytoplasmic components and nucleic acids (Kim, 2007; Kumar *et al.*, 2008). In the present study, the ecofriendly biosynthesis of

silver nanoparticles using the culture supernatant of *Bacillus* sp. Strain DRI-6 was mediated. Synthesized nanoparticles were characterized by UV-Visible spectroscopy, XRD, FTIR, SEM and TEM analysis. Furthermore, the antimicrobial activity of synthesized silver nanoparticles against *S. aureus*, *Klebsiella pneumoniae*, *E.coli* and *Pseudomonas* sp. was evaluated.

Materials and Methods

Bacterial Strain Used

The bacterial strain used in this study was isolated from environmental samples including contaminated water samples, effluent samples and soil samples collected from in and around Kanchipuram. Based on the morphological, cultural, biochemical characteristics and 16 s rDNA sequencing, the isolate was identified as *Bacillus* sp. strain DRI-6.

Synthesis of Ag NP's from Culture Supernatant of *Bacillus* sp. Strain DRI-6

The aqueous solution of 1 mM silver nitrate (AgNO₃) was prepared and used for the synthesis of silver nanoparticles. 15 ml of culture supernatant of *Bacillus* sp. strain DRI-6 was added into 200 ml of aqueous solution of 1 mM silver nitrate for reduction into Ag⁺ ions and kept for 15-20 minutes. Culture supernatant acts as reducing and stabilizing agent. The prepared Ag NP's were further characterized (Karthika *et al.*, 2015).

Characterization of synthesized Ag NP's

The techniques used for characterization were as follows:

UV-VIS spectroscopy

Biogenic synthesis of Ag NP's solution with the culture supernatant of *Bacillus* sp. strain

DRI-6 was observed by UV-Vis spectroscopy. Samples were monitored as a function of time of reaction using Shimadzu 1601 spectrophotometer in the 300–800 nm range operated at a resolution of 1 nm. The double distilled water used as a blank reference.

Fourier Transform Infra-Red Spectroscopy (FTIR)

The purified suspension of silver nanoparticles was freeze dried to obtain dried powder. Then, the dried nanoparticle samples, prepared as KBr discs were analyzed by FT-IR Spectrometer for the detection of different functional groups from the region of 400-4000 cm^{-1} .

X-Ray Diffraction (XRD) Analysis

Purified and dried pellet of synthesized Ag NP's were subjected to XRD analysis. For XRD studies, dried NPs were coated on XRD grid, and the spectra were recorded by using Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30 mA with Cu K α 1 radiation.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The particle size and morphology of the silver nanoparticles were examined using Scanning electron microscopic observations. SEM measurements were performed on a JEOL JSM 6390 instrument operated at an accelerating voltage at 15kV. The shape and size of Ag NP's was determined by transmission electron microscopy. The images were obtained at a bias voltage of 200 kV used to analyze samples.

Antibacterial activity of Ag Nanoparticles

The antibacterial effect of Ag NP's was examined against *Staphylococcus aureus*,

Klebsiella pneumoniae, *Escherichia coli*, and *Pseudomonas aeruginosa* by disc diffusion method. The synthesized nanoparticles were diluted with distilled water (15 $\mu\text{g}/\text{ml}$) and placed onto each wells and incubated for 24 hours. Following incubation, the zone of inhibition against nanoparticle was observed and measured (Karthika *et al.*, 2015).

Results and Discussion

Nanobiotechnology combines biological principles with physical and chemical procedures to generate nano-sized particles with specific functions. Nanobiotechnology represents an economic alternative for chemical and physical methods of nanoparticles formation (Ahmad *et al.*, 2005). The biosynthesis of metallic nanoparticles is an active and pronounced area of research in nanotechnology. The synthesis of metal nanoparticles depends on the nitrate reductase enzyme present in the microbes. The mechanism of the biosynthesized nanoparticles involves the reduction of silver ions by the electron shuttle enzymatic metal reduction process. NADH and NADH-dependent enzymes are important factors in the biosynthesis of metal nanoparticles (Kalimuthu *et al.*, 2008). The microbes are known to secrete the cofactor NADH, and NADH-dependent enzymes like nitrate reductase might be responsible for the bioreduction of metal ions and the subsequent formation of silver nanoparticles.

Biogenic Synthesis of Ag NPs using the culture supernatant of *Bacillus sp.* DRI-6

Biogenic synthesis of silver nanoparticles was carried out by using the culture supernatant of *Bacillus sp.* Strain DRI-6. On mixing the culture supernatant of *Bacillus sp.* with silver nitrate solution (1 mM), a change in the color from pale yellow to dark brown was observed. Similarly, Kushwaha *et al.* (2015) reported the biosynthesis and characterization

of Ag NPs from *E. coli*. The brown color confirms the reduction of Ag^+ which indicates the formation of Ag nanoparticles. Various microbes are known to reduce metal ions to the metals. The formation of extracellular silver nanoparticles by photoautotrophic cyanobacterium *Plectonema boryanum* had been described (Langke *et al.*, 2007).

Characterization of Biogenic Ag Nanoparticles

UV-vis spectrophotometer Analyses

The corresponding UV-Vis absorption spectrum showed absorption in the form of a sharp peak between 200-250 nm which indicates the synthesis of silver nanoparticles (Fig 1). The absorption behavior arises due to surface Plasmon resonance (SPR), which originates from coherent oscillations of electrons in the conduction band of nanoparticles induced by the electromagnetic field. Similar results were reported with the silver nanoparticles synthesized with the culture supernatant of *Bacillus licheniformis* and *Streptomyces* sp. JAR1 (Kalimuthu *et al.*, 2008; Chauhan *et al.*, 2013).

FTIR of Ag Nanoparticles

The FTIR spectroscopy is used to probe the chemical composition of the surface and capping agents for the synthesis of NPs (Fig 2). The synthesized Ag NPs showed the presence of bands due to heterocyclic amine, O-H free bond (3280 cm^{-1}), alkanes, O-H bend (2916 cm^{-1}), Carboxylic acid, OH (very broad) (2812 cm^{-1}), arene, = C-H and Carboxylic acid derivative, C-O-H bending (1417 cm^{-1}). Hence, it proves that synthesized Ag NPs have been synthesized with the culture supernatant of *Bacillus* sp. Strain DRI-6 involved in the biological reduction of the AgNO_3 .

X-ray Diffractometer of Ag Nanoparticles

The crystal structure of the AgNPs was analyzed by X-ray diffractometer. X-ray diffraction is a very important method to characterize the structure of crystalline material and used for the lattice parameters analysis of single crystals, or the phase, texture or even stress analysis of samples. X-ray diffractogram of the synthesized Ag NPs showed distinct diffraction peaks at 38.30° , 44.44° , 64.61° and 76.88° which were indexed to the planes 111, 200, 220 and 311 respectively (Fig 3). The sharp peaks and absence of unidentified peaks confirmed the crystallinity and higher purity of prepared NPs.

SEM & TEM Analysis

The morphology and size details of the nanoparticles were analyzed by SEM analyses. The formation of silver nanoparticles as well as their morphological dimensions in the SEM study demonstrated that the average size was from $30 \pm 3\text{ nm}$ with inter particle distance, whereas the shapes were slightly oval to spherical (Fig 4). TEM images revealed that the morphology of Ag NPs are nearly spherical and some non-spherical in nature having particle size less than 100 nm (Fig 5).

Antibacterial activity of Silver Nanoparticles

Exploration of nanoparticles (NPs) as medicines / therapeutic agents is one of the major significance of nanomedicine (Kim *et al.*, 2010; Irache *et al.*, 2011). Ag NPs synthesized using *Bacillus* sp. DRI-6 exerted maximum antibacterial activity against *E.coli* (17 mm) and *Klebsiella pneumoniae* (13 mm) (Table 1). Similar study was carried out by Sadhasivam *et al.* (2010).

Table.1 Antibacterial activity of biogenic Ag NPs against the selected bacterial isolates

S. No	Bacterial strains	Zone of Inhibition
1.	<i>Staphylococcus aureus</i>	9 ± 0.5 mm
2.	<i>Klebsiella pneumoniae</i>	13 ± 0.4 mm
3.	<i>Pseudomonas aeruginosa</i>	8 ± 0.6 mm
4.	<i>Escherichia coli</i>	17 ± 0.8 mm

Fig.1 UV-Vis absorption spectrum of Ag Nanoparticles

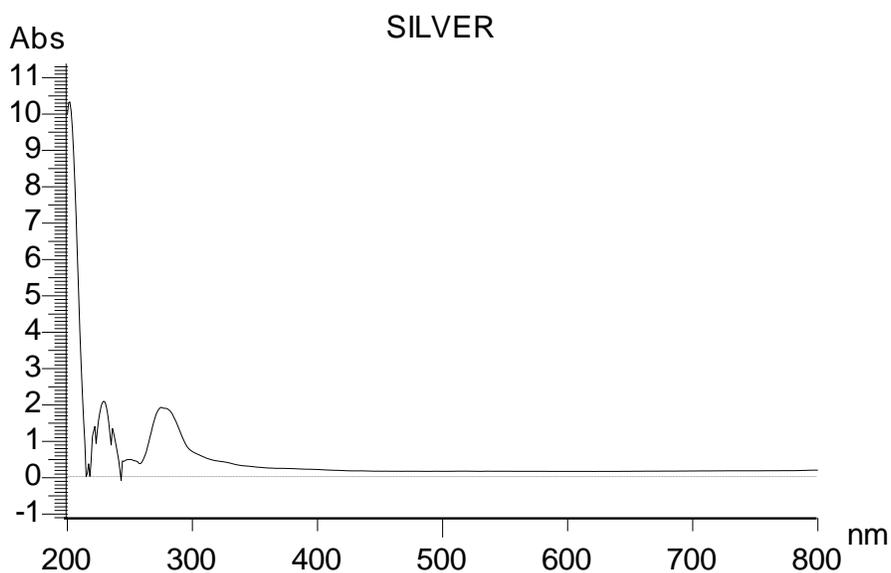


Fig.2 FT IR analysis of biogenic Ag Nanoparticles

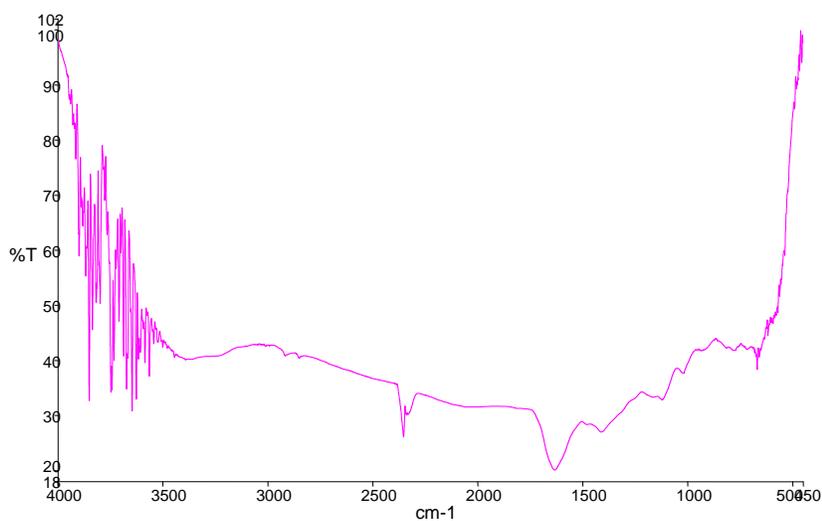


Fig.3 XRD Analysis of Biogenic Ag Nanoparticles

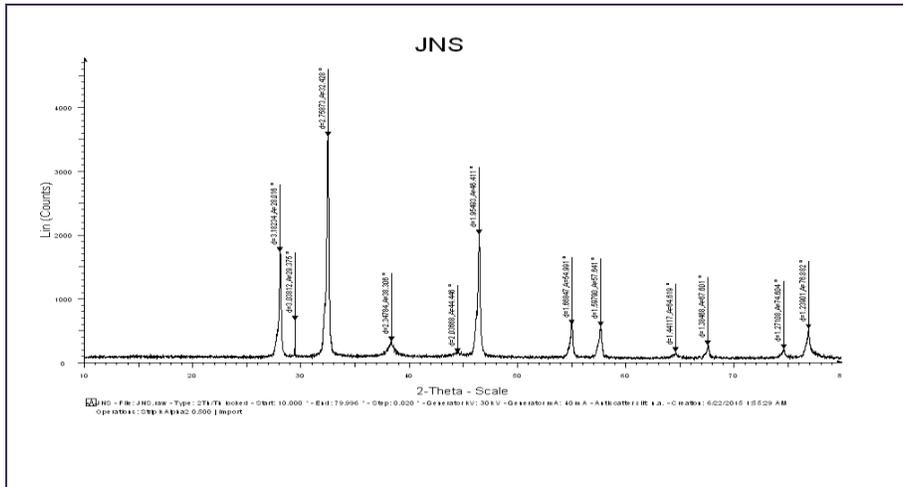


Fig.4 SEM micrographs of biogenic Ag nanoparticles

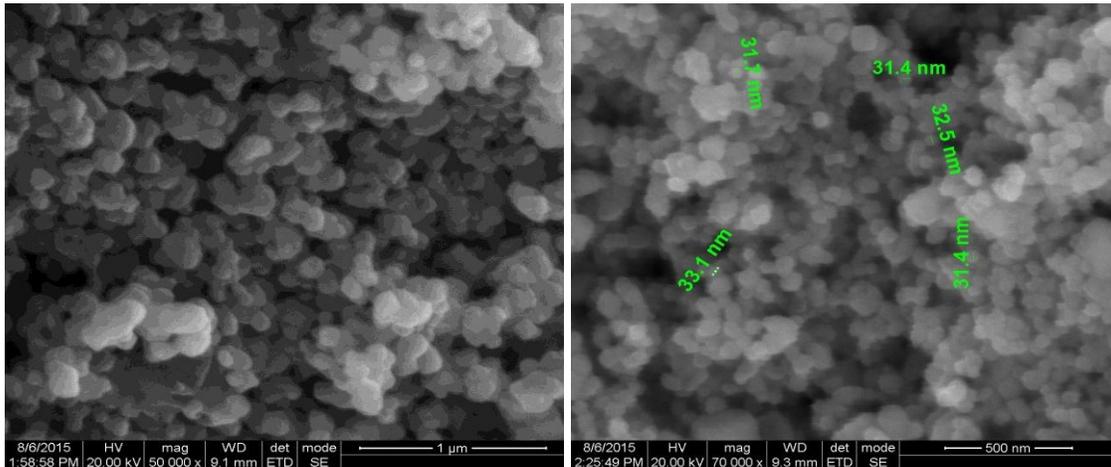
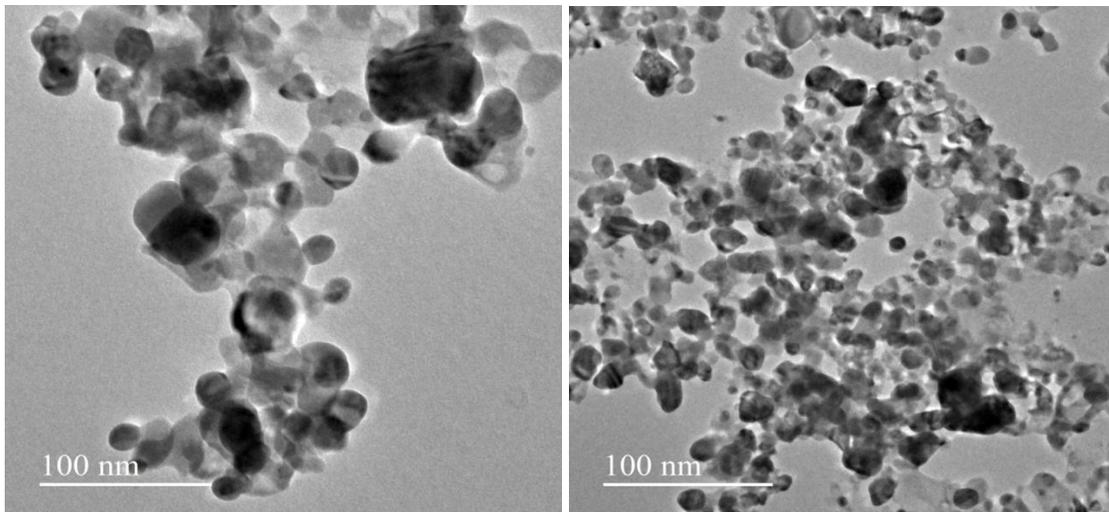


Fig.5 TEM micrographs of biogenic Ag nanoparticles



Silver ions have long been known to exert strong inhibitory and bactericidal effects as well as to possess a broad spectrum of antimicrobial activities. And the acting mechanism of silver has been known in some extent (Rai *et al.*, 2009). Ag^+ inhibits phosphate uptake and exchange in bacterial cells and causes efflux of accumulated phosphate as well as of mannitol, succinate, glutamine, and proline (Schreurs and Rosenberg, 1982).

Tenover (2006) proposed three different mechanisms for the antibacterial activity of Ag NPs. Firstly, Ag NPs attach to the surface of the cell membrane and disturb its power functions, such as permeability and respiration. The binding of the particles to the bacteria depends on the interaction of the surface area available. With a smaller particle size, a large surface area will have a stronger bactericidal effect. Secondly, Ag NPs are able to penetrate the bacteria by possibly interacting with sulfur- and phosphorus-containing compounds such as DNA and cause further damage (Gibbins and Warner, 2005). Thirdly, the silver nanoparticles release silver ions, which contribute to the bactericidal effect (Feng *et al.*, 2000).

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