

BIOSYNTHESIS OF SILVER NANOPARTICLES USING ACTINOBACTERIA AND EVALUATING ITS ANTIMICROBIAL AND CYTOTOXICITY ACTIVITY

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ABSTRACT

Objective: The biosynthesis of silver nanoparticles (Ag NPs) was carried out by using actinobacteria isolated from medicinal garden soil sample, VIT University.

Methods: AgNPs synthesized by mixing culture supernatant with 1mM of AgNO₃ incubated at room temperature under dark condition.

Results: Based on morphological characteristics, 14 strains were selected and screened for the production of AgNPs. Among 14 strains only one strain (PSBVIT-13) showed positive result for biological synthesis of Ag NPs. The biosynthesized Ag NPs were confirmed and characterized by using UV, X-ray diffraction analysis. The shape of synthesized AgNPs was characterized using SEM and confirmed as small spherical in nature. The biosynthesized AgNPs showed activity against *L. monocytogenes* (17±0.15), *S. typhi* (8.63±0.25), *S. aureus* (6.17±0.15), *B. cereus* (6.8±0.2) and *P. aeruginosa* (9.2±0.2). Furthermore, the cytotoxicity effects of biosynthesized AgNPs were tested on brine shrimps. Ag NPs showed 70% of inhibition at the concentration of 50µg/mL and complete inhibition was observed from the concentration of 75 to 100µg/mL.

Keywords: AgNPs, Ag NO₃, Actinobacteria, Antimicrobial activity, Brine shrimp

INTRODUCTION

Nanotechnology is a science of creating new substances and instruments having new and unique properties by self- assembling individual atoms, molecules and molecule clusters into different structures [1]. Nanotechnology has maximized the applications by minimising the size of particles [2]. Their unique size dependent properties make these materials superior and essential in many areas of human activity. Nanotechnology has numerous applications in various fields like medicine, food industries, agricultural fields and many more [3, 4 5]. Many environmental and technological challenges are being solved by nanoparticles of desired size and composition [6].

Currently several techniques are available for the production of silver nanoparticles such as chemical reduction of silver ions in aqueous solutions with or without stabilizing agents, thermal decomposition in organic solvents, chemical reduction and photo reduction in reverse micelles, and radiation chemical reduction have been reported[7]. Most of these methods are extremely expensive and also cause toxic effect to environment. Biosynthesis of Ag NPs is an eco-friendly approach by using different biological sources such as plants and microorganisms like bacteria, fungi and actinobacteria [8]. Synthesizing nanoparticles with desired size and composition are of great interest, as they provide solutions to various environmental and technological challenges. Many microorganisms are reported as being capable of producing Ag NPs. Very few studies have been reported on actinobacteria being capable of synthesizing nanoparticles[9].

Silver has been used for medicinal purpose in the field of health care from ancient period. Ag NPs have been found to be a potent antimicrobial agents and replacing elemental silver as antimicrobial agent. The efficient antimicrobial property of Ag NPs in comparison to other salts is due to their extremely large surface area which leads to more contact with microorganisms [10, 11]. Ag NPs can kill bacteria by attacking their respiratory chain and cell division[12]. Thus it has a great application in the field of medicine and medical research[13].

Ag NPs having many commercial applications in both industrial and as well as health care sector, such as intercalating material for electrical batteries, bio labelling, and optical receptors[14]. The biosynthesized Ag NPs are widely characterized based on their physical and chemical properties. Actinobacteria are gram positive

filamentous bacteria which are widely distributed in both terrestrial and marine environment and have long been exploited commercially as an amusing source of unique secondary metabolites, such as antibiotics, enzymes and enzyme inhibitors[15, 16]. The emergence and proliferation of drug resistance in microorganisms causes resistance to various drug molecules[17]. Hence, the treatments for several diseases are becoming tough task to cure and human mortality rate is also high. Drug resistance in microbial population indicates to develop unique and more effective compounds. The present work was focused on the biosynthesis of silver nanoparticles from actinobacteria and to elucidate its cytotoxic and antimicrobial activity against drug resistance microorganisms.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from medicinal garden of VIT University, Vellore Tamil Nadu India (12.93°N 79.13°E). Soil samples were collected into sterilized container from the depth of 10-25cm and immediately transported to Molecular and Microbiology Research Laboratory and stored in refrigerator at 4°C until further processing.

Isolation of actinobacteria

Collected soil samples were serially diluted from 10⁻¹ to 10⁻⁷ and 100µl of diluted samples were inoculated into different types of media like Actinobacteria Isolation Agar, Kuster's agars and Starch Caesin agar by spread plate technique. All these media are supplemented with cyclohexamide to avoid fungal contamination. Inoculated plates were incubated at room temperature for 7 to 30 days. After incubation period actinobacteria colonies were separated and purified from mother plate[18].

Biological synthesis of silver nanoparticles

The isolated actinobacterial colonies were inoculated into production broth (SS Media) containing soluble starch-25 g, glucose-10 g, yeast extract-2 g, CaCO₃- 3 g, trace elements-1 mL, distilled water-1000 mL. Flasks were lodged on the rotatory shaker at a speed of 120 rpm at room temperature for 7 days. After incubation, the medium was harvested and centrifuged to remove cell debris. The collected supernatant were challenged with 1mM silver nitrate (AgNO₃) as precursor and the flasks were incubated at room temperature in a shaker under dark condition and observed for color change.

Characterization of Biosynthesized Silver Nanoparticles

UV visible spectroscopy

The bioreduction of AgNO_3 in solution was monitored by periodic sampling of aliquots (0.1 mL) of aqueous component and measuring the UV-vis spectra of the solution in 10-mm-optical-path-length quartz cuvettes (U-900, Japan) at a resolution of 1 nm between 200 and 800 nm with a scanning speed of 1856 nm/min. The nanoparticle solution was diluted to 20 times with deionized water to avoid errors due to high optical density of the solution. Presence of Ag NPs was confirmed by comparing obtained peaks with surface Plasmon resonance of Ag[19].

X-ray diffraction (XRD) studies

The bio reduced silver nanoparticles solution was drop-coated onto glass substrate and powder X-ray diffraction measurements (PANalytical X'pert PRO X-ray diffractometer Netherlands). The pattern was recorded by $\text{Cu K}\alpha$ 1 radiation with λ of 1.5406 Å and nickel monochromator filtering the wave at tube voltage of 40 kV and tube current of 30mA. The scanning was done in the region of 2θ from 30° to 80° at 0.02min and the time constant was 2s. The average particle size of the Ag NPs formed in the bio reduction process was determined using Scherr's formula: $d = (0.9\lambda \times 180^\circ) / \beta \cos \theta\pi$.

Scanning Electron Microscopy and Energy-Dispersive Spectroscopy

The morphology of the AgNPs was examined using scanning electron microscopy. The films of the samples were prepared on a carbon coated copper grid by dropping a small amount of the sample and then allowed to dry prior measurements. EDS analysis of the particles revealed a strong signal for Ag at 2.1 keV, characteristic of silver nanoparticles along with the C and O signatures that might be from the stabilizing protein on its surface, an additional peak for Cu was also observed due to the copper grids on which the nanoparticle samples were analyzed.

Antimicrobial activity

Test organism

Clinical isolates were collected from Narayani Hospital, Ariyur, Vellore District, Tamil Nadu, India. Bacterial isolates includes *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Bacillus cereus* and *Listeria monocytogens*. The test organisms were maintained in glycerol stock and stored at -20°C .

Antibiogram

The four clinical isolates were screened for their sensitivity towards standard antibiotics included, Ampicillin (10mcg/disc), Methicillin (10mcg/disc), Vancomycin (30 mcg/disc), Penicillin (10U/disc), Chloramphenicol (30mcg/disc), Drug sensitivity test was performed by disc diffusion method on Mueller Hinton agar (MHA) plates.

Bacterial test pathogens were prepared by inoculating into nutrient broth overnight. Isolates were lawn cultured on Mueller Hinton agar plates using sterile cotton swabs. The standard antibiotics discs were placed on the agar surface using a sterile forceps. Plates were incubated at 37°C for 24 hours and were observed for zone of inhibition.

Antimicrobial assay

Antimicrobial activity of biosynthesized was analyzed for comparative study using agar well diffusion method[20]. The clinical isolates *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Bacillus cereus* and *Listeria monocytogens* were inoculated in nutrient broth and incubated at 37°C for 24hours. After incubation the bacterial cultures were inoculated on Muller Hinton agar (MHA) by using sterilized cotton swabs. In each of these plates, wells were cut out using a sterilized gel borer and 100 μl of biosynthesized Ag NPs were used as a test sample against clinical isolates. Inoculated plates were incubated at 37°C for 24 hours. After incubation, all plates were examined for the presence of zone of inhibition around the wells.

Cytotoxicity bioassay

The brine shrimp lethality assay is an *in vitro* tool for preliminary assessment of cytotoxicity of Ag NPs. The brine shrimp lethality bioassay is a technique used to determine cytotoxic ability of various bioactive compounds [21]. Brine shrimps (*Artemiasalina*) were hatched using brine shrimp eggs in glass flask (1L) filled with sterile seawater under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were collected and placed in test tube containing 4.5 mL of sterile seawater. In each test tube, 0.5 mL of synthesized Ag NPs was added in different concentration (25, 50, 75 and 100 μg) and maintained at room temperature for 24 hours under the light. After 24hours the viable larvae were counted[22].

RESULTS AND DISCUSSION

Isolation and Screening of Actinobacteria for the Synthesis of Nanoparticles

A total of 14 (PSBVIT-1 to PSBVIT-14) actinobacterial strains were isolated from collected soil sample. The isolated actinobacteria are inoculated into production media for screening of biosynthesis of silver nanoparticles. Among 14 strains tested, only PSBVIT-13 strain showed ability to synthesise Ag NPs. The current study was focused on the extracellular synthesis of Ag NPs from actinobacteria. The Ag^+ ions reduction was evidently noticeable when AgNO_3 was added to the supernatant of actinobacteria, and the color changed from yellow to dark brown and in control, there was no color development (Fig. 1). The similar reports of color change during extracellular biosynthesis of Ag NPs were also reported in other studies[23, 24].

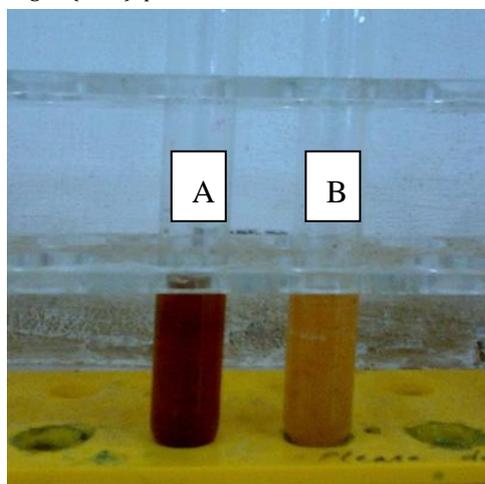


Fig. 1: PSBVIT-13 mediated Ag NPs showing the change of broth color to dark brown. (A) Ag NPs production (B) control broth.

Characterization of biosynthesized Ag NPs

UV and X-ray diffraction (XRD) studies

The absorption spectrum of the Ag NPs showed a surface plasmon absorption band with a maximum of 428nm, indicating the presence of Ag NPs. The sharp narrow absorption peak located between 420 and 450nm for Ag NPs was observed in the present study(Fig 2). Likewise, Bhainsa et al 2006 reported that the biosynthesized Ag NPs are primarily confirmed by using UV-vis spectroscopy and peak was noted at about 420 nm [25].

This absorption peak depends on particle size and stabilising molecules [26]. The obtained XRD spectrum of Ag NPs from the present study was matched with JCPDS card No.010893722 which exhibits the silver peaks observed at 2θ values of 38.28°, 44.27°, 64.64° and 77.66° (Fig. 3). Otari et al 2012 confirmed the biosynthesized Ag NPs by using XRD and the silver peaks noted at 2θ values of 37.8°, 44.1°, 62.9° and 75.9° and JCPDS card 89-3722 [27], which exhibits similar results to current study.

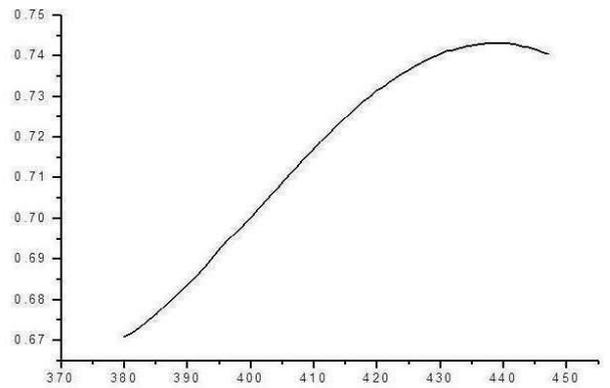


Fig. 2: UV-Vis absorption spectrum of Ag NPs synthesized by PSBVIT-13 treating 1mM AgNO₃ solution with actinobacteria supernatant

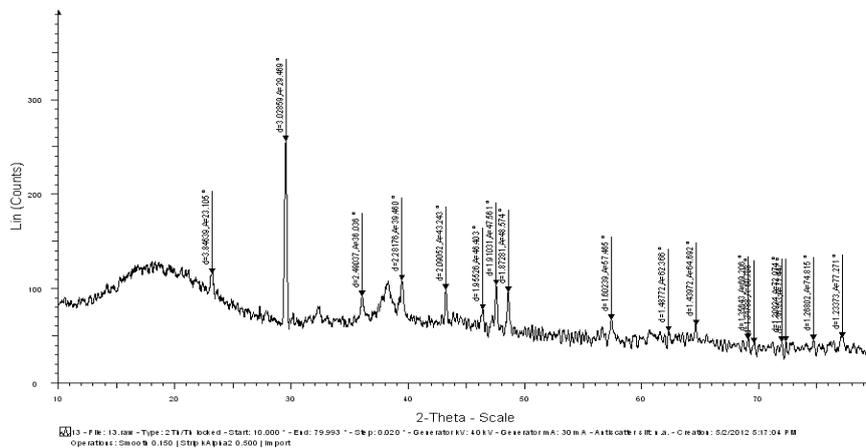


Fig. 3: XRD pattern developed using the Ag NPs synthesized by PSBVIT-13 using 1mM AgNO₃ solution.

SEM and EDS analysis

Vishnu kirthi et al 2012 reported that the fungal mediated biosynthesized Ag NPs are characterized by SEM and confirmed the shape to be round, spherical with aggregates [28]. In this current study also the SEM image showed biosynthesized Ag NPs are round spherical, oval aggregates, in singular form. While EDS

analysis of the particles revealed a strong signal for Ag at 2.1 keV, characteristic of silver nanoparticles along with the C and O signatures that might be from the stabilizing protein on its surface, an additional peak for Cu was also observed due to the copper grids on which the nanoparticle samples were analyzed. The EDS gives the elemental composition of the test substance (Fig. 4).

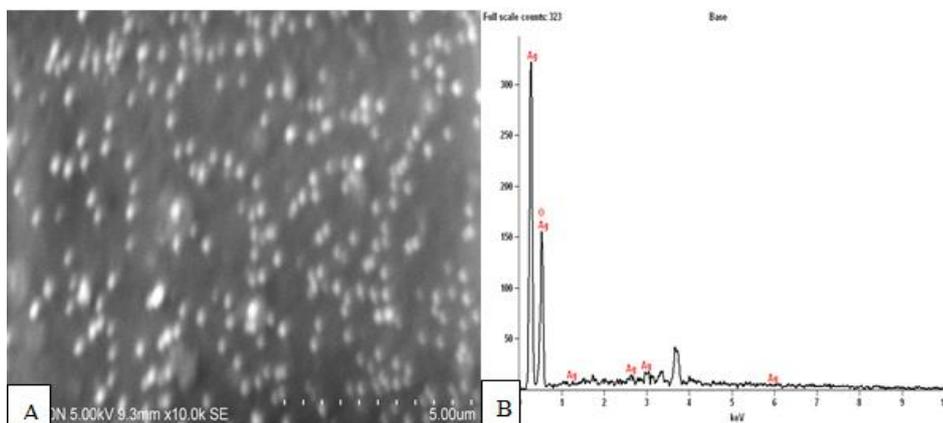


Fig. 4: SEM (A) and EDS (B) analysis of Ag NPssynthesized by PSBVIT-13

Antimicrobial and Cytotoxic Activity of biosynthesized Ag NPs

The clinical isolates were screened for antibiogram by disc diffusion method on MH agar plates. The result exhibits that tested drugs did not showed any zone of inhibition against the test organisms. It concludes that tested clinical isolates are resistant to common antibiotics. The antimicrobial activity of biosynthesized Ag NPs

against clinical isolates were carried out by well diffusion method on Mueller hinton agar plates. The synthesized silver particles showed activity against tested organisms (Table 1).

The result obtained in this study indicates that biologically synthesized silver nanoparticles possess tremendous antimicrobial properties. Sadhasivam et al. 2010 reported antimicrobial activity of

biosynthesized Ag NPs from *Streptomyces hygroscopicus* against *B. subtilis*, *E. coli*, *E. fecalis* and *Calbicans*[29]. The brine shrimp lethality assay is a tool for investigation of in vitro cytotoxic studies. The brine shrimp lethality assay was carried out by using *A. salina* and it directly denotes the antitumor and toxic effect of tested

sample [30, 31]. In this study, we found that 50µg/mL Ag NPs showed 75% of inhibition and complete inhibition observed from the concentration of 75 to 100µg. The toxicity strength of Ag NPs against the brine shrimp caused 100% mortality after 24 h of exposure.

Table 1: Antimicrobial activity of biosynthesized Ag NPs

	Zone of inhibition (mm)				
	<i>L. monocytogenes</i>	<i>S. typhi</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>P. aeruginosa</i>
PSBVIT13	17±0.15	8.63±0.25	6.17±0.15	6.8±0.2	9.2±0.2

CONCLUSION

This study concludes that the biosynthesized Ag NPs from actinobacteria can be a prominent source for the development of various nanomedicines.

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