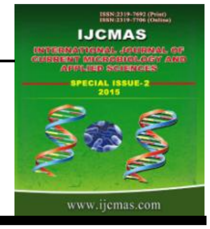

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Original Research Article

Biosynthesis, Characterization and Study of Antimicrobial Effect of Silver Nanoparticles by *Actinomycetes* spp

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A B S T R A C T

Keywords

Kaas Plateau, Tamhini Ghat, Antibiotic Producing Organisms, Silver Nanoparticles, UV-Vis Spectrophotometer, FTIR, SEM, Antibacterial effect

Silver nanoparticles have become prominent in the field of medicine due to their antimicrobial properties. The biosynthesis of silver nanoparticles is preferred over physical and chemical processes as this is an ecofriendly approach. The present study investigated the synthesis of silver nanoparticles from *Actinomycetes*. *Actinobacteria* are gram positive filamentous bacteria which have been explored commercially as a source of secondary metabolites such as antibiotics, enzymes and enzyme inhibitors. Four isolates of *Actinomycetes sp*. showing antibiotic producing activity were obtained from soil samples from Kaas plateau, Satara and one isolate from Tamhini Ghat and were identified using morphological and cultural characteristics. Biosynthesis of silver nanoparticles (Ag NPs) by *Actinomycetes sp.* was achieved by addition of 1mM silver nitrate to the culture supernatant and observed by change in colour after incubation and characterized by means of UV-Visible spectrophotometer, FTIR and Scanning Electron Microscopy (SEM). Antibacterial, effect of the synthesized nanoparticles were studied by disc diffusion assay. The study concludes that biologically synthesized nanoparticles have potential application in the field of medicine due to rapid, eco-friendly synthesis process.

Introduction

Actinobacteria are Gram positive, aerobic bacteria with high G+C content. They are one of the major groups of soil population and are widely distributed. Actinobacteria will provide a valuable resource for novel products of industrial interest, including antimicrobial agents.

Actinobacteria are 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 (Priyaraghini *et al.*, 2013). They play a major role in recycling of organic matter, production of novel pharmaceuticals, nutritional materials, cosmetics, enzymes, antitumor agents, enzyme inhibitors, immune modifiers and vitamins. Specifically, compared with bacteria, fungi and *Actinomycetes* are known to secrete much higher amounts of proteins, there by significantly increasing the productivity of this biosynthetic approach. In addition, *Actinomycetes* are classified as prokaryotes and can be manipulated genetically without much difficulty in the future in order to achieve better control over size and polydispersity of the nanoparticles (Dattu Singh *et al.,* 2014).

Nanotechnology is enabling technology that deals with nanometer sized object. A nanoparticle is microscopic particle with 10 to 1000 nm in diameter (Sohail Yasin *et al.,* 2013). There are various categories of metallic nanoparticles including silver, gold, alloy and other metal nanoparticle (Dattu Singh *et al.,* 2014).

In recent years Nanotechnology has been an area of intense scientific research, due to a wide variety of potential application in biomedical, optical and electronic field and has made tremendous progress and gained a lot of attention for developing nanosized materials showing novel properties different from their macroscopic counterparts.

These novel properties are often associated with the increased surface to volume ratio. Of the different metallic nanoparticles Silver nanoparticles are widely studied owing to their many applications. The conventional chemical and physical method of synthesis of nanoparticles raises concerns for negative impact on the environment. This has created a lot of interest in 'Green Nanotechnology (Devina Merin *et al.,* 2010).

Efforts are been made to investigate environmentally benign materials for synthesis of nanoparticles like plant extracts (Udaya Prakash *et al.,* 2014), bacteria (Dattu Singh *et al.,* 2014; Priyaraghini *et al.,* 2013) and fungi, etc.

Many microorganisms are known to produce nanocrystalline materials with novel properties. Of these, prokaryotic bacteria have been explored most for synthesis of nanoparticles by intracellular or extracellular mechanisms. The exact mechanisms of the synthesis of nanoparticles are still to be elucidated.

The present study investigates silver nanoparticle synthesis using *Actinomycetes spp.* isolated from Kass Plateau and Tamhini Ghat, Maharashtra, India and evaluation of antimicrobial and antidiabetic activities of the synthesized nanoparticles.

Materials and Methods

Collection of samples

The investigation was carried out on soil samples collected from Kaas Plateau and Tamhini Ghat, Maharashtra, India. The samples were collected and transported to lab in Polythene bags.

Isolation of *Actinomycetes spp.*

The *Actinomycetes* were isolated by serial dilution and plating of aliquots on CSA plates. 1 gm of soil sample was added to glass test tube containing 10 ml sterile saline. Serial dilution of that sample were done from 10^{-2} to 10^{-6}.

0.1 ml of each aliquot of dilutions was spread on CSA plate. The plates were incubated as 37^0C for 24 h. colonies were selected and maintained on CSA plates.

Morphological characterization of bacterial isolate

Morphological characterization was done by slide culture technique. The isolate was determined by using Bergey's Manual of Determinative Bacteriology, 9th edition (Holt, 1994).

Synthesis of silver nanoparticles

The organism was inoculated into sterile LB and incubated at 37ºC on closed rotary shaker at 100 rpm for 72 hrs. After incubation broth was centrifuged at 7500 rpm for 15 min. The pellet and supernatant were collected separately in different test tubes. 10 ml of 1mM silver nitrate ($AgNO_3$) was added into 50 ml of culture supernatant. Two controls were kept as sterile LB, supernatant without $AgNO_3$ (Abdeen *et al.*, 2014).

Characterization of silver nanoparticles

UV-Vis spectroscopy

The bio reduction of silver ions was monitored by visual observation of colour change to dark brown band further was confirmed by sharp peaks shown by the absorption spectrum of this solution and recorded by using UV-Vis spectrophotometer. A small aliquot of sample was taken in a quartz cuvette and observed for wavelength scanning between 200-800 nm. The UV-Vis spectrum of silver nanoparticles was obtained by using BioEra's UV-Visible spectrophotometer. Sterile Luria broth was used as reference (Dattu Singh *et al.,* 2014).

FTIR analysis of silver nanoparticles

The silver nanoparticles were subjected to Infrared spectroscopy (FT-IR 8400, Shimadzu) in KBR pellet, in the 4000 to 400 cm^{-1} spectral region at a resolution of 4 cm^{-1}. The liquid sample was added in NaCl cuvettes (Abdeen *et al.,* 2014; Deepa *et al.,* 2013)

SEM analysis

The liquid sample was air dried to obtain the SEM analysis of the nanoparticles. Air dried sample was subjected to Bruker AXS GmbH model D5005.

Determination of Antibacterial effect

The organisms selected for the present study were *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Bacillus* isolated from hospital environment. Pure cultures were maintained on sterile Luria agar slants by frequent subculturing, each organism was subcultured on fresh media prior to testing. Loopful of organism was inoculated in sterile Luria broth and incubated at 37^0 C for 24 hrs. These cultures were used as inoculums in the experiment.

The bactericidal effects of AgNPs on Gram-negative bacteria were studied. The antimicrobial effect of nanoparticles was investigated against three antibiotics (Rifampicin 25µg, Amoxicillin 25µg, Penicillin G 10 IU) using disc diffusion method. 0.1 ml of bacterial culture was spread on sterile Muller-Hinton agar plates.

The soaked and dried discs of 5mm diameter of Whatman filter paper no.1 were placed on seeded plates and gently pressed down to ensure contact (Priyaraghini *et al.,* 2013). Comparative susceptibility of Ag-NPs, $AgNO_3$, Antibiotic, and Antibiotic combined with Ag-NPs was studied after incubation for 24hrs at 37^0C. Zone of Inhibition was measured after incubation (Devika *et al.,* 2012).

Results and Discussion

Isolation of *Actinomycetes*

The *Actinomycetes spp*. was successfully isolated from soil sample (Figure 1).

Characterization of isolated *Actinomycetes spp.*

The isolate (TG-1) characterized by slide culture technique by referring Bergey's Manual of Determinative Bacteriology 9th Edition it may be Genus of *Streptomyces*. (Holt, 1994).

Synthesis and characterisation of silver nanoparticle

The synthesis of NPs was detected by observing the change in colour of reaction mixture from yellow to brown. The formation of brown colour can be taken as indication of synthesis of silver nanoparticles as it is known that silver nanoparticles appear brown colour in aqueous solutions due to surface Plasmon Resonance Effect.

In the present study culture supernatant incubated with $AgNO_3$ showed colour change while culture supernatant incubated without addition of $AgNO_3$ showed no change in colour. Thus NPs has been synthesized in sample and further taken for optical analysis (Figure 2).

UV-Vis Spectroscopy

Synthesis of Silver nanoparticles was confirmed by taking UV-Visible spectra of reaction mixture after regular time intervals at a range of 200nm-800nm.

The spectrum showed a peak at 400nm which is assigned to SPR effect of silver.

FTIR analysis

Further characterization of nanoparticles was done by FTIR to identify possible interaction of biomolecules with silver nanoparticles.FTIR provides the information about functional groups present in the sample which is responsible for the transformation of $AgNO_3$ from simple inorganic to 3 elemental silver. The FTIR studies showed sharp absorption peaks located at 1636.3cm^{-1} and 3358cm^{-1}.

The peak appeared at 3358 cm^{-1} shows that the stretching of bonded hydroxyl (-OH) group and H-bonded. It is the characteristics of N-H stretching in primary/secondary amines. The band seen at 1636.3 cm^{-1} is characteristics of -C=O carbonyl groups and -C=C- stretching (Figure 3).

SEM analysis

The scanning electron micrograph of the silver nanoparticles synthesized after treatment of 1mM silver nitrate solution with aqueous filtrate for 72 hrs, which clearly shows surface deposited silver nanoparticles. The silver nanoparticles synthesized by isolates are irregular in shape (Figure 4).

Antibacterial effect

Antibacterial effect of nanoparticles and antibiotics was evaluated by disc diffusion assay against known human pathogens. The zone of inhibition showed by antibiotics with NPs was relatively larger than the alone antibiotics and NPs. This shows a possibility of synergistic action of nanoparticles and antibiotics (Table 1).

In the present study isolates of *Actinomycetes* were obtained from Tamhini Ghat, Maharashtra, India. Devina Merin *et* *al.* (2010) reported that upon addition of Ag^+ ions into the cell free culture in the dark, samples changed the colour from almost colourless to dark brown with intensity increasing during the period of incubation. It showed no colour change of the cell filtrate when incubated in the same condition. The appearance of a yellowish brown colour in solution was a clear indication of the formation of silver nanoparticles in the reaction mixture. A characteristic surface Plasmon absorption peak at 420 nm was observed at 24 hrs which are similar to our study.

The mechanism involved in formation of metallic nanoparticles may have developed for survival or resistance to metals. Some bacteria reduce metal oxides by producing and secreting small, diffusible redox compounds that can serve as electron shuttle between the microbes and the ion substrate. Though the exact mechanism of silver nanoparticle synthesis is not elucidated it is hypothesized that NADH dependent nitrate reductase enzyme plays an important part in extracellular synthesis of nanoparticles. Nanoparticles were synthesised when supernatant was allowed to react with silver nitrate. This indicates that *Actinomycetes spp* might secrete some extracellular protein or enzyme which is responsible for reduction of Ag ions. The bioreduction of silver from ionic to native form is indicated by change in colour. This change in colour is due to the surface plasmon resonance (SPR) phenomena. The electron cloud at nanometer dimension can oscillate on the surface of the particles and absorb electromagnetic radiation at particular energy. The resonance developed is known as surface plasmon resonance (Smitha *et al.*, 2008).

Similar studies on green synthesis of nanoparticles use UV-Vis spectroscopy, FTIR, and Electron microscopy for characterization of nanoparticles. UV-Vis spectroscopy analysis in present study showed peak at 400nm which is concurrent with SPR of silver. This indicates formation of silver nanoparticles.

Balaji *et al.* (2009) reported FTIR spectroscopic studies on silver nanoparticles obtained from the fungus, *Cladosporium cladosporioides*. Their study confirmed that the carbonyl groups from the amino acid residues and peptides of protein have strong ability to bind silver. The proteins could possibly form a coat covering the metal nanoparticles to prevent their agglomeration and aid in its stabilization in the medium. Hence biological molecules could possibly function in the formation and stabilization of the silver nanoparticles in aqueous medium.

Devika *et al.* (2012) reported that scanning electron microscope surface morphology image showed relatively spherical shape nanoparticles formed with the diameter range 40-50 nm. Deepa *et al.* (2013) reported SEM micrograph of silver nanoparticles synthesized from marine *Actinomycetes*, taken at different magnifications shows size of nanoparticles ranging from 20-30nm. In this current study SEM image showed biosynthesized silver nanoparticles are irregular shape.

The antimicrobial activity of synthesized nanoparticles was investigated against various known pathogens and fungi. The study concluded that nanoparticles showed variable effectiveness against different species of bacteria and exhibited no effect against fungi. Highest inhibition zone was observed against *Pseudomonas aeruginosa* when compared against other Gram negative bacteria. When silver nanoparticles were combined with antibiotic Amoxicillin the effectiveness increased.

Table.1 Antibacterial effect of silver nanoparticles against Gram negative pathogenic bacteria

Study No.	Organism	Name of Antibiotic	Zone of diameter in mm			
			Silver nitrate	Nanoparticles + Antibiotic	Nanoparticles	Antibiotic
1	*Escherichia coli*	Penicillin G	6	10	8.5	8
		Rifampicin	6.5	8.5	9	9
		Amoxicillin	9	9.5	9	8.5
2	*Klebsiella pneumoniae*	Penicillin G	7.5	7	6.5	6
		Rifampicin	7.5	8	6	9
		Amoxicillin	6	Irregular zone	9	7.5
3	*Pseudomonas aeruginosa*	Penicillin G	6.5	11.5	6.5	11
		Rifampicin	6.5	13.5	7.5	20
		Amoxicillin	10	17.5	7.5	6.5

Figure.1 Isolated colonies of *Actinomycetes spp.*

Figure.2 Synthesis of silver nanoparticles of *Actinomycetes spp.* treated with $AgNO_3$.
(a) Supernatant without $AgNO_3$, (b) formation of AgNPs

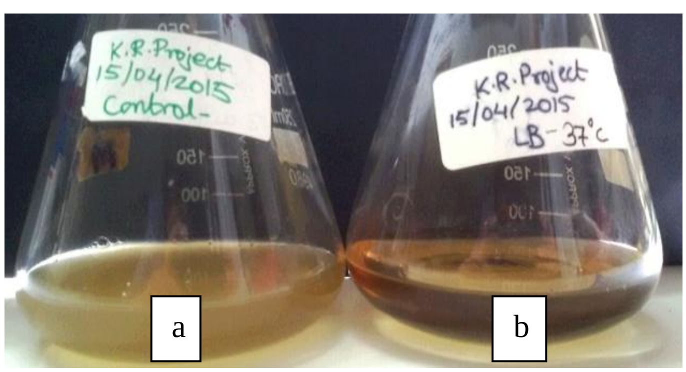


Figure.3 FTIR spectra of Ag NPs synthesized from *Actinomycetes spp.*

Silver Nanoparticles (%T)

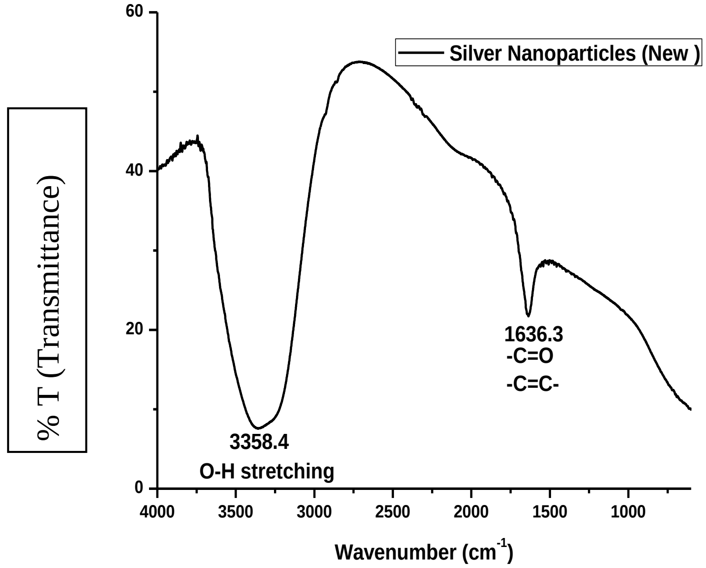
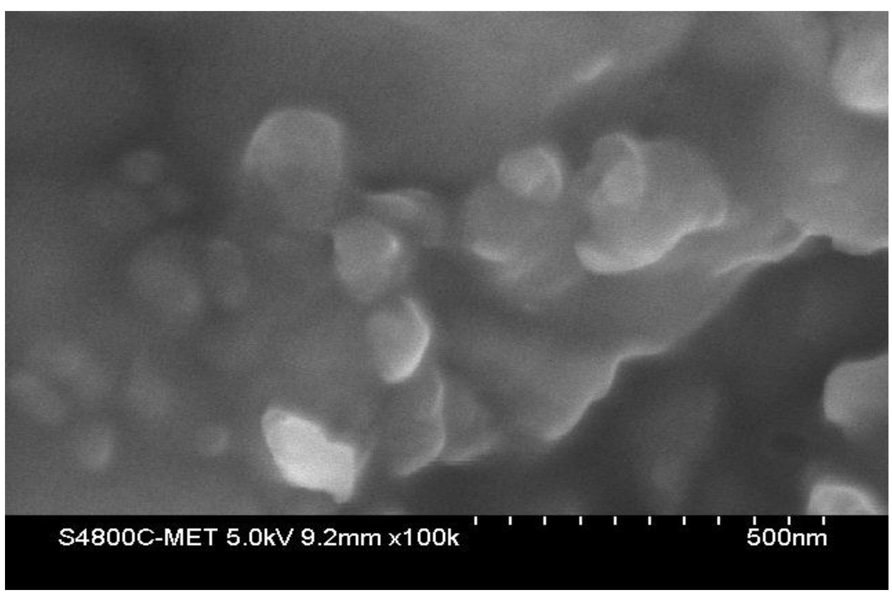


Figure.4 Scanning electron microscope (SEM) Surface morphology image of AgNPs

SEM Analysis



These studies suggest that the synthesized nanoparticles are effective against Gram negative organisms and can be explored as carriers. The similar study was carried out by Devika *et al.* (2012) and they got similar results. So the nanoparticles and antibiotics acts synergistically against Gram negative organisms.

The present study deals with green synthesis of nanoparticles using *Actinomycetes sp*. and investigation of their antimicrobial activities against various known pathogens.

The present study concludes that silver nanoparticles synthesized by *Actinomycetes* have a potential as antimicrobial compound against hospital isolates mostly *Pseudomonas aeruginosa,* and may be used in treatment of diseases caused by them. Silver nanoparticles when combined with antibiotic show maximum zone of inhibition. Thus they may have potential in drug delivery.

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