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Biosynthesis of Silver nanoparticles from *Actinomycetes* for therapeutic applications

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ABSTRACT

Silver is composed of a large percentage of silver oxide due to their large ratio of surface to bulk silver atoms. Silver nanoparticles have been a potent antibacterial, antifungal, anti-viral and anti-inflammatory agent. A simple, eco-friendly, inexpensive biosynthetic method was employed to synthesize silver nanoparticles. The present study aimed at the comparative study of silver nanoparticles synthesized through microbial and chemical methods. A microbial route to synthesize silver nanoparticles by *Actinomycetes sp.* was done. Actinomycetes are aerobic, Gram-positive bacteria, generally exhibiting branched filamentous growth and contain high guanine plus cytosine content in their DNA. Chemical methods were employed to synthesize silver nanoparticles. Chemical reduction of silver ions (Ag^{+}) using sodium borohydride in aqueous solution generally yields silver nanoparticles with particle diameters of several nanometers. It is observed that reduction is slow in chemical methods as compared to rapid microbial synthesis of silver nanoparticles. The obtained silver nanoparticles were characterized using UV-vis spectroscopy and TEM. TEM images of microbially synthesized silver nanoparticles were of smaller size (10-20 nm) compared to chemical methods (60-80 nm). The microbially synthesized silver nanoparticles using *Actinomycetes* were found to be highly toxic to bacteria and it was found that smaller silver nanoparticles synthesized by microbial route had a greater antibacterial activity when compared to their chemical moieties.

Keywords: *Silver nanoparticles; Microbial synthesis; Green synthesis; Chemical synthesis; Actinomycetes; Antibacterial activity.*

INTRODUCTION

Silver nanoparticles are in high demand due to their widespread use. Among noble-metal nanomaterials silver nanoparticles have received considerable attention due to their attractive physicochemical properties. The surface plasmon resonance and large effective scattering cross section of individual silver nanoparticles make them ideal candidates for molecular labelling [1].

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Ionic silver is highly toxic to most bacterial cells and has long been used as a potent bactericidal agent [2]. However, several silver-resistant bacterial strains have been reported and even shown to accumulate silver nanoparticles in their periplasmic space [3-5].Silver nanoparticles are used as antibacterial agent because of their high reactivity that is due to the large surface to volume ratio. Antibacterial activity of the silver-containing materials can be used, for example, in medicine to reduce infections as well as to prevent bacteria colonization on prostheses [6], catheters [7,8], vascular grafts [9], dental materials [10], stainless steel materials and human skin[11,12].

Microorganisms are being used as ecofriendly nanofactories for bioproduction and synthesis of different compounds of nanometer size [13-14]. Both microorganisms and metals are interactive and the microorganisms have the ability to extract or accumulate metals. Biological synthesis of such nanomaterials has gained significant interest due to the use of mild experimental conditions of temperature, pH and pressure. Biological synthesis could present extra advantages over chemical methods such as higher productivity and lower cost. Bacteria when exposed to metals or other toxic substances beyond a certain level they develop many mechanisms such as efflux, alteration in the solubility and toxicity by change in redox state of the metalion, extracellular complexation or precipitation of metals and the lack of specific metal transport systems [15-17]. Various microorganisms for instance *Bacillus subtilis* [18,19] *Pseudomonas stutzeri* strains have been used to reduce silver ions to silver nanoparticles[20]. Fungi like *Fusarium oxysporum and Vericillium* were used to produce Magnetite, Silica and Titania *Klebsiella aerogenes* was manipulated to produce CdS nanoparticles extracellularly [21- 23].

Chemical reduction is the most frequently applied method for the preparation of silver nanoparticles as stable, colloidal dispersions in water or organic solvents. Commonly used reductants are borohydride, citrate, ascorbate, and elemental hydrogen. The reduction of silver ions (Ag^+) in aqueous solution generally yields colloidal silver with particle diameters of several nanometers. The large scale synthesis of silver nanomaterial by chemical method suffers from issues such as polydispersity and stability, especially if the reduction is carried out in aqueous media.

The extracellular biological synthesis of AgNPs could be an attractive and ecologically friendly alternative method for the preparation of large quantities because it offers the advantage of easy downstream processing. Moreover, bacteria are easy to handle and can be manipulated genetically without much difficulty. Considering these advantages, microbial synthesis could prove to be an excellent alternative for the extracellular synthesis of AgNPs. The present work involves synthesis of silver nanoparticles by three methods and further evaluating the antimicrobial activity of these biologically synthesized nanoparticles.

EXPERIMENTAL

Microbial synthesis of silver nanoparticles using Actinomycetes sp.

- ***Source of actinomycetes***

The actinomycete strains were isolated from heavy metal polluted and non-polluted soil samples. The isolated strains were maintained in Potato Dextrose Agar slants (supplemented with cyclohexamide [25 µg/ml] and nalidixic acid [10 µg/ml] as anti fungal and antibacterial compounds respectively) at 27^{o}C as well as subcultured to regulate its viability during the study period.

- ***Identification of actinomycetes***

In the present investigation, four genera of *actinomycetes* which included 10 different species were isolated from the heavy metal polluted and non-polluted soil samples. The species identified in the non-polluted soil, were *Streptomyces* sp. I, *Streptomyces* sp. II, *Streptomyces* sp. III, *Rothia* sp., *Actinomadura* sp. and *Rhodococcus* sp. In the heavy metal polluted soil sample four species were identified which were, *Streptomyces* sp .I, *Streptomyces sp. II*, *Streptomyces* sp. III and *Rhodococcus* sp. The results revealed that *Streptomycessp*, was the predominant *actinomycetes* in both the soil samples.

- ***Screening of actinomycetes (Nitrate reduction test)***

Screening of actinomycetes was performed using nitrate reduction test. Nitrate broth

was prepared and sterilized properly. The isolated ten strains (P1, P2, P3, P8, NP1, NP2, NP4, NP5, NP7, NP10) grown in PDA broth were subcultured individually in 50 ml nitrate broth in 100 ml Erlenmeyer flask and incubated at 37^{o}C for 24 - 48 hours. At the end of incubation, 1 ml of alpha naphthylamine reagent and 1 ml of sulfanilic acid reagent were added to the test medium and the color change was observed. If there was no change in color, zinc metal dust was sprinkled and observed for any notable changes.

- ***Production of biomass***

For the production of biomass, the *actinomycetes* strains such as *Streptomyces* sp. I(P1), *Rhodococcus* sp. (P8) and *Streptomyces* sp. II(NP1) were grown aerobically in actinomycetes broth. The culture flasks were incubated on room temperature at 37^{o}C. After 15 days of incubation period, biomass was harvested using Whatman filter paper. The harvested biomass was washed with double distilled water and the biomass was filtered. The filtered biomass was immersed in double distilled water for 24 hours. After incubation, the biomass was separated from the layer of water (cell free filtrate) for further studies.

Actinomycete mat (20 gm) was obtained from *actinomycete* broth and used for the synthesis of silver nanoparticles. The harvested cell filtrate was suspended in sterile double distilled water for 1 day. After one day incubation, the cell filtrate (biomass) was obtained by passing it through Whatman filter paper. 20 gm of biomass (fresh weight) was grind well using mortar and pestle and mixed with 200 ml of Millipore water in a 500 ml Erlenmeyer flask and agitated in the same condition for 72 hour at 37^{o}C. The same was repeated for the cell free filtrate (filtered water) along with experimental flask (20 ml filtered water in 200 ml of Millipore water). Biomass and water samples were randomly named as B and W. The biomass strains were named as BP1, BP8 and BNP1. The filtered water samples were named as WP1, WP8 and WNP1.For the synthesis of silver nanoparticles, 50 ml of 1 mM $AgNO_3$ solution was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated at 37^{o}C in darker condition. The same was done for the cell free filtrate (50 ml of 1 mM $AgNO_3$ solution with 50 ml filtered water). Simultaneously, control without silver ions was also run along with the experimental flasks. Colour change was noted at specific intervals (12 hours and 72 hours). The nanoparticles were characterized by UV-visible spectroscopy and Scanning Electron Microscope (SEM) analysis.

Chemical synthesis of silver nanoparticles

About 10 ml of 1.0 mM silver nitrate was added drop wise (about one drop/sec) to 30 ml of 2.0 mM solution of sodium borohydride. The reaction mixture was stirred vigorously on a magnetic stirrer plate. The solution was turned light yellow after the addition of 2 ml of silver nitrate and brighter yellow when all of the silver nitrate had been added. After which the stirring was stopped and the stirrer bar was removed. The presence of silver nanoparticles can be identified by color change.

Characterization of synthesized silver nanoparticles

- ***UV- Vis spectroscopy***

Silver nanoparticles synthesized by all the three methods were analyzed for UV-Vis spectroscopy. The UV-Vis spectroscopy measurements of silver nanoparticles were recorded on Systronic double beam spectrophotometer: 2202. Microbially synthesized silver nanoparticles were measured in a wavelength of 420 nm. Chemically synthesized silver nanoparticles were measured in a wavelength ranging from 200-1100 nm.

- ***Transmission Electron Microscopy***

The size and morphological characterization of the silver nanoparticles were carried out using a Hitachi H 7650 transmission electron microscope (TEM) operating at 200 kV. TEM samples were prepared by dispersing nanoparticles in acetone for 30 min by ultrasonic vibration. The aqueous dispersion was dropped on a carbon coated copper TEM grid with filter paper underneath to absorb the acetone and dried in vacuum.

- ***Antibacterial activity of silver nanoparticles against human pathogens***

The identified *actinomycetes* were tested for their antibacterial activity by disc diffusion method against pathogenic organisms like *Staphylococcus aureus*, *Klebsiella pneumoniae*,

Proteus vulgaris, *Pseudomonas aeruginosa* and *Escherichia coli*.

RESULTS AND DISCUSSION

The appearance of a dark brown color in the biomass after reaction with the Ag+ ions is a clear indicator of the reduction of metal ions and formation of silver nanoparticles. Silver nanoparticles exhibit light yellow to brown for silver due to excitation of surface plasmon vibrations in the particles. Microbially synthesized Silver nanoparticles are found to have characteristic absorption peak at 415 nm (Figure 1) indicating the formation of nano silver particles.

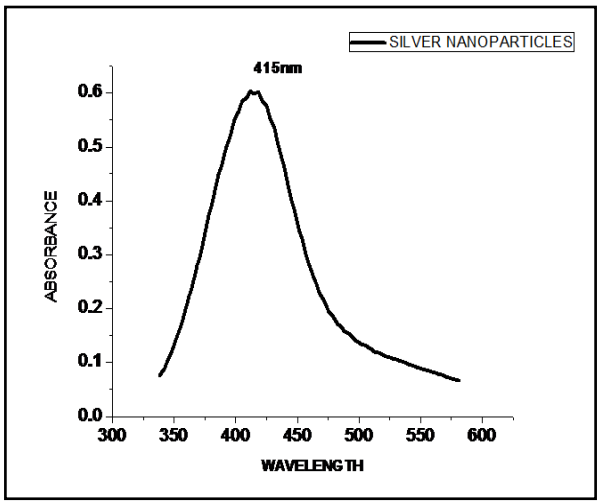

Fig. 1.UV –vis spectroscopy of microbially synthesized silver nanoparticles

TEM measurements were used to determine the morphology and shape of nanoparticles. Silver nanoparticles were synthesized by the cell free filtrate of *Streptomyces* sp. II, after reacting with silver nitrate. The well dispersed nanoparticle was confirmed by the occurrence of silver nanoparticles which showed that the size of particles range from 65 to 80 nm. The nanoparticles were observed as spherical in shape. Low magnification TEM micrographs revealed that the particles are spherical in shape and uniformly distributed (monodispersed) without significant agglomeration as represented by Figure 2a. The particle size of microbially synthesized silver nanoparticles shows that the particle size is smaller(10 to 20 nm) compared to the chemically synthesized silver nanoparticles(60-80 nm) as denoted by the Figure 2b.

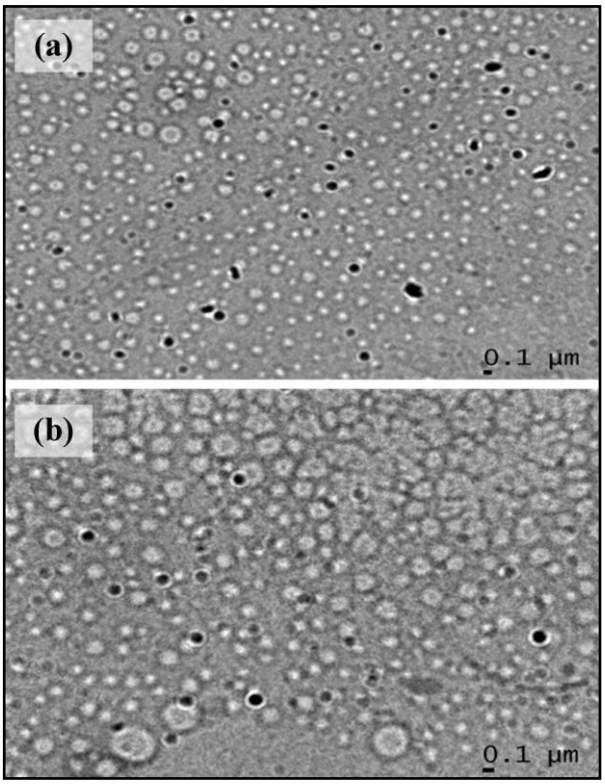

Fig. 2. TEM images of microbially and chemically synthesized silver nanoparticles

The antibacterial activity of microbially silver nanoparticles was investigated against various pathogenic bacteria of Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus vulgaris*) strains using disc diffusion method. The highest antimicrobial activity was observed against *Pseudomonas aeruginosa* followed by *Staphylococcus aureus* and *Klebsiella pneumoniae* and the least was noticed against *Proteus vulgaris* and *Escherichia coli* (Figure 3).

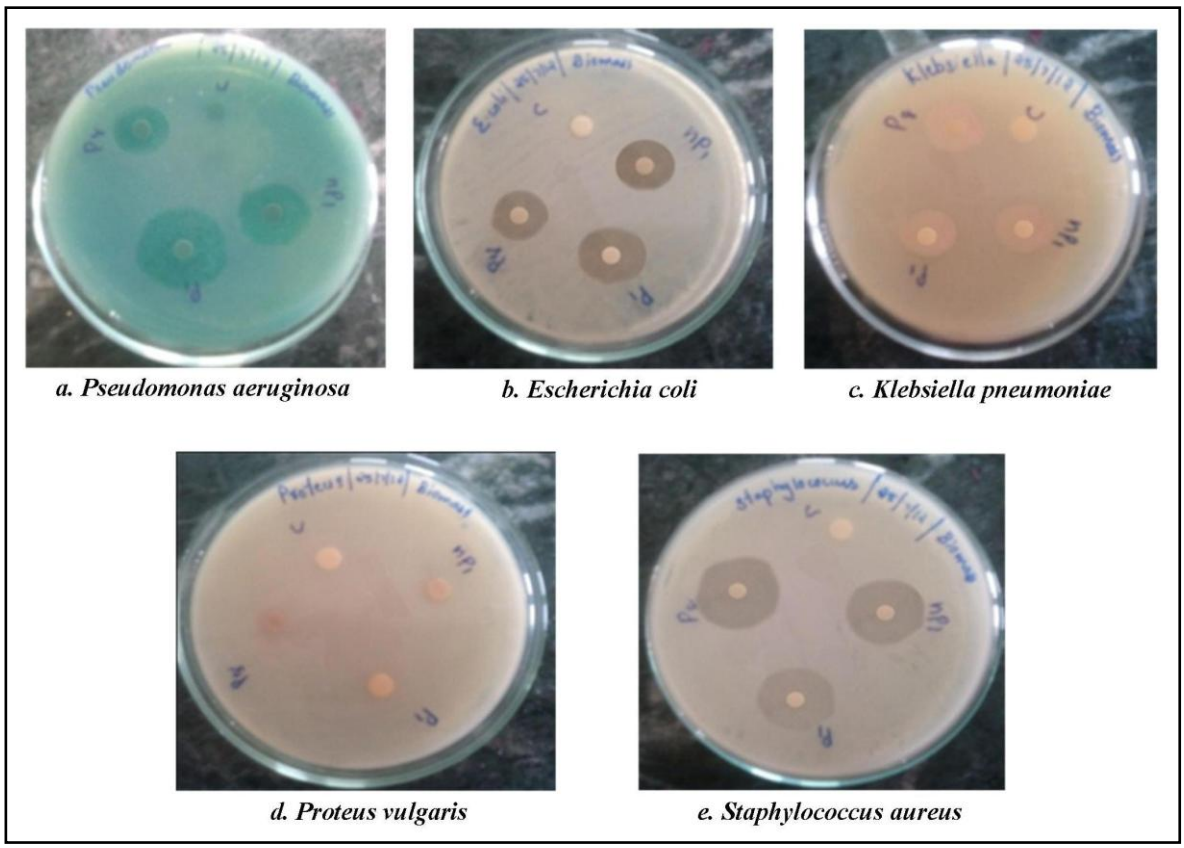

Fig. 3. Antibacterial activity of microbially synthesized silver nanoparticles agains thumanpathogens

The principle of preparation of silver nanoparticles by using microorganism is a bioreduction process; the silver ions are reduced by the extracellular reductase enzymes produced by the microorganisms to silver metal in nanometer range. It is concluded from protein assay of microorganisms that thereductase involved in bioreduction for preparation of silver nanoparticles is an NADH-dependent reductase. The enzyme reductase gains electrons from NADH and oxidizes it to NAD+. The enzyme is then oxidized by the simultaneous reduction of Silver ions forming silver metal in nanoform. The nanoparticles are formed on the surface of mycelia not in the solution. So the possible mechanism is trapping of the Ag+ ions on the surface of the Actinomycete cells possibly via. Electrostatic interactions between the Ag+ and negatively charged carboxylate groups in enzymes present in the cell wall of mycelia. The silver ions are reduced by enzymes present in the cell wall leading to the formation of the silver nuclei, which subsequently grow by further reduction of Ag+ ions and accumulation on these nuclei. The TEM analysis shows the presence of some silver nanoparticles both in the cytoplasmic membrane and in the cytoplasm [24].It has been studied elsewhere that when metallic nanoparticles are formed they are stabilized by the proteins. Proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins [25, 26]. Chemical synthesis results in the formation of yellowish brown colour which results from absorption by colloidal silver nanoparticles in the visible (380-450 nm) region of the electromagnetic spectrum. The colour formation was mainly due to the surface Plasmon resonance of deposited silver nanoparticles and silver nanoparticles exhibit striking colors due to excitation of surface Plasmon vibrations in the particles [27]. In chemical nanoparticle synthesis, a stabilizer is necessary to prevent the aggregation of fine particles to make them stable for a long period of time but with use of biological systems, it is clear from the

Transmission electron spectroscopy study that even aggregated nanoparticles don‘t have direct contact with one another. This is due to the fact that nanoparticles are stabilized in solution by capping proteins, which are secreted from microorganisms. One important enzyme that may be responsible for this is Cytochrome C. The silver nanoparticles formed by this process are quite stable due to capping by bacterial proteins for a period of 5 months at 25°C[28].

The antibacterial activity of silver nanoparticles was investigated against various human pathogenic organisms using well diffusion method. The synthesized silver nanoparticles were more effective against gram positive bacterial strain than the gram negative bacteria. Silver nanoparticles have antimicrobial effect on *Staphylococcus aureus*. The reason for different sensitivity between Gram-positive and Gram-negative bacteria could be explain to the morphological differences between these microorganisms [29]. Gram-negative bacteria, having an outer polysaccharide membrane and them carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, The Gram-positive strains should more susceptible having only an outer peptid oglycan layer which is not an effective permeability barrier. These results suggest the Biosynthesized silver nanoparticles are highly antagonistic in nature and they showed high antibacterial activity because silver nanoparticles allowed them to easily interact with other particles and increased their antibacterial activity due to its small size compared to the chemical synthesized components.

CONCLUSIONS

Microbially synthesized silver nanoparticles were about 10-20 nm in size which showed highest antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Escherichia coli*. The “biogenic” approach is further supported by the fact that the majority of the bacteria inhabit ambient conditions of varying temperature, pH, and pressure. The particles generated by these processes have higher catalytic reactivity, greater specific surface area, improved contact between the enzyme and metal salt in question due to the bacterial carrier matrix and smaller size of silver nanoparticles. The smaller silver nanoparticles showed stronger antibacterial activity compared to the chemically synthesized silver nanoparticles (60-80 nm). The bacterium was highly resistant to silver cations. These silver nanoparticles were of high purity, making them potentially useful for biological applications. The application of silver in combination with microbial system would be effective in enhancing its anti-microbial activity.

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