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Research Article

BIOGENIC SYNTHESIS OF NANOSILVER AND ITS ANTIBACTERIAL EFFECT AGAINST RESISTANT GRAM NEGATIVE PATHOGENS

S.DUGAL*, S.CHAKRABORTY

Dept.of Microbiology, Sophia College, University of Mumbai, Maharashtra, India. *Email: suparnadugal@gmail.com

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ABSTRACT

Objective: The rapidly advancing field of nanomedicine has emerged as an extremely useful tool to combat the development of drug resistant organisms. In this study, *E.coli*, a Gram negative bacterium, was utilized for the extracellular biosynthesis of silver nanoparticlesfor use as a novel biocide against resistant Gram negative bacteria.

Method: Biologically synthesized nanoparticles (BAgNPs) were characterized by UV visible spectroscopy, nanoparticle analyzer and atomic absorption spectroscopy. Analysis of the functional groups was done by FTIR spectroscopy. Antimicrobial tests were done by the agar cup diffusion assay, growth curve study and macrobroth dilution assay. Clinical test strains of extended-spectrum beta lactamase (ESBL) producing *Escherichia coli* and resistant *Pseudomonas aeruginosa* were selected as model organisms representative of resistant Gram negative pathogens.

Results: BAgNPs were mostly spherical in shape and exhibited a maximum absorption peak between 420-440nm which corresponds to their surface plasma resonance. The average particle size and zeta potential of the particles was found to be 75 nm and -10mV respectively, while the concentration of nanosilver in solution was recorded to be 315ppm.The test cultures exhibited low susceptibility to antibiotics like trimethoprim, ampicillin, bacitracin and carbenicillin compared to BAgNPs. Study of the growth pattern revealed both cultures to exhibit delayed growth in the presence of nanosilver.Determination of minimum inhibitory concentration and minimum bactericidal concentration of the particles revealed that *Pseudomonas* exhibited greater susceptibility to BAgNPs compared to ESBL producing *E. coli.*

Conclusion: The current study demonstrates the production of nanosilver in a simple and cost-effective manner, and suggests its potential use for controlling drug resistant clinical isolates of *E.coli* and *Pseudomonas*.

Keywords: Silver nanoparticles, Antibacterial, *E.coli*, *Pseudomonas*, Biological synthesis.

INTRODUCTION

The growing trend of antimicrobial drug resistance in virtually all organisms responsible for hospital acquired infections has become a major public health concern worldwide. Due to their rapidly evolving adaptive strategies microorganisms have been known to successfully flourish in hospitals and community settings.Several researchers are investigating various alternative strategies to combat this problem. Gram negative pathogens like *Proteus, Pseudomonas, Escherichia coli* and *Klebsiella* have been associated with increasingly resistant diseases of the urinary tract and cases of aggravated infection in burn and wound afflicted patients. [1] In addition, they have also been responsible for associated sequelae in intensive care and immunocompromised patients. [2]

Nanomedicine has been touted as the solution against the spiraling increase in drug resistance. Nanoparticles (NPs) exhibit either entirely new or improved properties compared to the original bulk material and present a higher surface area to volume ratio which is related to enhanced antimicrobial activity.

The synthesis of nanoscale metallic particles using biosystems has been explored for production of cadmium, gold, zinc and silver nanoparticles [3]. The antibacterial activity of silver is well documented [4] and has been known to be nontoxic to humans in low concentrations. [5] Silver has a far lower propensity to induce microbial resistance than antibiotics. Silver ions uncouple the respiratory chain from oxidative phosphorylation and can lead to the collapse of the proton motive force across the bacterial cytoplasmic membrane [6]. Organisms have been explored by investigators as nanofactories for the synthesis of silver nanoparticles using both extracellular and intracellular methods. Extracellular synthesis is preferred due to the ease of control over the process, the possibility of large scale synthesis and easy downstream processing [7]. Organisms which synthesize nanosilver extracellularly have been studied and they include sources such as *Bacillus licheniformis* [8], *Escherichia coli* [9], *Aspergillus fumigates* [10], *Pseudomonas aeruginosa* [11] and *Klebsiella pneumonia* [12].

The current research focuses on the development and characterization of biogenically synthesized nanosilver using *Escherichia coli* and further evaluating its antibacterial activity against drug resistant clinical isolates of *Pseudomonas aeruginosa* and ESBL producing *Escherichia coli*.

METHODS AND MATERIAL

Cultures of antibiotic resistant *Pseudomonas aeruginosa a*nd ESBL producing *Escherichia coli* were obtained from local hospitals. Microbial suspensions were obtained from a single colony isolated on agar plates and inoculated in nutrient broth (HiMedia) for overnight cultures. After incubating microbial cells at 37^0C overnight, optical density (OD) of the suspension at 600 nm was adjusted to 1.0 using a colorimeter (ErmaInc.Colorimeter). The suspension was diluted with phosphate-buffered saline (pH 7.4) to 1:100 and suspended to final concentration of 1.0×10^7 cells/mL.

To prepare silver nanoparticles a culture suspension of 24hour old *Escherichia coli*113D (laboratory strain) was used. Silver nitrate was purchased from SD Fine Chemical Ltd.

Preparation of silver nanoparticles

5ml culture suspension of *Escherichia coli* 113D adjusted to 0.1 OD was inoculated in 150ml nitrate broth and incubated at 37^0C for 48hrs under shaker conditions. The broth was centrifuged twice at 3000 rpm for 15mins and the supernatant collected. Controls were maintained and consisted of tubes containing nitrate broth without the culture supernatant. 1mM $AgNO_3$ was added to both the test and the control and incubated at 55^0C in the dark for 24 hrs. Following incubation the yellow color of the culture supernatant turned brown indicating formation of silver nanoparticles.

Quantification and characterization of silver nanoparticles

Characterization of the nanoparticles was done using UV-visible spectrophotometer (ELICO SL 207 MINI). For this, 0.1ml sample of the silver nanoparticles diluted with 10ml of Nitrate broth was used while Nitrate broth without the culture supernatant served as the blank.

Quantification of the prepared silver nanoparticles was carried out by atomic absorption spectroscopy (Perkin Elmer AA 700) using air-acetylene flame. Prior to analysis, the sample was digested using nitric acid on hot plate for 3hrs. The dry residue obtained was diluted with 5% nitric acid and filtered through 0.45µ filter and used for the analysis.

Further, the morphology of the BAgNPs was studied using scanning electron microscope equipped with an Energy Dispersive X ray Analyzer (Icon Analytical). Each specimen was dispersed ultrasonically to separate individual particles before analysis

The zeta potential and the particle size distribution were confirmed with a nanoparticle analyzer (HORIBA- SZ100Z).Functional groups were analyzed by SHIMADSU 8400 FTIR Spectrophotometer.

Testing the cultures for antibiotic resistance

Antimicrobial resistance of the test cultures obtained from hospitals was further investigated by the Kirby Bauer method [13]. Standard discs (HiMedia) of 25µg clotrimazole,5µg trimethoprim, 10µg ampicillin and 100µg carbenicillin was used for checking antimicrobial resistance of ESBL producing *E.coli*, while the culture of pathogenic *Pseudomonas* was tested against 100µg carbenicillin and 10units bacitracin.

Assay of antimicrobial activity of silver nanoparticles

In vitro antimicrobial activity of silver nanoparticles was first investigated by the agar cup diffusion method [14]. Mueller Hinton Agar plates were swabbed with the test culture and 40 µl BAgNPs was added to wells. Wells containing nutrient broth and 1mM $AgNO_3$ served as controls. Sizes of zones of inhibition were measured after incubation.

Determination of minimum inhibitory concentration of BAgNPs against drug resistant cultures

Using stock solution of the prepared silver nanoparticles, various dilutions were prepared. 0.1 ml of the 24 hr old test culture was added to all dilutions. Positive and negative controls were maintained. Tubes were incubated at 37^{o}C for 24 hrs and the lowest concentration that did not show growth corresponded to the minimum inhibitory concentration (MIC).

Determination of minimum bactericidal concentration of BAgNPs

0.1 ml of culture from the tube indicating minimum inhibitory concentration and all higher concentrations beyond it were surface spread on nutrient agar and incubated at 37^{o}C for 24 hrs. The minimum concentration showing no growth corresponded to the minimum bactericidal concentration (MBC).

Effect on growth pattern

To study the effect of BAgNPs on the growth of the test cultures, culture suspensions were added to flasks containing the nanoparticles (sublethal concentration) and kept on shaker at 37^{0}C. Aliquots were withdrawn at different time intervals and optical density measured. Controls consisted of cultures inoculated in flasks without the nanoparticles.

All experiments were done in triplicate and repeated three times. Mean values have been reported.

RESULTS AND DISCUSSION

Antibiotic resistance has become a serious problem threatening human and animal health world-wide. [15] Among the emerging resistant pathogens, spread of infections caused by Gram negative multidrug resistant and ESBL producing hospital isolates is of major concern. ESBLs are defined as beta-lactamases capable of hydrolyzing oxyiminocephalosporins [16] *E.coli*, an organism responsible for urinary tract infection, has the ability to produce ESBLs in large quantities. Production of ESBL enzymes confers multiple drug resistance, making infections difficult to treat [17]. ESBL-producing *E.coli* infections are growing globally and are said to be harder to treat than infections caused by methicillin resistant *Staphylococcus aureus*. *Pseudomonas* is an opportunistic pathogen responsible for infections in immunocompromised patients. These organisms have shown a steady increase in resistance over the past few decades and are difficult to treat in a majority of hospitalized individuals.

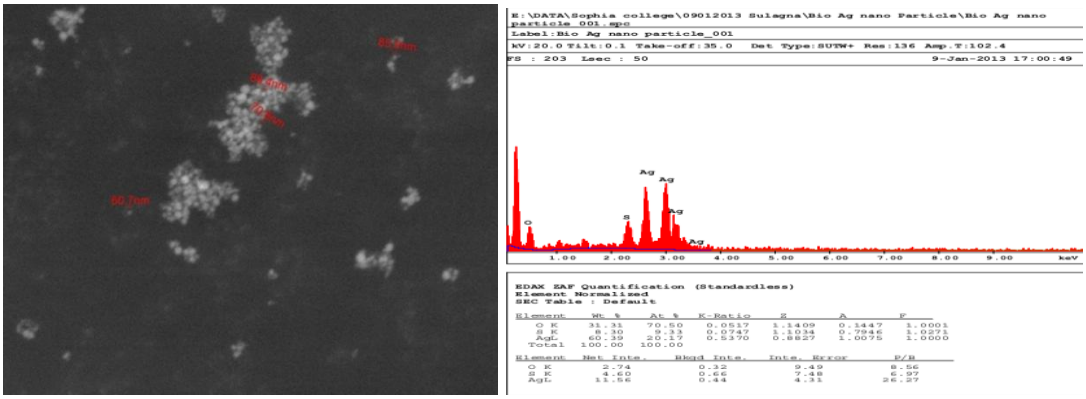
Silver has been known for its inhibitory and bactericidal effect and possesses greater toxicity against a broad spectrum of sessile bacteria and fungi, compared to many other metals [18].Silver has far lower propensity to induce microbial resistance than antibiotics, since the mode of action of the antibiotics to prevent bacterial growth is quite different from the mechanism by which NPs act on microbes. Silver exerts its antibacterial activity by interacting with DNA as well as with sulfur containing proteins present in the bacterial cell membrane. Compared to the original bulk metal, nanoparticles of silver possess a higher surface area to volume ratio and hence provide enhanced contact, mediating the killing of bacteria [19]. Silver nanoparticles are known to penetrate bacterial cells and accumulate in the bacterial cytoplasmic membrane causing significant increase in permeability and cell death. Such characteristics have led to attention being drawn to them recently [19]. Silver nanoparticles have been tested alone and in combination with various classes of antibiotics for use against pathogenic micro-organisms. [20, 21, 22]

The problem with the chemical and the physical methods of synthesis of silver nanoparticles is that the process is expensive and the nanoparticles may have toxic substances absorbed onto them. To overcome this, development of a reliable green chemistry process for the biogenic synthesis of nanomaterials is an important aspect of current nanotechnology research.The present study investigates (i) the use of *Escherichia coli* as a potential biofactory for the rapid synthesis of extracellular silver nanoparticles (ii) the in vitro antimicrobial effect of the BAgNPs towards ESBL producing *E.coli* and resistant *Pseudomonas* selected as model organisms representing resistant Gram negative bacteria and (iii) the dose of silver nanoparticles necessary to obtain the desired cidal activity.

Rapid biosynthesis of nanoparticles was observed when aqueous silver nitrate was exposed to a culture supernatant of *E. coli.* The reduction process was fast and formation of a brown homogenous solution indicated the formation of nanosilver. The synthesized nanoparticles exhibited maximum absorption peak between the range of 420-440nm on analysis with UV visible spectroscopy.

Scanning electron micrograph revealed spherical nanoparticles ranging in size from 50-70nm [Figure1].The scanning electron microscope was equipped with an Energy Dispersive X ray Analyzer which helped in the mapping of elements present in the sample. Data analysis of the composition of the analyte revealed that it contained mainly silver, with trace amounts of sulfur and oxygen as contaminants.

Quantification of the BAgNPs by Atomic Absorption spectrophotometry revealed the concentration of silver in the solution to be 315ppm. Further characterization using Nanoparticle Analyzer based on Dynamic light scattering study (DLS) confirmed the size of the particles to be 75nm. Zeta potential analysis is important since it indicates the potential interaction between particles and bacterial cell surfaces. The mean surface charge of the synthesized BAgNPs was found to be -10mV. Though bacterial cell surfaces are also known to possess a negative surface charge, it is obvious that an affinity existed between the two, which resulted in the subsequent killing of the cells. Previous researchers too have observed a similar anomaly in the surface charge and interaction between NPs and bacterial cells [23], the possible mechanism behind which requires further confirmatory studies. The reduction process of silver nitrate by *E.coli* was explored by FTIR measurements which helped to identify the possible interactions between the silver salt and protein molecules. The amide linkages between amino acid residues produced signature bands in the infrared region of the electromagnetic spectrum. Bands observed at 3338.5cm^{-1}were attributed to stretching vibrations of the primary and secondary amines respectively while corresponding bending vibrations were observed at 1635cm^{-1}& 1186cm^{-1}(Figure2). These observations hence confirm the presence of protein in samples of BAgNPs. Binding of proteins through their free amine or cysteine groups to NPs aids in their stabilization [24].



(A) **(B)**

Fig. 1: (A) It shows the SEM image of BAgNPs (B) EDS Spectra of the synthesized BAgNPs

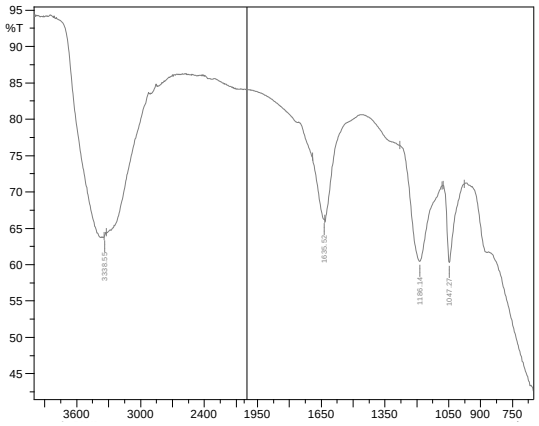


Fig. 2: It shows FTIR spectrum obtained for BAgNPs

The antimicrobial susceptibility profile of the resistant clinical isolates used in the study was further checked by the Kirby Bauer method against some standard antibiotics. The culture exhibited resistance towards all of these antibiotics as shown in Table1. It was noted that the resistance observed to different drugs was due to varied genetic and biochemical mechanisms acquired by these organisms. The bactericidal activity of BAgNPs was evaluated against Gram negative isolates by agar cup diffusion method. ZOIs obtained are depicted in Table 1.

Table 1: It shows the susceptibility profile of cultures.

Culture	Trimethoprim	Ampicillin	Clotrimazole	Bacitracin	Carbenicillin	BAgNP	$AgNO_3$
E.coli	No inhibition *R	6mm *R	No inhibition *R	Not performed	15mm *R	18±2mm	13±2 mm
Pseudo-monas	No inhibition *R	6mm *R	Not performed	6mm *R	Not performed	28±2 mm	15±2 mm

*R=Resistant, **NP=Not performed. Nutrient broth (control) showed no inhibition.

In the current study values of MIC of BAgNPs against ESBL producing *E.coli* and *Pseudomonas* was found to be 9.843ppm and 4.92ppm respectively. Hence it is evident from the agar cup studies and macrobroth dilution technique that *Pseudomonas* exhibited greater susceptibility to the NPs than ESBL producing *E.coli*. It was noted that for both the clinical isolates the inhibitory concentration of AgNPs was the same as the bactericidal concentration. Since, the MBC/MIC ratio was ≤ 4, silver nanoparticles can be considered potent bactericidal agents against the test organisms.

The dynamics of bacterial growth was studied using nutrient broth containing sub lethal concentration of nanoparticles was added. A time dependant change in bacterial growth was monitored at regular intervals of 2hrs (upto 22hrs). Bacterial cell growth enhanced the turbidity of the liquid medium resulting in increased absorption. Due to their inhibitory action, nanoparticles caused delayed bacterial growth which was evident in the growth curve of both the cultures.

CONCLUSION

The results of the current investigation suggest that BAgNPs exhibit excellent bactericidal effect towards drug resistant clinical isolates of *E.coli* and *Pseudomonas*. However, since one of the major concerns despite the beneficial aspects of nanotechnology is now focused on the unknown effects on biological systems [25], further genotoxic and cytotoxic studies would help to evaluate the use of biological nanoparticles as a valuable cidal agent in clinical settings.

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