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Silver oxide nanoparticle synthesis from *Bacillus* species and its anti-bacterial action against clinical pathogens

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ABSTRACT

Silver nanoparticles inhibit microbial growth and have been gaining importance as a solution for numerous biomedical applications. In this study, synthesis of silver nanoparticles is carried out using *Bacillus* species isolated from silver fabrication area. Nanoparticle Tracking Analysis and Scanning Electron Microscopy revealed the size of particles ranged from 39 to 67.4 nm and spherical shape. XRD spectra revealed nanoparticles are of silver oxide. The anti-bacterial activity of silver oxide nanoparticles of *Bacillus* species was prominent against the three clinical strains of major Urinary Tract Infection (UTI) causing *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Keywords: Urinary Tract Infection, nanoparticles, biomedical applications

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INTRODUCTION

Silver nanoparticles have been emerging as a solution for numerous biomedical problems ranging from the enhancement of antibiotic action, control of the malarial vector *Anopheles culicifacies, to non-cytotoxic wound dressing to protect against the pathogenic bacteria inside biofilms*[1,2,3]. In addition, silver nanoparticles have also been used to control the growth of lactic acid bacteria during winemaking[4].

A wide range of organisms can synthesize silver nanoparticles which including actinomycetes, algae, bacteria, fungi, viruses, yeasts, and plants[5,6,7,8,9]. Among them, bacterial nanoparticle synthesis is preferred for environment safety reasons because no hazardous chemicals are required, it can be performed in standard laboratory conditions, and downstream processing is relatively easy. Many bacterial species have been exploited for silver nanoparticle synthesis for antimicrobial actions. Silver nanoparticle synthesized from phototrophic bacteria *Rhodopseudomonas* inhibited *Klebsiella pneumonia, Bacillus subtilis, Pseudomonas putida, Escherichia coli* and *Staphylococcus aureus*. The size of the nanoparticles ranged from 6-10 nm[10]. The cubical hexagonal nanoparticles synthesized from *Pithophora oedogonia* in the range of 25-44nm significantly inhibited *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Shigella flexneri*, *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus*[11]. *Present study contributes to research in this direction.*

Regarding Urinary tract infections (UTI) it is stated that *Escherichia coli* and *Staphylococcus spp* account for about 80% of urinary tract infections specifically in women below 50 years of age[12]. Along with *E. coli* and *Staphylococcus* spp; *Pseudomonas also is one of the major causative agents of UTI*[13]. *Pseudomonas* alone accounts more than 25% of urinary tract infections[14]. Of the overall health care infections 12.9% infections were UTI of which 67.7% patients had urinary catheters[15]. To control UTI it is better to target catheter for prevention of infection. Catheter associated UTI (CAUTI) also causes the cost of treatment to increase by more than $2800[16]. In order to prevent catheter associated UTIs beside appropriate use, aseptic insertion, periodic removal of urinary catheters and hand hygiene; the catheters may also be coated with silver nanoparticles. The inhibition of catheter associated UTI pathogens by silver nanoparticles is therefore necessary to study.

Bacilli are a good choice for economic nanoparticle synthesis considering their wide distribution in nature and growth on agricultural waste media[17]. The stable nanoparticles ranging from 42-94 nm from *Bacillus* species have been characterized by Das et al, (2014)[18]. *Bacillus* species from

various environments has variability in the nanoparticle synthesized. The size of silver nanoparticle ranged from 50 to 120 nm. The average silver nanoparticle size was reported to be 90 nm as studied by Vithiya et al, (2014)[19].

Species of *Bacillus* being spore bearing can withstand most harsh environment and can utilize wide range of organic compounds to predominate other bacterial genera. Because of this ability these can be grown in agricultural waste media to avail economic synthesis of silver nanoparticles. Hence *Bacillus* species are undertaken for nanoparticle synthesis.

MATERIALS AND METHOD

Sample aquisition, cultivation and synthesis of silver nanoparticles

Samples were collected aseptically from silver fabrication area in Amravati. The samples were diluted in ddH_2O and incubated in water bath at 80 °C for 15 min to induce spore formation. Spore bearing *Bacilli* were cultivated on nutrient agar. Overnight cultures in nutrient broth were established for individual colonies for all species. 100 ml of 1 mM silver nitrate solution were inoculated either with 1 ml supernatant or cell pellet obtained from centrifugation (15 min, 10,000 rpm) of the overnight culture,and kept stationary or on a rotary shaker (200 rpm) for 48 h at 25 ^{o}C. Silver nanoparticle synthesis was confirmed based on the opacity of the culture. The flask with maximum intensity was chosen for further characterization of both bacteria and silver nanoparticles. The bacterium was identified by morphological and biochemical characterization.

Characterization of Silver nanoparticles

UV- Visible spectroscopy

The samples of stationary and rotary reaction mixtures were subjected to UV-visible analysis (Systronics Pvt. Ltd, India). The UV-visible spectrum of the aqueous solution containing silver nanoparticles shows a characteristic absorption peak at 420 nm due to surface plasmon resonance phenomena which is characteristic for silver nanoparticles[20].

X-ray diffraction analysis

X-ray diffraction (XRD) is a non-destructive analytical method for characterization of nanoparticles in the reaction mixtures. Diffraction occurs when the waves collide with particles whose repeating distance is approximately equal to the wavelength. This means that the X-ray can be easily diffracted from materials which are crystalline and have repetitive atomic structures. The nanoparticle suspension was filtered, freeze-dried and powdered prior to XRD analysis. The spectra were recorded in automatic X-ray Diffractometer X-ray generator. The diffracted intensities were recorded from 30 to 90 2Ɵ angles. The spectra were recorded in

automatic X-ray Diffractometer (Rigaku miniflex-II diffractometer). The diffracted intensities were recorded from 30^0 to 90^0 2 θ angles[21].

Nanoparticle tracking analysis (NTA)

The basic principle behind particle illumination is the specially aligned and focused laser beam. Due to this, the extremely small particle (down to 10 nm) can be observed individually. The procedure follows dilution of 5 µl sample in 2 ml of nuclease free water which is injected into the chamber[22].

Liquid preparations of silver nanoparticles were used to perform NTA (NanoSight-LM 20) analysis through which laser beam (40mW at *k*=635 nm) was allowed to pass. Particles present within the track of the laser beam were observed via a dedicated non-microscope optical instrument (LM-20, NanoSight Pvt. Ltd., UK) fitted with a Charge Coupled Device (CCD) camera. The motion of the particles in the field of view was recorded (at 30 fps) and the video and images generated were analyzed for the size distribution of nanoparticle in preparations.

SEM studies

Silver nanoparticles synthesized from bacteria were freeze dried and mounted on specimen stubs with double-sided adhesive tape, coated with gold in a sputter coater and examined under scanning electron microscopy (SEM), Philips® XL 30, at 12–15 kV with a tilt angle of 45°.

Bacterial action of silver nanoparticles against UTI pathogens

Previously studied biofilm forming bacteria from catheters were used. The method of bacterial extraction follows collection of catheters aseptically in sterile containers. Small sections 1-2 cm and 3-4 cm from tip of catheter were cut, followed by washing with sterile distilled water and were aseptically suspended in 10 ml Quarter strength Ringers solution. This was subjected to sonication for 5 min at 35 kHz in a transonic water bath and vortexed for two minutes to attain disruption of colonizing bacterium. Bacteria were inoculated on UTI chromogenic media (Hi-media make). Predominant bacteria characterized belonged to *E. coli*, *S. aureus* and *P. aeruginosa*. Fresh bacterial cultures with standardized dilutions were spreaded on Muller Hinton Agar (MHA) plates. Plates were allowed to stand for 10-15 min so that bacteria adhere to the agarized media. Wells of 6 mm diameter were made in each plate and three dilutions of nanoparticle were added. Tetracycline was kept as positive control and distilled water as negative control. Inoculated plates were incubated for 24 h at 37 °C, zone of inhibition was recorded and size of zones was measured. Likewise three dilutions of liquid nanoparticles were tested against each bacterial pathogen.

RESULTS AND DISCUSSION

Cultivation and Identification of Bacteria:

In present study synthesis of silver nanoparticles is carried using *Bacillus* species at stationary and rotary phases. *Bacillus species* was isolated from silver fabrication area in Amravati, India. Cultures were maintained by serial sub-culturing on nutrient agar slants and efficient bacterium was characterized (Table1). Further, culture flask at submerged condition showed maximum synthesis of silver nanoparticles.

Table 1: Characterization of bacterium under study

Cultural characteristics							**Morphological properties**				**Biochemical characteristics**														**Identified Bacterial species**
Colour	Size	Margin	Shape	Elevation	Opacity	Consistency	Gram reaction	Shape	Endospore	Motility	Catalase	Oxidase	Indol	MR	VP	Citrate	Arabinose	Glucose	Fructose	Lactose	Mannitol	Rhamnose	Xylulose	Sucrose	Salicilin	
White	1 mm	Regular	Rod	Convex	Opaque	Soft	+	Rod	+	N	+	+	-	-	-	+	-	+	+	-	-	-	+	+	-	*Bacillus* species

Biosynthesis of Silver oxide nanoparticles

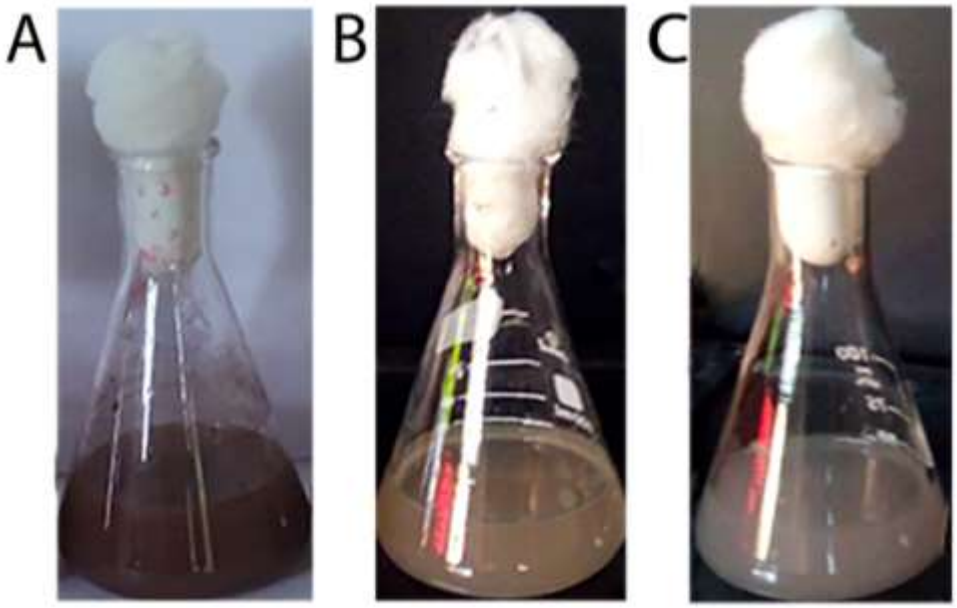

Figure 1 Silver oxide nanoparticle synthesis at 24 hours after incubation.

A) Flask inoculated with bacterial cell pellet on rotary shaker B) Slight coloration in flask inoculated with supernatant C) Flask on incubated on shaker without inoculation as negative control

The bacterial cell pellet when mixed with 1 mM $AgNO_3$ was initially colourless. After 10 h, it started to become brown and after 24 h, it shows characteristic brick red coloration as a result of nanoparticle synthesis (Figure 1A). Likewise, Flask incubated with supernatant also showed slight brown color formation (Figure 1B). On the other hand, no colour change was observed in the negative control i.e. flask without inoculation (Figure 1C). These results suggest the synthesis of silver oxide nanoparticles in the flasks with bacillus inoculums.

Characterization of Silver nanoparticles:

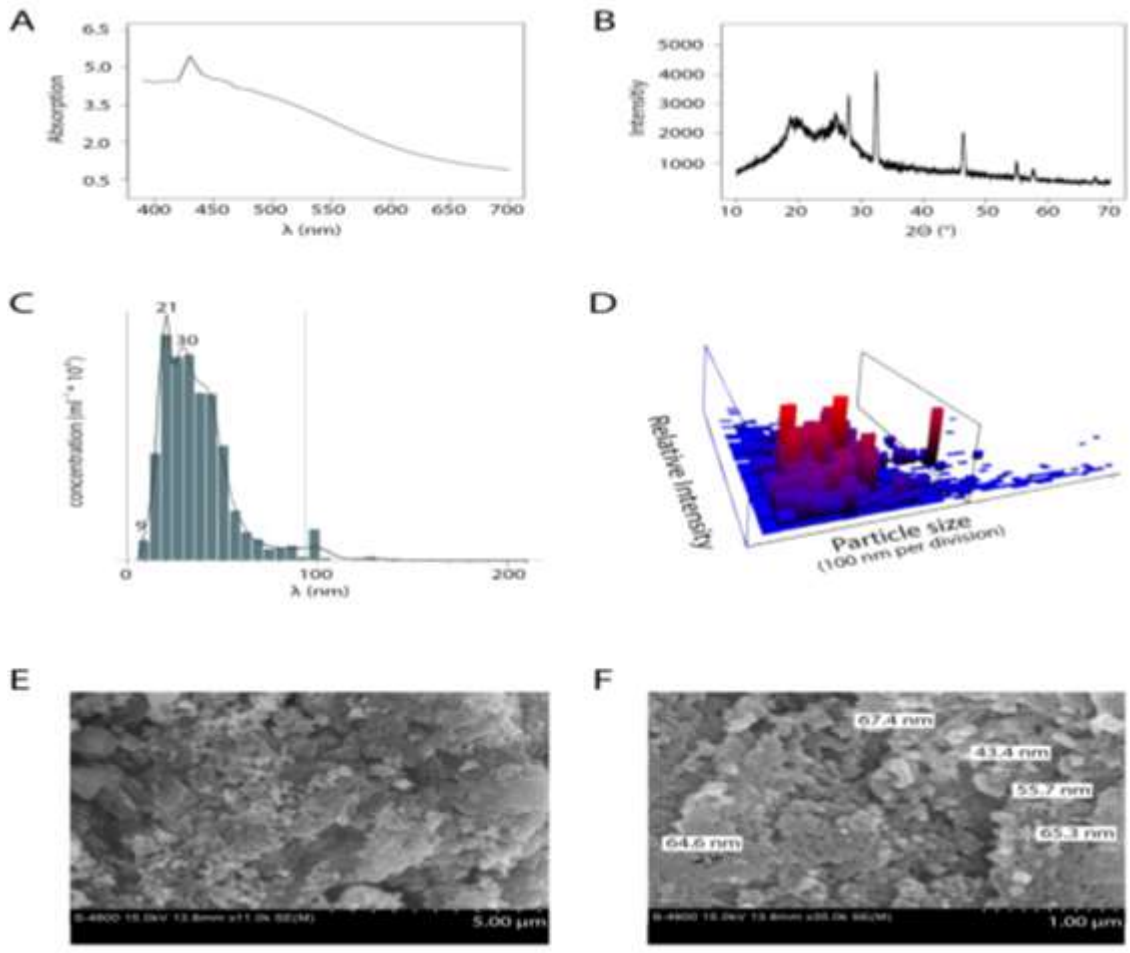

Figure 2: Characterization of Silver nanoparticles.

A) UV-Visible spectra of AgNPs B) XRD pattern of resulting AgNPs C) Nanoparticle tracking analysis NTA (NanoSight-LM 20) histogram D) Particle size analysis E) SEM images of resulting nanoparticles at 5µ scale F) SEM images of resulting nanoparticles at 1µ scale

In order to confirm our preliminary observations, the silver nanoparticle solution was observed in UV–visible spectra, the change in colour of this solution was recorded in spectrophotometer. Due to surface plasmon resonance effects, Silver nanoparticles show characteristic peaks

between 350 and 450 nm[20]. Consistently, the maximum absorption was observed at 420 nm, which indicates the biosynthesis of silver nanoparticles (Figure 2A).

Figure 2B shows prominent peaks of silver oxide nanoparticles. The peaks in XRD were at 27.98^0 and 32.44^0. The values are very much near to silver oxide nanoparticles synthesized by *Lactobacillus mindensis*[23]. The peak found at 2 θ =32.44^0 had β of 0.54^0. The average particle size using Debye- Scherrer equation was found to be 33.7 nm. The shoulder peaks at 2 θ =46.32^0 and 2 θ =54.88^0 might be of few aggregated nanoparticles. The average particle size observed by NTA was found to be 39 nm. The concentration of nanoparticles was found dense below 50 nm (Figure 2 C and D).

Previous studies have reported the synthesis of silver nanoparticles of size ranging from 42 to 94 nm in *Bacillus* species[18,24]. In our study, the size of Silver nanoparticles was in the range from 21 to 100nm with the average particle size of 39nm (±24) as determined by the nanoparticles tracking analysis. The size of nanoparticles by SEM was found to be 43.4 to 67.4 nm and the shape was circular (Figure 2 E and F).

The disparity in the size of the nanoparticles determined by different method was evident. The methods rely on different physical principles for size determination. Variation in the size of silver nanoparticles was reported by Gade et al., (2008)[25]. NTA and transmission electron Microscopy (TEM) was used for the size determination wherein size variation was observed for same sample. NTA uses hydrodynamic radius for the size determination, whereas electron microscopy uses dry particles. The account of different analytical methods used for the size determination of metallic nanoparticle dispersions is given by Mahl et al., (2011)[26]. Overall, this result confirms synthesis of circular silver oxide nanoparticles ranging from 21 to 67.4 nm by *Bacillus cereus*.

Anti-bacterial activity of Silver nanoparticles

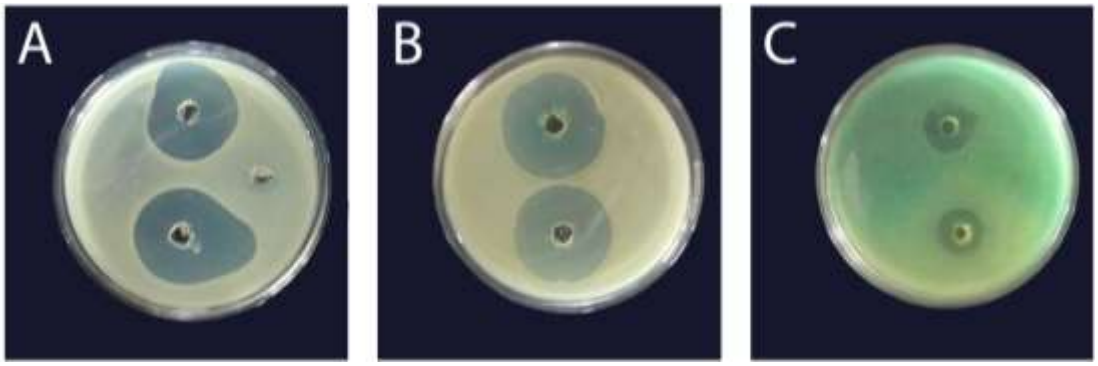

Figure 3 Anti-bacterial activities of Silver nanoparticles.

Silver nanoparticles inhibit the growth of A) *Escherichia coli* B) *Staphylococcus aureus* and C) *Pseudomonas species.*

Table 2: Antimicrobial action of Silver nanoparticles

Quantity of synthesized silver nanoparticle solution (µl)	Zone of inhibition (mm)		
	E coli	S aureus	P aeruginosa
100	32	38	18
50	17	19	12
25	11.2	10.8	9.5

Agar well diffusion method was used to test the antimicrobial sensitivity. Three clinical isolates i.e. *E.coli, S. aureus* and *P. aeruginosa* were selected which represent Gram negative as well as Gram positive species. These clinical isolates were inhibited by Silver nanoparticles at the minimum concentration of 25µl. The zone of clearance was on an average 10.5mm deep for three isolates. Maximum zones of inhibition were observed at 100 µl ranging from 18 to 38 mm (Table 2 and Figure 3 A to C).

In similar studies green silver nanoparticles from carob leaf extract were studied for antibacterial action against pathogenic *E. coli* wherein zone of inhibition (8 to 12mm) greater than standard antibiotic was reported[7]. AgNPs were also reported as promising agent against *Bacillus subtilis* and *Klebsiella planticola. Comparative studies of chemically and biologically synthesized nanoparticle for antibacterial activity have already proved that green AgNPs exceeds over chemical AgNPs*[8,9]*. This is a first time of antibacterial action of silver oxide nanoparticles of Bacillus cereus against three major UTI causing clinical isolates of E. coli, S. aures* and *P. aeruginosa* is reported. The results are contrary to those reported by Sinha et al, (2014) wherein strong activity of AgNPs was observed against *P. aeruginosa* whereas our results states *P. aeruginosa* was marginally affected by AgNPs[11]. Existence of efflux mechanism in some bacteria is reported as the cause behind antimicrobial resistance to nanoparticles which might be present in species of *Pseudomonas* under study[27]. Silver oxide nanoparticles were efficient in killing *E.coli* and *S.aurues* isolates as compared to *P. aeruginosa*.

CONCLUSION

A non-pathogenic bacterium i.e. *Bacillus* species isolated from silver fabrication area was exploited for synthesis of silver nanoparticles. AgNPs were characterized by UV-Visible spectroscopy, nanoparticle tracking analysis, XRD and SEM. The size of AgNPs ranges from 43.4 to 67.4 nm. The bacteriagenic AgNPs were potent inhibitors of three major UTI pathogens *E.coli, S.aures* and *P. aeruginosa*. AgNPs have shown stronger inhibitory actions against UTI

isolates and finds applicability in coating of medical equipments like urinary catheters. A possible use in one of the cause of UTI infection i.e. sanitary pads may be impregnated with silver nanoparticles is suggested.

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