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Antimicrobial Activity of Biosynthesized Silver Oxide Nanoparticles

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Increasing resistance of microorganisms to antimicrobial agents has increased the rates of mortality and morbidity from treatment failures indicating a serious threat in near future. *Bacillus thuringiensis* SSV1 was isolated from soil and growth media was optimized with efficient carbon, nitrogen and metal sources. Stable spherical shaped silver oxide nanoparticles of 10-40 nm diameters were synthesized extracellularly using bacterial culture supernatant. The antimicrobial activity of silver oxide nanoparticles of various concentrations was investigated against pathogenic bacteria. The results showed that silver oxide nanoparticles are as effective as silver nanoparticles against bacteria.

Key words: Optimization, *Bacillus thuringiensis*, Silver oxide nanoparticles, Antimicrobial activity.

Silver forms various phases like Ag_2O, AgO, Ag_3O_4 and Ag_2O_3 by interacting with oxygen. Thus, silver oxide nanoparticles are very interesting owing to this multivalency[1] which gives many interesting applications ranging from ultra-high density optical memories to single molecule detection[2]. Ag_2O has various applications in oxidation catalyst, sensors, fuel cells, optical data storage systems, *etc*[3,4].

The antibacterial effects of silver have been noticed since antiquity and silver is used for controlling bacteria growth in variety of applications like dental works, catheters burn wounds[5,6,7]. Silver and silver based compounds are highly toxic to microorganism and have shown strong biocidal effects on as many as 12 species of bacteria including *E.coli*[8]. Recently silver oxide nanoparticles exhibiting antimicrobial activity have been synthesized[9]. Presently the investigation of this phenomenon has gained importance because of increase in bacterial resistance to antibiotics caused by their overuse. Antibacterial activity of the silver nanoparticles can be used to reduce infections and to prevent colonization on prostheses, catheters, vascular graft, dental material, stainless steel material and human skin[5,6,7,8]. Due to the large surface to volume ratio nanoparticles play a crucial role in inhibiting bacterial growth in aqueous and solid media. Silver containing materials can be employed to eliminate microorganisms on textile fabrics or they can be used for water treatment.

It has been shown that bacteria are able to reduce the inorganic metals to nanoparticles[5,9,10]. However, a number of issues have to be addressed before such a biosynthesis approach can compete with the existing physical and chemical methods[11]. Till date no standard protocol or mechanism has been established for biosynthesis of nanoparticles. The carbon/energy source and nitrogen sources are necessary for growth and product formation in microbial cultivation. The nature and characteristics of these substrates has

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a predominant role to play in the metabolism of microorganism.

In this study silver oxide nanoparticles were prepared by simple and cost effective manner. Carbon and nitrogen sources are screened for enhanced growth and silver oxide nanoparticle production. Antimicrobial activity of the silver oxide nanoparticles were studied against human pathogenic bacteria.

MATERIALS AND METHODS

Chemicals

All salts and media constituents *viz.*, starch, sucrose, glucose, sorbital, maltose, peptone, yeast extract, beef extract, KNO_3, $(NH_4)_2SO_4$, NaCl, K_2HPO_4, KH_2PO_4, $MgSO_4$, NaOH, $CaCl_2$ *etc*. were purchased from Hi-media. $AgNO_3$ were purchased from SD fine chemicals, India.

Micro-organism and culture condition

Bacillus thuringiensis SSV1 (GenBank accession no. JX157140) was isolated from soil sample and grown on nutrient agar slants at 37°C for 24 h. The preserved culture was revived on fresh agar slants after every week for whole experiment. The pure cultures of pathogenic strains of *S. aureus, B. cereus, E. coli, Pseudomonas sp., P. mirabilis, E. faecalis* were obtained from, Clinical Pathology Department, CMC Hospital, Vellore, Tamil Nadu, India.

Inoculum preparation

The inoculum was prepared by adding loop full of pure culture *Bacillus thuringiensis* SSV1 into 100 ml of sterile nutrient broth medium at initial pH 7.0 into 250 ml shake flask and incubated at 37°C on a shaking incubator for 24 hours. The inoculums level was maintained at 2% (v/v) from the seed culture broth throughout the study.

Biosynthesis of silver oxide nanoparticles

The optimized culture media (100 ml) was centrifuged at 10,900 g for 15 min to obtain *Bacillus thuringiensis* SSV1 culture supernatant. 100 ml of cell free culture supernatant was added to 200 ml of 1 mM aqueous silver nitrate solution and kept in dark. After visible color change, the solution was centrifuged at 10,900 g for 15 min. The pellet was washed thrice with double distilled water to remove the media components and dried.

Characterization of Silver oxide nanoparticles

Extracellular synthesis of silver oxide nanoparticles was primarily monitored by UV-visible spectroscopy. Morphology and composition of the silver oxide nanoparticles were examined by X- ray diffraction analysis (Advance Powder X-ray diffractometer, Bruker, Germany model D8), Atomic Force Microscopy (Hitachi S4800).

UV-visible spectroscopy

The bio reduction of the Ag+ ions was monitored by reading absorbance of the samples at 2ml of the sample at different time intervals measured by 425nm using UV- visible spectroscopy. (PHARMASPEC UV–1700 series, SHIMADZU CORPORATION Ltd., KYOTO, JAPAN) with samples in quartz cuvette.

X- ray diffraction analysis

The silver oxide nanoparticles solution were obtained and was purified by repeated centrifugation at 10,900 g for 10 minutes followed by re-dispersion of the pellets of silver oxide nanoparticles with deionized water. After freeze drying the purified silver oxide solution the structure and composition were analyzed by using X ray diffraction methods. The dried mixture of silver oxide nanoparticles was collected for the determination of the formation of silver oxide nanoparticles by x-ray diffractometer operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in θ-2θ configuration.

Atomic force microscopy

Purified Silver oxide nanoparticles in suspension was also characterized their morphology using a VEeco diNanoscope 3D AFM. A small volume of sample was spread on a well-cleaned glass cover slip surface mounted on the AFM stub, and was dried with nitrogen flow at room temperature. Images were obtained in tapping mode using a silicon probe cantilever of 125µm length, resonance frequency 209-286 kHz, spring constant 20-80 nm-1 minimum of four images for each sample were obtained with AFM and analyzed to ensure reproducible results.

Selection of most suitable carbon and nitrogen sources

Different sources of carbon, nitrogen, metals were screened by the classical one variable at a time (OVAT) method to identify the most efficient carbon, nitrogen and metal source for bacterial growth. 1% of inoculum was added into 50 ml of the medium containing one of the carbon

sources *viz*., glucose, starch, sucrose, mannose, maltose (each at 1% w/v), one of nitrogen sources *viz*., peptone, yeast extract, beef extract, KNO_3 (each at 0.5% w/v) and one of the metal sources *viz*., NaCl-10 g/l, $MgSO_4.7H_2O$-0.2g/l; $CaCl_2$-0.2 g/l, KH_2PO_4-10 g/l; K_2HPO_4-2.5 g/l at initial pH 7.0 in 100 ml shake flasks. The flask was incubated at 37ÚC on a shaking incubator for 24 hours and cell densities were determined at 600 nm using UV-visible spectrophotometer (Gene Quant 1300).

Determination of antimicrobial activity by well-diffusion method

The silver oxide nanoparticles were tested for antimicrobial activity by well-diffusion method. The pure culture was sub-cultured into nutrient broth at 24 hrs. Wells of 6-mm diameter were made on Muller-Hinton agar plates using gel puncture and strains were swabbed uniformly onto the individual plates using sterile cotton swabs. 10 µL of the silver oxide nanoparticle (with stock concentration of 4 mg/ml, 0.4 mg/ml, 40 µg/ml, and 4 µg/ml) solution was poured into each of appropriate wells *i.e.,* at a final working concentration of 1.6 mg/ml, 0.16 mg/ml, 0.016 µg/ml, and 0.0016 µg/ml. After incubation at 35°C for 24 hours, the zone of inhibition was measured.

RESULTS AND DISCUSSION

Biosynthesis of silver oxide nanoparticles

In the presence of the supernatant, silver oxide nanoparticles were produced after 72 hours as was noted by the visible color change (Figure 1). It is an established fact that the culture supernatant turns from yellow color to brown color when silver oxide nanoparticles are produced[12]. Earlier it was revealed that the production of silver oxide nanoparticles takes place owing to reduction of silver nitrate mediated by some component present in the bacterial culture supernatant[12]. The characteristic brown color of colloidal silver oxide solution is due to the excitation of surface plasmon vibrations and provides a convenient spectroscopic signature of their formation [10, 12,13]. Silver oxide nanoparticle synthesis was further confirmed by UV- Visible spectrophotometric analysis. From the spectra, the silver oxide nanoparticle production was confirmed by the peak occurring around at 420 nm as observed in Figure 2.

The structural analysis of the Ag_2O NPs was performed using XRD. The peaks around 3.40 keV correspond to the binding energy of silver ion-ligand. Figure 3 shows the X-Ray diffraction peaks obtained at angles 2¸ of 27.79° (110), 32.29° (110), 38.13° (200), 46.35° (211), 54.83° (220), 64.49° (311), 67.37° (222) and 76.77° (311) are attributed solely to the face centered cubic crystalline silver oxide (Ag_2O) content of the sample (corresponding to JCPDS file no. 41-1104). The diffraction pattern therefore confirmed that the products were a mixture of silver oxide and small amounts of metallic silver. Similar results were reported earlier[9.10,13].

Atomic force microscopy has provided further insight into the morphology and size details of the Ag_2O NPs as presented in Figure 4. The particle size of the silver nanoparticles ranges in size from 10-40 nm. The morphology of the nanoparticles is almost spherical as observed in the micrograph.

The growth of *B. thuringiensis* SSV1 on the different carbon and nitrogen sources is

Fig. 1.

illustrated in the Figure 5. It is evident from the plots that sucrose supports growth more effectively (9.1488 g/L) as compared to other carbon sources (glucose, starch, mannose, and maltose) when peptone is used as the nitrogen source. In contrast to this, Patil *et al*., reported that sucrose did not show any effect on biomass growth[14]. Bacterial growth is better with beef extract as nitrogen source (10.1871 g/L) than other nitrogen sources used in this study *viz*., peptone, yeast extract, ammonium sulfate, ammonium nitrate, KNO_3 and urea. But metal sources did not show much effect on growth of the bacteria in contrast to a previous study[15]. Earlier Sarrafzadeh reported that metal ions did affected bacterial growth[16]. Similarly, Singer *et al* reported that glucose, sucrose and mineral salts in medium did not show any effect on growth[17].

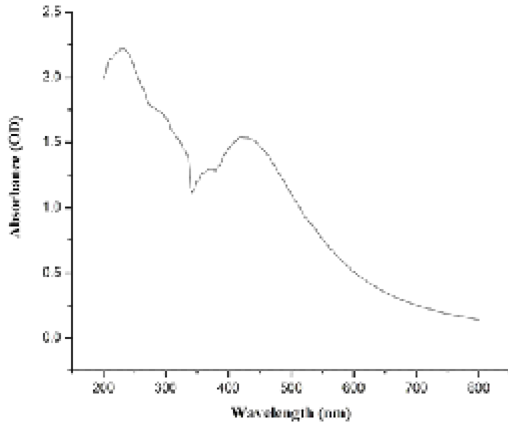

Fig. 2.

Antimicrobial activity of Silver oxide nanoparticles

The antibacterial activity of silver oxide nanoparticles was investigated by well-diffusion method. The inhibition zone caused by silver oxide nanoparticle solution against pathogens is shown in Figure 6. No bacterial growth was observed at higher nanoparticle concentration. The highest

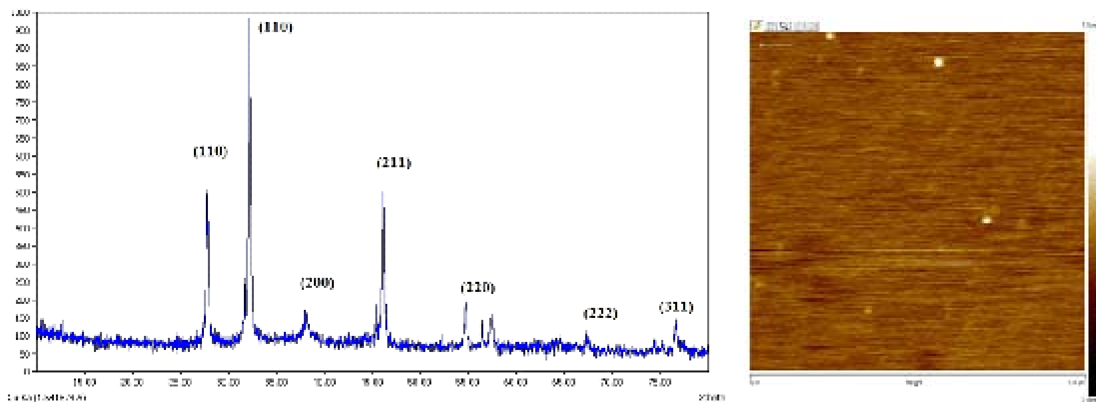

Fig. 3.

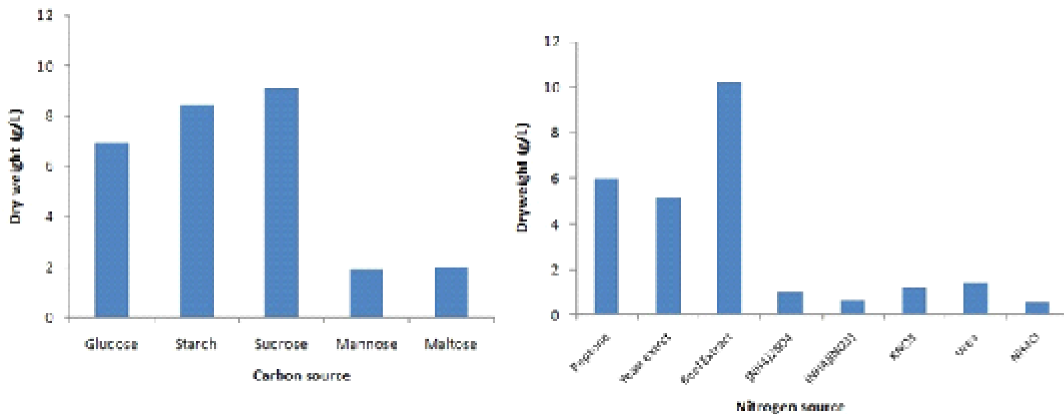

Fig. 4.

zone of inhibition was observed against all the strains at 1.6 mg/ml concentration except in case of *B. cereus*. Silver oxide nanoparticles did not show any inhibition to bacterial growth at lower concentration of 0.0016 µg/ml against any of the strains used in this study. There is only a few report available on biosynthesis of silver oxide nanoparticle[18], but its antibacterial activity was not reported earlier. Many reports on antibacterial activity of silver nanoparticle are available in literature[5,6]. Srinivas *et al.*[19] reported antimicrobial activity of silver nanoparticles from strawberry leaf extract using disc diffusion method. Their results showed that silver nanoparticles were very much effective in both gram positive and gram negative bacteria (such as *S.aureus, E.coli, P. putida, K.pneumonia, B.subtilis*) at concentration of 1mg/ml. Panacek *et al*[20] reported that 1.69 mg/mL concentration of silver nanoparticles had shown antibacterial performance. To the best of our knowledge, this is the first report on silver oxide nanoparticles showing antibacterial effect against pathogenic microorganisms at such a low concentration of 1.6 mg/ml.

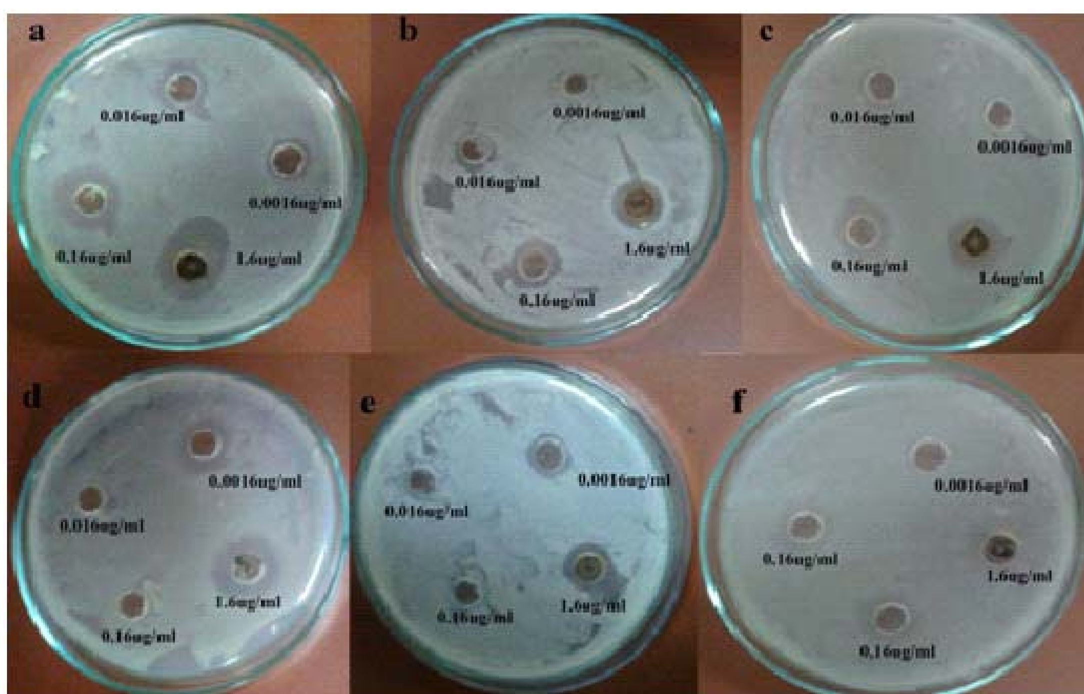

Fig. 5.

CONCLUSION

Bacillus thuringiensis SSV1 was found to synthesize silver oxide (Ag_2O) nanoparticles extracellularly in a size range of 10 – 40 nm. The nanoparticles were found to be spherical and monodispersed. Sucrose and beef extract were found to be the best carbon and nitrogen sources respectively for the growth of *Bacillus thuringiensis* SSV1 whereas metal ion sources did not show any impact on its growth. The silver oxide nanoparticles had shown inhibitory effect against antibiotic resistant human pathogens namely, *E. coli, Pseudomonas sp., P. mirabilis, E. faecalis* (Gram negative) and *S. aureus* (Gram positive).

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