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# Novel microbial route to synthesize silver nanoparticles using spore crystal mixture of *Bacillus thuringiensis*

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Metallic nanoparticles are traditionally synthesized by wet chemical techniques, where the chemicals used are often toxic and flammable. In the present study, the spore crystal mixture of *Bacillus thuringiensis* was used for the synthesis of silver nanoparticles. Nanoparticles were characterized using UV-Vis absorption spectroscopy, XRD and TEM. X-ray diffraction and TEM analysis showed the average particle size of 15 nm and mixed (cubic and hexagonal) structure. This is for the first time that any bacterial spore crystal mixture was used for the synthesis of nanoparticles. Further, these biologically synthesized nanoparticles were found to be highly toxic against different multi drug resistant human pathogenic bacteria.

**Keywords**: Antimicrobial activity, *Bacillus thuringiensis*, Spore crystal mixture

Nanoparticles exhibit completely new or improved properties and have applications in diverse fields[1, 2]. Silver nanoparticles have applications in bimolecular detection, diagnostics, antimicrobials and therapeutics, catalysis, micro-electronics, photonics, optics, DNA sequencing, surface-enhanced Raman spectroscopy and pharmaceuticals[3-5]. However, there is need for economic, commercially viable and environmentally clean synthesis route.

A number of chemical, physical and biological approaches are available for the synthesis of silver nanoparticles[4,6]. The chemical and physical methods are harmful in one or the other way as the chemicals used are toxic, flammable, do not dispose off in the environment easily[7]. Chemical synthesis methods lead to presence of some toxic chemicals absorbed on the surface of nanoparticles that may have adverse effect in the medical applications.

Efforts have been made for the search of methods utilizing the biological system for nonmaterial synthesis. Environmentally benign materials like plant leaf extract[8], plant fruit extract[9], bacteria[10], fungi[11], yeast[7] and enzymes[12] for the synthesis of silver nanoparticles have been used. Green synthesis provides advantage over the chemical and physical method as it is cost effective, environment friendly, single step method, easily scaled up for large scale synthesis and it does not require the use of high pressure, energy, temperature and toxic chemicals.

Many microbes are known to produce nanostructured mineral crystals and metallic nanoparticles with properties similar to chemically synthesized materials, while exercising strict control over size, shape and composition of the particles. Among the microorganisms, prokaryotic bacteria have received the most attention in the area of bio-synthesis of nanoparticles. Exact reaction mechanisms leading to the formation of silver nanoparticles by the silver resistant bacteria is yet to be elucidated.

The present article deals with the synthesis of silver nanoparticles, reducing the silver ions present in the solution of silver nitrate by the spore crystal mixture of *Bacillus thuringiensis* (Bt). The spore crystal mixture of Bt has been used because it is easily available throughout the year, and doesn't have any seasonal dependence unlike the plant based extracts. Further, these biologically synthesized nanoparticles have been found highly toxic against different multi drug resistant human pathogens.

## Materials and Methods

*Bacterial strain and preparation of spore crystal mixture*—Bt strain IS1 used in this study, was isolated from soils of Bikaner (Rajasthan), characterized by microscopic and sequencing of partial 16s rDNA

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region (Genebank Accession no. FJ755916) and maintained in the laboratory. Bt strain was grown in T3 medium[13] at 30°C until cell lysis (48~72 h). The spore crystal mixture was centrifuged at 10,000 x *g* for 5 min and washed twice in 0.5 *M* NaCl and TE buffer [Tris 10m*M*, EDTA 1 m*M*, *p*H 8.0]. Finally, the spore crystal mixture was resuspended in a 2 ml of sterile milli-Q water.

*Synthesis of silver nanoparticles*—Aqueous solution of silver nitrate (1m*M* $AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. Spore crystal mixture (1 ml) was added into 99 ml of aqueous solution of 1 m*M* silver nitrate for reduction and kept at room temperature for 5 h.

*UV-Vis spectra analysis*—Reduction of pure $Ag^+$ ions was monitored by measuring UV-Vis spectrum between 300-600 nm range of the reaction medium at 5 h after diluting a small aliquot of the sample into distilled water. The spectral analysis was done by using UV-2450 spectrophotometer (Shimadzu, Japan). It is generally recognized that UV-Vis spectroscopy could be used to examine size and shape controlled nanoparticles in aqueous suspensions[14].

*XRD measurement*—Silver nanoparticle solution, thus, obtained was purified by repeated centrifugation at 5000 rpm for 20 min followed by redispersion of the pellet of silver nanoparticles into 10 ml of deionized water. The dried mixture of silver nanoparticles was collected to test the formation of Ag nanoparticles by an X'Pert Pro X-ray diffractometer (PAN analytical BV, The Netherlands) operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in a θ- 2 θ configuration. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer formula.

$$D = 0.94 \lambda / \beta \cos \theta$$

where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the diffraction angle. To eliminate additional instrumental broadening, FWHM was corrected using the FWHM from a large grained Si sample.

$$\beta \text{ corrected} = (FWHM^2_{sample} - FWHM^2_{si})^{1/2}$$

This modified formula is valid only when the crystallite size is smaller than 100 nm[15].

*TEM analysis of silver nanoparticles*—Transmittance Electron Microscopic (TEM) analysis was done using Joel JEM-1400 TEM machine. The samples were prepared on a carbon coated copper grid by dropping small amount of the sample on grid, extra solution was removed using a blotting paper and then the film on TEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

*Antibacterial assay*—Antibacterial assays were done on multi drug resistant (MDR) human pathogens obtained from SMS Medical College and Hospital by standard disc diffusion method[16]. Susceptibility of these microbial strains to different antibiotics was tested. Luria Bertani (LB) broth/agar medium was used to cultivate bacteria. Fresh overnight cultures of inoculum (100 µl) of each culture was spread on to LB agar plates. Sterile paper discs of 5 mm diam. (containing 50 mg/l silver nanoparticles) were placed in each plate.

## Results and Discussion

Silver nanoparticles exhibit yellowish brown colour in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles[14]. As the spore crystal mixture was mixed in the aqueous solution of the silver ion complex, it started to change colour from watery to yellowish brown due to reduction of silver ion, which indicated formation of silver nanoparticles. After 5h incubation, reduction of silver ions present in the aqueous solution of silver complex during the reaction with ingredients present in Bt spore crystal mixture observed by UV-Vis spectroscopy revealed the presence of silver nanoparticles (Fig. 1). Absorption spectra of silver nanoparticles formed in the reaction medium had absorbance peak at 450 nm, broadening of peak indicated that the particles were polydispersed.

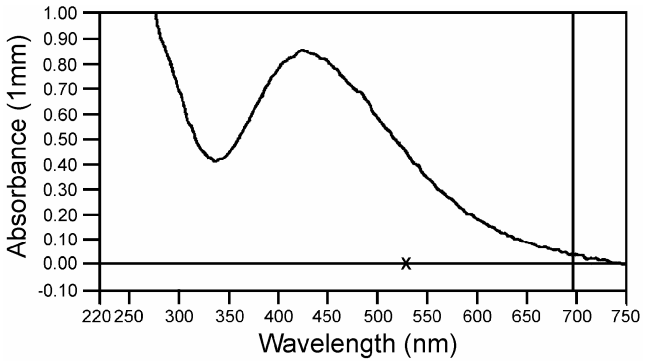


Fig. 1—UV-Vis absorption spectra of silver nanoparticles synthesized by Bt spore crystal mixture after 5 h.

Biosynthesized silver nanostructure by employing Bt spore crystal mixture was further confirmed by the characteristic peaks observed in XRD image (Fig. 2) and the structural view under the transmittance electron microscope (Fig. 3). The XRD pattern showed four intense peaks in the whole spectrum of $2\theta$ value ranging from 10 to 80. The four intense peaks observed in the spectrum agree to the Braggs's reflection of silver nanocrystals reported in the literature[17]. The typical XRD pattern (Fig. 2) revealed that the sample contains a mixed phase (cubic and hexagonal structures) of silver nanoparticles. The average estimated particle size of this sample was 15 nm derived from FWHM of peak corresponding to 111 plane (Fig. 2). TEM image showing the high density silver nanoparticles synthesized by Bt spore crystal mixture confirmed the development of silver nanostructures. Micrograph showed nanoparticles with variable shapes. The size of the particles ranged from 10 to 20 nm. Majority of the silver nanoparticles were scattered with only a few of them showing aggregates of varying sizes as observed under TEM (Fig. 3).

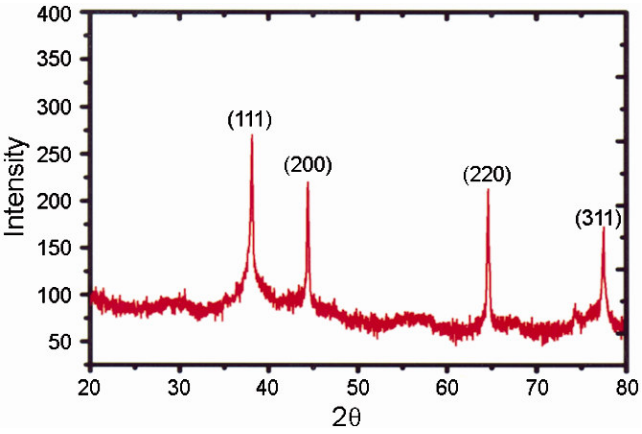


Fig. 2—XRD patterns of silver nanoparticles.

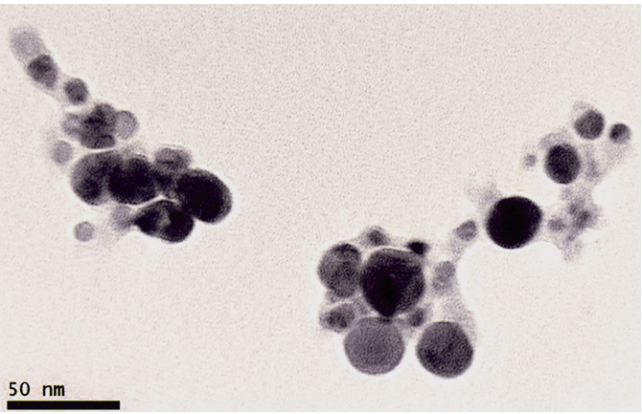


Fig. 3—TEM micrograph of silver nanoparticles

Further the nanoparticles synthesis by a novel microbial route was found highly toxic against multi drug resistant human pathogenic bacteria at a concentration of 50 ppm (Fig. 4). Silver nanoparticles exhibited antibacterial activity against multi drug resistant (MDR) human pathogenic *Escherichia coli*, *Pseudomonas aeruginosa* and *Streptococcus aureus* as it showed a clear inhibition zone whereas the standard antibiotics did not show any inhibition (Table 1). Several studies propose that silver nanoparticles may attach onto the surface of bacterial cell membrane disturbing their permeability and respiration functions. Smaller silver nanoparticles having the large surface area available for interaction would give more bactericidal effect than the larger silver nanoparticles[3]. Silver nanoparticles synthesized via green route were highly toxic to pathogenic bacteria, hence has a great potential in biomedical applications.

The present study showed a simple, rapid and economical route to synthesize silver nanoparticles. Biological synthesis provides particles with good control over size distribution and shape. The main reason for this may be that the processes devised by nature for the synthesis of inorganic metals on nano scales have contributed to the development of a relatively new and unexplored area of research based on the use of different microbes[18].

Table 1—Resistance profiling of MRD pathogens against standard antibiotics

| Microorganisms | Resistance pattern of antibacterial agents |
|---|---|
| *E. coli* | Pc, Do, Cfx, Cpm, C, T, A, Cfs, Gf |
| *S. aureus* | Do, Cfs, G |
| *P. aeruginosa* | Gm, Pc, T, Gf |

Antibacterial Agents: Pc-Piperacillin (100 mcg); Do-Doxycycillne (30 mcg); Cfx-Cefixime (10 mcg); Cpm-Cefepime (30 mcg); C-Chloramphenicol (30 mcg); T-Tetracycline (30 mcg); A-Ampicillin (25 mcg); Cfs-Cefoperazone (30 mcg); Gf-Gatifloaxacin (10 mcg); G-Gentamycin (50 mcg)

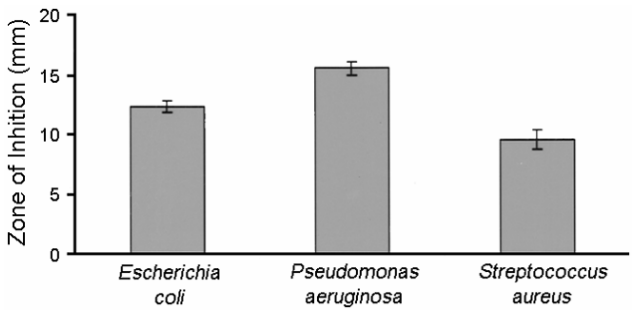


Fig. 4—Antibacterial activity of silver nanoparticles against some pathogenic microorganisms (Error bars represent S.D.)

In this study, we demonstrated the extracelluar synthesis of silver nanoparticles by Bt spore crystal mixture. Reduction of the metal ions through various plants and fungus extracts leading to the formation of silver nanoparticles are fairly well-defined. But the capabilities of the bacteria and algae as a capping and reducing agent are not well known. The previous studies have suggesed that maximum silver nanoparticle synthesis occurs at stationary phase of *Bacillus licheniformis*[19]. Pugazhenthiran *et al*.[20] have reported that the silver resistant *Bacillus* sp. when cultured with silver nitrate synthesize silver nanoparticles in the periplasmic space of the cell. Silver nanoparticles (2–5 nm size) are synthesized extracellularly by a silver tolerant yeast strain MKY3, when subjected to silver nitrate in the log phase of growth[7]. Early studies reveal that *Bacillus subtilis* 168 is capable in reducing $Au^{3+}$ ions to 5–25 nm octahedral gold particles within the bacterial cells under ambient conditions[21]. Xie *et al.*[22] have reported the extract of unicellular green alga *Chlorella vulgaris* is involved in the shape-controlled synthesis of silver nanoplates due to the presence of different proteins.

Most of the previous research on synthesis of silver nanoparticles using plant extracts employed a broth resulting from boiling fresh plant leaves. This approach suffers from few drawbacks mainly that fresh leaves would not be readily available for the bioreduction throughout the year, secondly, it is difficult to control some parameters accurately such as the optimum boiling time for aqueous leaf extract preparation[23].

The green chemistry approach toward the synthesis of silver nanoparticles has many advantages such as, ease with which the process can be scaled up, economic viability, etc. Applications of such eco-friendly nanoparticles in cancer treatment, drug delivery, sensors, commercial appliances and other medical and electronic applications, makes this method potentially exciting for the large-scale synthesis of other inorganic nanomaterials[24]. Toxicity studies of silver nanoparticles on human pathogen opens a door for a new range of antibacterial agents.

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## References

1 Jahn W, Chemical aspects of the use of gold clusters in structural biology, *J Struct Biol,* 127 (1999) 106.

2 Murphy C J, Sustainability as an emerging design criterion in nanoparticle synthesis and applications, *J Mater Chem*, 18 (2008) 2173.

3 Rai M, Yadav A & Gade A, Silver nanoparticles as a new generation of antimicrobials, *Biotechnol Advs,* 27 (2009) 76.

4 Panáek A, Kvítek L, Prucek R, Kolár M, Veeřová R, Pizúrová N, Sharma V K, Nevěná T & Zbořil R, Silver Colloid Nanoparticles: Synthesis, Characterization, and Their antibacterial Activity, *J Phys Chem B,* 110 (2006) 16248.

5 Vaidyanathan R, Kalimuthu K, Gopalram G & Gurunathan S, Nanosilver-The burgeoning therapeutic molecule and its green synthesis, *Biotechnol Adv*, 27 (2009) 924

6 Goia D V & Matijevic E, Preparation of monodisperse metal particles, *New J Chem,* 22 (1998)1203.

7 Kowshik M, Ashtaputre S, Kharrazi S, Vogel S, Urban J, Kulkarni S K & Paknikar K M, Extracellular synthesis of silver nanoparticles by a silver-tolerant yeast strain MKY3, *Nanotechnology,* 14 (2003) 95.

8 Song J Y & Kim B S, Rapid biological synthesis of silver nanoparticles using plant leaf extracts, *Bioproc Biosyst Eng*, 32 (2009) 79.

9 Jain D, Daima H K, Kachhwaha S & Kothari S L, Synthesis of plant-mediated silver nanoparticles using papaya fruit extract and evaluation of their anti microbial activities. *Dig J Nanomater Biostruct,* 4(3) (2009) 557.

10 Saifuddin N, Wong C W & Yasumira A N, Rapid biosynthesis of silver nanoparticles using culture supernatant of bacteria with microwave irradiation**,** *E - J Chem*, 6(1) (2009) 61.

11 Bhainsa K C & D'Souza S F, Extracellular biosynthesis of silver nanoparticles using the fungus *Aspergillus fumigatus* , *Coll Surf B: Biointerfac*,47 (2006) 160.

12 Willner B, Basnar B & Willner B, Nanoparticle-enzyme hybrid systems for nanobiotechnology, *FEBS J,* 274 (2007) 302.

13 Martin P A W & Travers R S, Worldwide abundance and distribution of *Bacillus thuringiensis* isolates, *Appl Envron Microbiol*, 55 (1989) 2437.

14 Wiley B J, Im S H, McLellan J, Siekkinen A & Xia Y, Maneuvering the surface plasmon resonance of silver nanostructures through shape-controlled synthesis, *J Phys Chem B*, 110 (2006) 15666.

15 Boulch F, Schouler M C, Donnadieu P, Chaix J M & Djurado E, Domain size distribution of Y-TZP nano-particles using XRD and HRTEM, *Image Anal Stereol,* 20 (2001) 157.

16 Maruzzella J C & Henry P A, The antimicrobial action of perfume oils, *J Am Pharmaceutic Assoc*, 28 (1958) 471.

17 Lu H W, Liu S H, Wang X F, Qian J, Yin J & Jhu J K, Silver nanocrystals by hyperbranched polyurethane-assisted photochemical reduction of $Ag^+$, *Mater Chem Phys,* 81 (2003) 104.

18 Mandal D, Bolander M E, Mukhopadhyay D, Sarkar G & Mukherjee P, The use of microorganisms for the formation of metal nanoparticles and their application, *Appl Microbiol Biotechnol*, 69 (2006) 485.

19 Kalimuthu K, Suresh Babu R, Venkataraman D, Bilal M & Gurunathan S, Biosynthesis of silver nano crystals by *Bacillus licheniformis*, *Coll Surf B: Biointerfac*, 65 (2008) 150.

20 Pugazhenthiran N, Anandan S, Kathiravan G, Udaya Prakash N K, Crawford S & Ashokkumar M, Microbial synthesis of silver nanoparticles by Bacillus sp. *J Nanopart Res*, 11 (2009) 1811.

21 Fortin D & Beveridge T J, From biology to biotechnology and medical applications, in: *Biomineralization,* edited by E Baeuerien (Wiley-VCH, Weinheim) 2000, 7.

22 Xie J, Lee J Y, Wang D I C & Ting Y P, Silver nanoplates: From biological to biomimetic synthesis, *ACS-Nano*, 1 (2007) 429.

23 Huang J, Li Q, Sun D, Lu Y, Su Y, Yang X, Wang H, Wang Y, Shao W, He N, Hong J & Chen C, Biosynthesis of silver and gold nanoparticles by novel sundried *Cinnamomum camphora* leaf, *Nanotechnology*, 18 (2007) 105104

24 Sharma V K, Yngard R A & Lin Y, Silver nanoparticles: Green synthesis and their antimicrobial activities, *Adv Coll Interfac Sci*, 145 (2009) 83.