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Research Article

BIOSYNTHESIS OF SILVER NANOPARTICLES USING THE BACTERIA *BACILLUS CEREUS* AND THEIR ANTIMICROBIAL PROPERTY

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ABSTRACT

The use of microorganisms in the synthesis of nanoparticles emerges as an eco-friendly and exciting approach. In this study, we report on the use of *Bacillus cereus* for the extracellular synthesis of silver nanoparticles (Ag NPs) from silver nitrate solution. Ag NPs were characterized by UV–vis spectroscopy, fourier transform infrared (FTIR) spectroscopy, and atomic force microscopy (AFM). UV–vis spectroscopy of Ag NPs exhibited peak at 440 nm which corresponds to the surface plasmon resonance of Ag NPs. FTIR spectroscopy confirmed the presence of protein as the stabilizing agent surrounding the Ag NPs. AFM of Ag NPs showed irregular shape with 62.8 nm in size. Antimicrobial activity of the silver bio-nanoparticles was performed by well diffusion method against *Staphylococcus aureus, Salmonella typhi*, *Klebsiella pneumoniae* and *Escherichia coli*. The highest antimicrobial activities recorded were against *Staphylococcus aureus* followed by *Klebsiella pneumoniae* and *Salmonella typhi*, where as *Escherichia coli* showed the lesser activity.

Keywords: *Bacillus cereus*, Silver nanoparticles, UV–vis spectroscopy, FTIR, AFM, Antimicrobial activity.

INTRODUCTION

Nanotechnology is emerging as a rapidly growing field with its application in science and technology for the purpose of manufacturing new materials at the nanoscale level[1]. Recently, biosynthetic methods employing microorganism such as bacteria[2] and fungus[3] or plants extract [4–6], have emerged as a simple and viable alternative to more complex chemical synthetic procedures to obtain nanomaterials. Different types of nanomaterials like copper, zinc, titanium[7], magnesium, gold[8], alginate[9] and silver have come up but silver nanoparticles have proved to be most effective as it has good antimicrobial efficacy against bacteria, viruses and other eukaryotic microorganisms[10]. Of these, silver nanoparticles are playing a major role in the field of nanotechnology and nanomedicine.

Several microorganisms have been utilized to synthesize silver nanoparticles (Ag NPs) intracellularly or extracellularly[2,11–15]. For instance, Ag containing nanocrystals of different compositions were synthesized by *Pseudomonas stutzeri* AG259 bacterium[2]. In *Fusarium oxysporum* fungus, the reduction of Ag^+ ions was attributed to an enzymatic process involving NADH-dependent reductase[14]. The white rot fungus, *Phanerochaete chrysosporium*, also reduced Ag^+ ion to form Ag NPs, a protein was suggested to cause the reduction[15]. Silver nanoparticles are synthesized by the biomass of the bacterium, *B. licheniformis*[16] and *Staphylococcus* aureus[17]. Silver has been known to possess strong antimicrobial properties both in its metallic and nanoparticle forms hence; it has found variety of application in different fields. The nanocrystalline silver dressings, creams, gel effectively reduce bacterial infections in chronic wounds[18]. The present work has focused on the development of an extracellular biosynthesis of silver nanoparticles using *Bacillus cereus* and the evaluation of their antimicrobial activity against various human pathogenic bacteria. The study also includes UV–vis spectroscopy, fourier transform infrared spectroscopy and atomic force microscopic for characterization of silver nanoparticles.

MATERIALS AND METHODS

Isolation of the bacteria

The soil samples were collected from Pichavaram mangrove ecosystem situated along the southeast coast of India. They were serially diluted in sterile distilled water and plated on nutrient agar plates. The plates were then incubated at 37ºC for 48 h. The morphological and physiological characterization of the isolate was performed according to the methods described in Bergey's manual of determinative bacteriology and Manual of clinical microbiology[19,20].

Biosynthesis of silver nanoparticles

The characterized isolate was inoculated into sterile nutrient broth and 2 g of wet biomass was harvested at different points of time. The biomass obtained was washed with phosphate buffer (pH 7.0) thrice and collected in a 500 ml Erlenmeyer flask. One mM solution of $AgNO_3$ was prepared using de-ionized water, and 100 ml of the solution was added to the biomass harvested at each point of time. The Erlenmeyer flasks were incubated at 37°C under agitation (200 rpm) for 24 h.

Sonication of samples

The cells from each Erlenmeyer flask were washed twice with 50 mM phosphate buffer (pH 7.0) and re-suspended in 1 ml of the same buffer. Ultrasonic disruption of cells was carried out with an ultrasonic processor over three 15 s periods, and with an interval of 45 s between periods. The sonicated samples were centrifuged at 15,000 rpm for 30 min at 4°C to remove cell debris. The supernatants were then used for the characterization of silver nanoparticles.

Evaluation of maximum nanoparticle synthesis

To determine the time point of maximum production of silver nanoparticles, the absorption spectra of the supernatants were taken 340 to 560 nm using a UV–vis spectrophotometer. The deionized water was used as the blank.

Characterization of silver nanoparticles

The supernatant from the maximum time point of production (of silver nanoparticles) was freeze-dried and the dried powder was diluted with potassium bromide in the ratio of 1:100 and recorded the spectrum in Fourier Transform Infrared spectrum (FTIR) in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} by using the diffuse reflectance accessory. The supernatant from the maximum time point of production of silver nanoparticles were also allowed to characterize by Atomic force microscopy (AFM) for its detail size, morphology and agglomeration of silver. AFM Image was taken with silicon cantilevers with force constant 0.02 – 0.77 N/m, tip height 10–15 nm, contact mode.

Antimicrobial activity by well diffusion method

The Silver nanoparticles (Ag NPs) synthesized from *Bacillus cereus* was tested for their antimicrobial activity by well diffusion method against pathogenic organism like *Staphylococcus aureus*, *Salmonella typhi, Klebsiella pneumoniae* and *Escherichia coli*. The pure cultures of organism were sub cultured on Muller-Hinton broth at 35°C on rotary shaker at 200 rpm. Wells of size 6 mm have been made on Muller-Hinton agar plates using gel puncture. Each strain was swabbed uniformly on the individual plates using sterile cotton swab. Using micropipette, 20µl (0.002mg) of the sample of nanoparticles solution was poured into wells on all plates. After incubation at 35°C for 18 h, the different levels of zone of inhibition were measured.

RESULTS

The bacteria were isolated from Pichavaram mangrove forest soil sample. Pure colonies were obtained and characterized as *Bacillus cereus* based on the results described in Bergey's manual of determinative bacteriology and Manual of clinical microbiology[19,20].

Silver nanoparticles biosynthesis

Figure 1 shows the conical flasks containing aqueous silver ions which were reduced to silver nanoparticles when added to the biomass of *B. cereus*. It is observed that the colour of the solution turned from whitish to brown after 24 h of the reaction, which indicated the formation of silver nanoparticles extracellularly.

The formation and stability of the reduced silver nanoparticles in the colloidal solution was monitored by UV–vis spectrophotometer analysis. The UV–vis spectra for supernatants from all harvested biomass samples (after incubation with silver nitrate and subsequent sonication) it showed a maximum absorbance at 440 nm, which increased with the time of incubation of silver nitrate with the biomass (Figure 2). The curve showed the increased absorbance in various time intervals (6 h, 12 h, 18 h and 24 h) and the peak at 440 nm which corresponds to the surface plasmon resonance of silver nanoparticles.

Figure 3 shows that the FTIR spectrum of silver nanoparticles. The two bands were present at 3441.71 cm^{-1} and 1650.10 cm^{-1}. The 1650.10 cm^{-1} band was identified as amide and this observation confirms the presence of protein in the sample of silver nanoparticles.

The result obtained from AFM gave the shape and size of the silver nanoparticles produced from *B.cereus*. Figure 4 shows the topographical image of irregular Ag NPs, it can be clearly seen as nano island formation apart from that there is prominent agglomeration of silver. The size of the Ag NPs was found to be 62.8 nm.

Antimicrobial activity of silver nanoparticles

The antimicrobial activity of silver nanoparticles synthesized by *B. cereus* was investigated against various pathogenic organisms such as *Staphylococcus aureus*, *Salmonella typhi, Klebsiella pneumoniae* and *Escherichia coli* (Figure 5). The diameter of inhibition zones (mm) around each well with silver nanoparticles solution is represented in Table 1. The highest antimicrobial activity was found against *Staphylococcus aureus* (25 mm) followed by *Klebsiella pneumoniae* (22 mm) and *Salmonella typhi* (21 mm). The lesser antimicrobial activity was found against *Escherichia coli* (18 mm).

Table 1: Zone of inhibition of silver nanoparticles against various pathogenic bacteria

Pathogenic bacteria	Zone of inhibition (mm)
Staphylococcus aureus	25
Klebsiella pneumoniae	22
Salmonella typhi	21
Escherichia coli	18

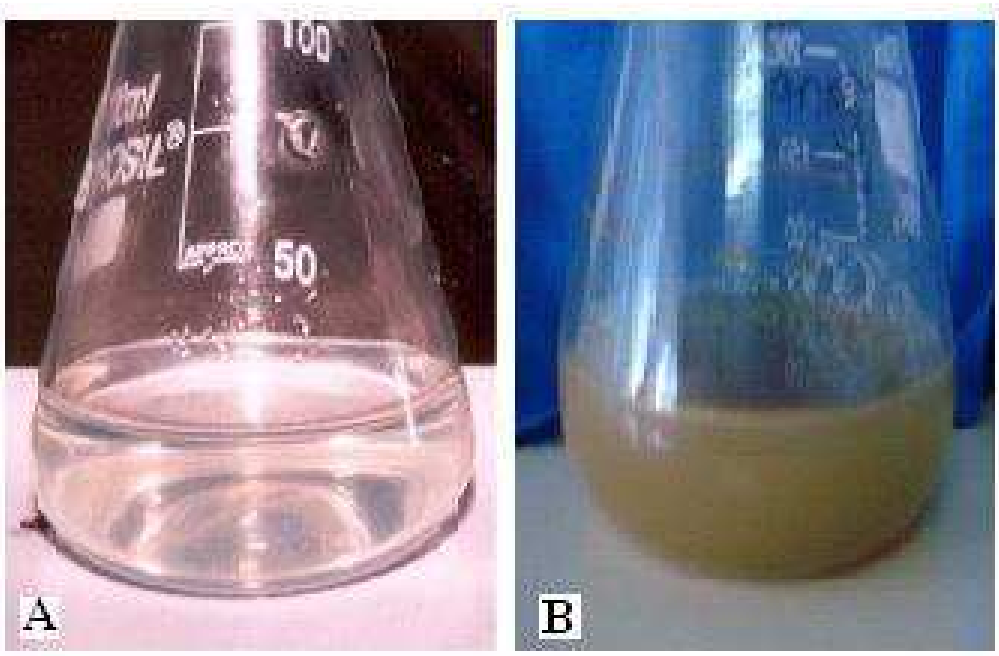


Fig. 1: Biosynthesis of silver nanoparticles visible observation. (A) Conical flask containing $AgNO_3$ solution without *B.cereus* for 24 h (no colour change), (B) Conical flask containing *B.cereus* with $AgNO_3$ solution for 24 h (brown colour)

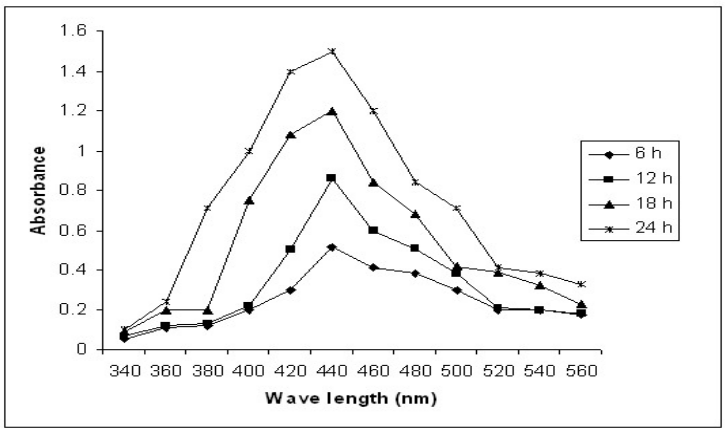


Fig. 2: UV–Vis spectra recorded at different time intervals.

The samples were collected at different time intervals of growth and the cells were incubated with $AgNO_3$. After incubation period, the cultures were sonicated and visualized in UV–vis spectra. The curve shows the increased absorbance in various time intervals (6 h, 12 h, 18 h and 24 h). The peak at 440 nm corresponds to the surface plasmon resonance of silver nanoparticles.

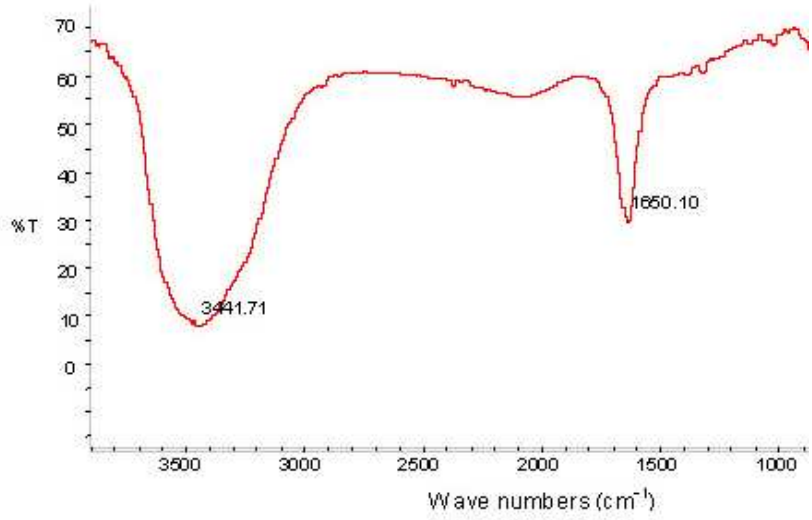


Fig. 3: FTIR spectrum of silver nanoparticles synthesized by *B.cereus*

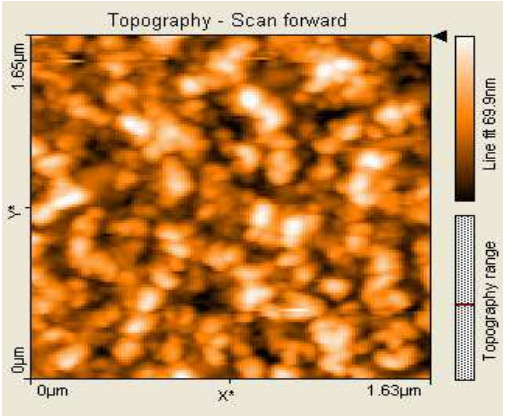
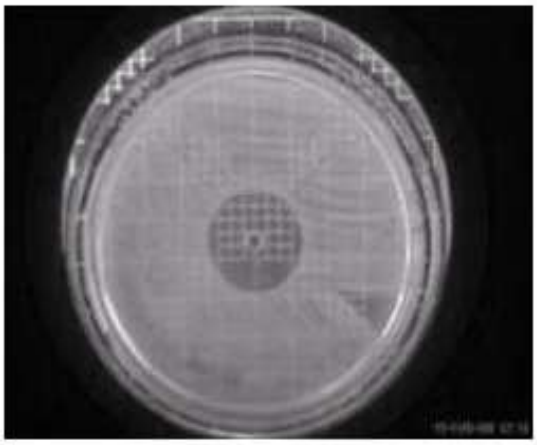
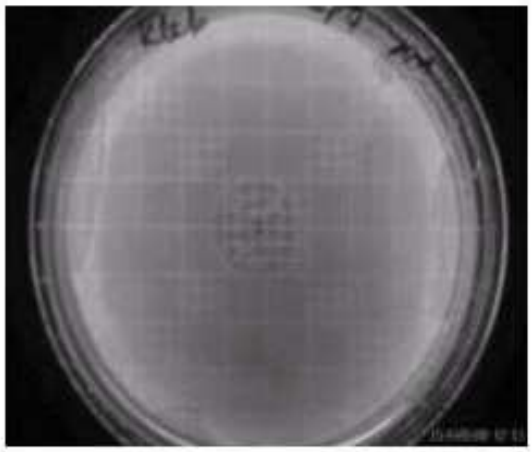


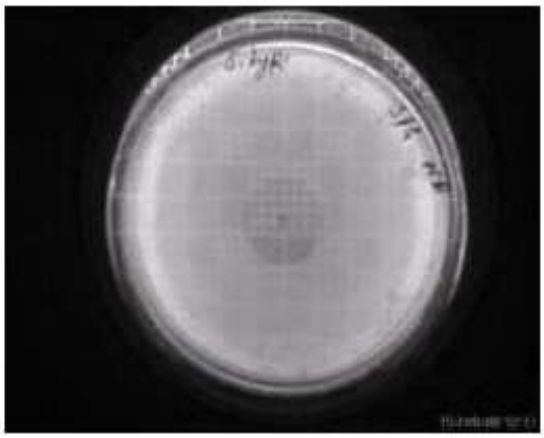
Fig. 4: AFM image of the silver nanoparticles synthesized by *B.cereus*



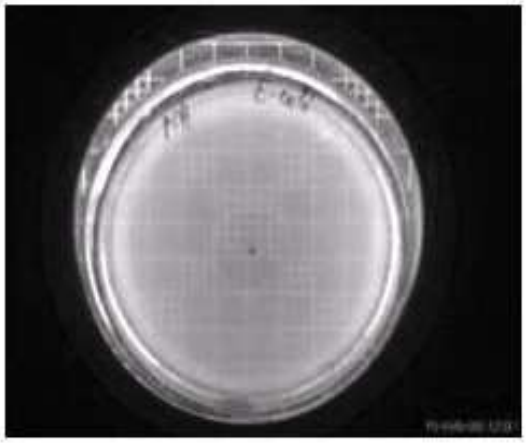
Bionanoparticles against *S. aureus*



Bionanoparticles against *K. pneumoniae*



Bionanoparticles against *S. typhi*



Bionanoparticles against *E. coli*

Fig. 5: Antimicrobial activity of silver nanoparticles against various pathogenic bacteria shown by well diffusion method.

DISCUSSION

The detailed study on biosynthesis of silver nanoparticles by *B. cereus* was carried out and is reported in this work. The characterization of Ag^+ ions exposed to this bacterial supernatant by UV–vis spectrophotometer confirmed the reduction of silver ions to silver nanoparticles. The peak was noticed at 440 nm which corresponded to the surface plasmon resonance of silver nanoparticles. It has been reported earlier that absorbance at 440 nm corresponds to surface plasmon resonance of Ag NPs[16]. The FTIR spectroscopy confirmed the presence of proteins in sample. The band at 1650.10 cm^{-1} was identified as amide. Previous studies have indicated that proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins and via the electrostatic attraction of negatively charged carboxylate groups in enzymes present in the cell wall of mycelia[21]. The bands at 1650 and 1540 cm^{-1} are identified as the amide I and II bands and arise due to carbonyl stretch and –N–H stretch vibrations in the amide linkages of the proteins, respectively. The positions of these bands are close to that reported for native proteins[22] and therefore, this evidence suggests that the stabilization of the silver nanoparticles by protein is a possibility.

The results showed that *B. cereus* could be used for the production of silver nanoparticles from silver nitrate. Moreover, biomass that is harvested at the stationary phase results in the maximum production of nanoparticles for the given incubation period. The enzyme involved in the synthesis of nanoparticles may be the nitrate reductase present in *B. cereus*. This enzyme is induced by nitrate ions and reduces silver ions to metallic silver. The possible mechanism that may involve the bioreduction of silver ions is the electron shuttle enzymatic metal reduction process earlier proposed for gold nanoparticles[23] and silver nanoparticles[16]. The earlier works reported that NADH and NADH-dependent nitrate reductase enzymes are responsible for the biosynthesis of metal nanoparticles[13]. *B. cereus* is known to secrete the cofactor NADH and NADH-dependent enzymes that might be responsible for the bioreduction of Ag^+ to Ag^0 and the subsequent formation of Ag NPs. The reduction may initiate by means of the electrons transfer from NADH by NADH-dependent reductase as electron carrier. Then the Ag^+ ions obtain electrons and thus reduced to Ag^0.

In the present study, 0.002 mg of the nanoparticles was taken as final product for antimicrobial assay. The antibiotic activity of the bio-nanoparticles for *Staphylococcus aureus* was maximum (25 mm) followed by *Klebsiella pneumoniae* (22 mm), *Salmonella typhi* (21 mm)

and *Escherichia coli* (18 mm). It was reported previously that *E. coli* being the model for Gram negative bacteria was found to be susecptible for silver nanoparticles thus confirming its atimicrobial property[24]. The silver nanoparticles have antimicrobial effect on *Staphylococcus aureus* and *E. coli*[25]. It was clear from the experiment that *Staphylococcus aureus* being Gram positive showed most susceptibility against the nanoparticles in comparision to *Klebsiella pneumoniae*, *Salmonella typhi* and *Escherichia coli* being Gram negative. The strongest reason about the susceptibility of *Staphylococcus aureus* against nanoparticles may be due to their cell wall plasmolysis or separation of cytoplasm from their cell wall[26]. The antimicrobial mechanisms of bio-nanosilver particles may differ from species to species of bacteria and also on the size of the nanoparticles.

CONCLUSION

In conclusion, synthesis of silver nanoparticles by *B. cereus* is an economical, efficient, eco-friendly and simple process. UV–vis spectrophotometer, FTIR and AFM techniques confirmed the reduction of silver nitrate to silver nanoparticles by the *B. cereus*. The zone of inhibition formed in the screening test indicated the antimicrobial activity against various human pathogenic bacteria. It shows that the silver nanoparticles synthesized by this process find use in the field of medicine. In future, our aim is to synthesize the silver nanoparticles by using different microbes and also to study the biochemical and molecular mechanism of nanoparticles formation by the cell filtrate in order to achieve better control over size and polydispersity of the nanoparticles.

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