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Green Synthesis of Silver Nanoparticles using *Bacillus subtillus* IA751 and its Antimicrobial Activity

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ABSTRACT

Nanobiotechnology has emerged as integration between biotechnology and nanotechnology for developing bioactive, biosynthetic and ecofriendly technology for synthesis of nanomaterials. This study has investigated extracellular biosynthesis of silver nanoparticles using *Bacillus subtillus* IA751. The synthesis of silver nanoparticles was rapid and within few hours silver ion makes contact with the cell filtrate and reduces Ag^{+} to Ag^{0}. The reaction time was shortened from a long period, nearly one month, to a couple of hours. As the bioreduction process went on, the color of the culture medium changed from pale yellow to brown. UV-visible spectrum of the liquid medium containing silver ion showed a peak at 450 nm corresponding to the plasmon absorbance of silver nanoparticles. The antimicrobial properties of silver nanoparticles were investigated using *Escherichia coli, Staphylococcus epidermidis, Staphylococcus coagualase* positive, *Serratia* spp. and *Salmonella typhi*. The bactericidal effect of silver nanoparticles was compared based on diameter of inhibition zone in disk diffusion tests. Bacterial sensitivity to nanoparticles was found to vary depending on the microbial species. Disk diffusion studies with *E. coli* and *S. coagualase* positive revealed greater effectiveness of the silver nanoparticles than other microorganisms.

Key words: Nanobiotechnology, surface plasmon resonance, extracellular, antimicrobial activity, nanoparticles, silver nitrate

INTRODUCTION

Nanotechnology provides the broad knowledge of applied science and technology to control the matter on the atomic and molecular scale. Nanobiotechnology is an important and emerging technical tool for development of eco-friendly, reliable methodology for synthesis of nanoscale materials using biological sources (Gilaki, 2010). In modern nanoscience and technology, the interaction between inorganic nanoparticles and biological structures are one of the most exciting areas of research. The biological synthesis process elucidates the importance of metal-microbe interaction in several biotechnological applications including the fields of bioremediation, biomineralization, bioleaching and microbial corrosion (Singh *et al*., 2011). In recent years the development of microbial sources has a potential effect on synthesis of metallic nanoparticles such as silver, cadmium sulfide, gold, tin and Ni (Bruins *et al*., 2000; Beveridge *et al*., 1997; Sastry *et al*., 2003). Silver nanoparticles interactions with bacteria are dependent on size and shape of nanoparticles (Savithramma *et al*., 2011).

A variety of different techniques have been reported previously for the synthesis of silver nanoparticles, methods are including chemical reduction of silver ions using aqueous solutions or non-aqueous solutions, electrochemical or ultrasonic-assisted reduction, photo induced or photo catalytic reduction (Zhang *et al*., 2007), microwave-assisted synthesis (He *et al*., 2004), irradiation reduction (Hornebecq *et al*., 2003), micro emulsion method (Zheng *et al*., 2004), biochemical reduction (Ahmad *et al*., 2003) and so on. The chemical reduction of silver ions is the best method for production of large quantities of nanoparticles in relatively short periods of time. The main drawback of using chemical reduction, most of the reactants used in the reaction system is toxic chemical agents which have potential risks for environment and health. Biological reduction is recently developed as a promising method because of its greater advantages such as sufficient material sources, mild reaction conditions, good dispersion of nanoparticles as well as few chemical additives and poisonous byproducts and also micro-organisms as possible eco-friendly nanofactories.

There are two different natures of biomass available in the biological synthesis namely, intra and extra cellular process. From the observation of both methods, the application of nanoparticles would be better realized if they can be synthesized outside the bacterial biomass. Early studies reveal that Bacillus subtilis 168 is able to reduce silver ions to produce octahedral gold particles of nanoscale dimensions (5-25 nm) within the bacterial cells by incubation of the cells with gold chloride solution (Chowdhury *et al*., 2011). A strain of *Bacillus* sp. isolated from atmosphere was reported to produce silver nanoparticles at their periplasmic phase after a week of incubation period (Chowdhury *et al*., 2011). The very recently Kalimuthu *et al*. (2008) shown that details of the extracellular growth of silver nanoparticles using B. licheniformis. To the best of our knowledge it has been realize that the reduction of the Ag^+ ions by the bacterial strain occurs through the release of reductase into solution.

This study is devoted to a nanobiotechnological aspect of the synthesis of silver nanoparticles, which can be used in biological sensors, drug and gene delivery and antimicrobial protection. The primary intend of this work was to study extracellular biosynthesis of silvernaoparticles using bacterial strain, measuring the UV-visible spectra of resulting silver nanoparticles. To our knowledge, extracellular synthesis of Ag nanoparticles and their antimicrobial activity by this bacterial strain has not been reported so far.

MATERIALS AND METHODS

Source of microorganism: The bacterial strain was obtained from the *Bacillus* Genetic Stock Center (BGSC), Department of Biochemistry in the college of Biological science at The Ohio State University. The obtained pure culture was maintained in nutrient broth agar medium (HiMedia, Mumbai, India) slant at 27°C as well as subcultured from time to time to regulate its viability in the microbiology laboratory (Department of Biotechnology, Manipal Institute of Technology, Manipal University, Manipal, India) during the study period (2010).

Production of biomass: The bacterial strain was cultured in nutrient broth medium, to produce biomass. The culture flasks were incubated on an orbital shaker at room temperature and agitated at 220 rpm. The biomass was harvested after 24 h of growth and centrifuged at 12000 rpm for 10 min. The supernatant material was collected for the further reaction to synthesis of nanoparticles.

Synthesis of silver nanoparticles and characterization of silver nanoparticle: The supernatant was added separately to the reaction vessel containing silver nitrate ($AgNO_3$) at a

concentration of 10^{-3} (1% v/v) and control (without the silver nitrate, only biomass) was also run along with the experimental condition. The reaction between this supernatant and Ag^+ ions was carried out in bright conditions for 24 h. The bioreduction of the Ag^+ ions in the solution was monitored and sample of 2 mL was withdrawn at different time intervals and the absorbance was measured at a resolution of 1 nm were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) with samples in quartz cuvette.

Particle sizing measurements: Particle size analyzing experiments were carried out by means of laser diffractometry (laser particle size analyzer); using an extremely compact optical bench, the CILAS 1064 integrates 2 sequenced laser sources pointed at 0 and 45°. Measurements were taken in the range between 0.04 up to 500 μm. Through the software, the distribution curve is represented by 100 classes above mentioned range.

Antibacterial activity: The antibacterial activities of silver nanoparticles were investigated by well diffusion method. Muller Hinton agar plates were prepared, sterilized and solidified. After solidification bacterial cultures were swabbed on these plates. The silver nanoparticle solution (5 μg mL^{-1}) was added to the wells and kept for incubation at 37°C for 24 h. Zone of inhibition was measured.

RESULTS AND DISCUSSION

The present research work has emphasized the extracellular biogenic synthesis of silver nanoparticles using Bacillus strain and investigation of antibacterial activity of synthesized nanoparticles with various pathogenic bacterial strains. The mechanism adopted for the formation of metallic nanoparticles using biological systems, the bioreduction of ionic strength from their native ionic strength. The reduction in ionic concentration leads to the formation size controlled and stable nanoparticles. The importance of interaction between microorganisms and metals has to be increased and well documented. The primary conformity of bioreduction of metallic ions represents the change in their native color (order of ionic strength has been reduced). To validate the extracellular formation of silver nanoparticles, pure silver nitrate solution (without the bacterial biomass) as positive control and the flasks containing the aqueous filtrate (without silver nitrate solution) as negative control were monitored visually. The aforementioned control flasks were observed no color change; they retain its original color. The silver nitrate reacted with the supernatant culture shows the change in color. The change of color in production medium was monitored by visual observation. Investigation on bioreduction process, the color of the bioreduction system changed from pale yellow to dark brown color. The appearance of the dark red color confirms the synthesis of silver nanoparticles in the reaction mixture. The color formation was mainly due to the surface plasmon resonance of deposited silver nanoparticles and it is well-known that silver nanoparticles exhibit striking colors (light yellow to brown) due to excitation of surface plasmon vibrations in the particles (Kapoor, 1998). Figure 1 represents the color formation in culture medium. Fig. 1a and b shows result of bacterial biomass with silver nitrate (formation of nanoparticles, dark brown color), Fig. 1c shows the positive control (pure silver nitrate solution) and Fig. 1d and e observation of negative control (without silver nitrate solution).

The basic characterization and stability of produced silver nanoparticles in reaction mixture was monitored by UV-vis spectral analysis. For UV-vis spectrum characterization process aliquot of sample from the fermentation broth was withdrawn without much change in the culture volume

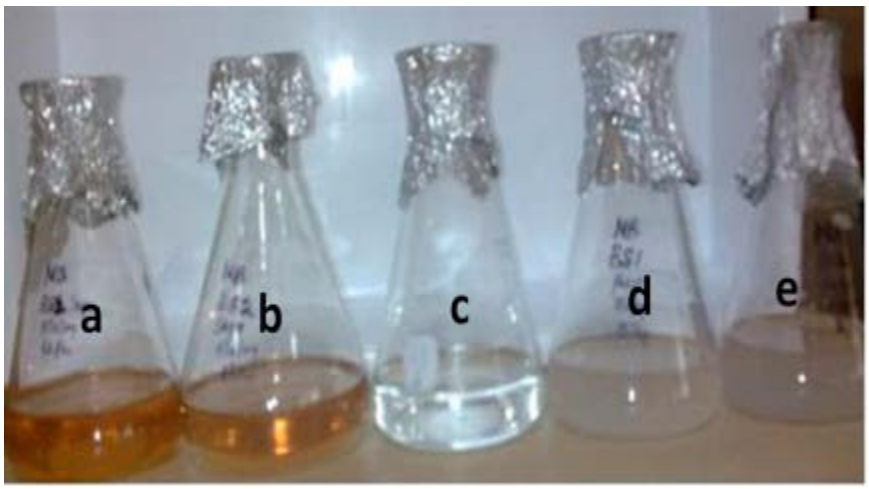

Fig. 1 (a-e): (a, b) the result of bacterial biomass with silver nitrate (formation of nanoparticles, dark brown color), (c) the positive control (pure silver nitrate solution) and (d, e) observation of negative control (without silver nitrate solution)

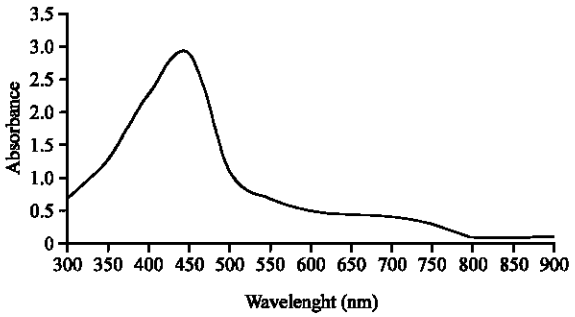

Fig. 2: UV-vis absorption spectra of silver nanoparticles synthesized by *Bacillus strain*. The absorption spectrum of silver nanoparticles exhibited a strong broad peak at 440 nm and observation of such a band is assigned to surface plasmon resonance of the particles

to maintain constant oxygen transfer. The light absorption pattern of the bacterial biomass was kinetically monitored in the range of 300 to 900 nm (Fig. 2). Sastry *et al.* (1998) proved that UV-vis spectroscopy is a very useful and accepted technique for analysis of synthesized nanoparticles. In the UV-vis absorption spectrum shown fairly strong and broad peak located at about the 450 nm was observed after the resuspended solution incubated with silver nitrate. The presence of the broad resonance indicates the aggregation of the silver nanoparticles in the solution. Various studies have established that the broad spectrum in this range assigned to a surface plasmon resonance of nano-sized silver metal nanoparticles with size ranges from 2 to 100 nm. Moreover, the corresponding position of the surface resonance band mainly depends on various factors such as the dielectrically constant of the medium, size, shape and stability of the particles, type of capping agent, as well as refractive index of the surrounding medium (Nair and Pradeep, 2002; Underwood and Mulvaney, 1994). Sastry *et al.* (2003) suggested that at 400 nm corresponded to

the transverse plasmon vibration in silver nanoparticles, whereas the peak at 450 nm due to excitation of longitudinal plasmon vibrations. Well know fact that the presence of the broad resonance band can indicate the polydispersity or shape of metal nanoparticles. And also one more possibility that scattering from the rough b ionanocomposites surface would contribute to the broadening of the resonance band (Mukherjee *et al*., 2001a, b). Laser diffraction particle size analyzer provides the detail about the particle nature, such as monodispersed, didispersed and polydispersed. Our investigation revealed that nanoparticles are in polydispersed mixture, with the various sizes range from 50 to 80 nanometers.

Antimicrobial activity: The antibacterial activity of silver nanoparticles were investigated against various human pathogenic organisms such as *Escherichia coli, Staphylococcus epidermidis*, *Staphylococcus coagulase* positive, *Serratia* spp. and *Salmonella typhi* using well diffusion method. In these tests, Muller Hinton agar plates were used and synthesized silver nanoparticles were supplemented in liquid systems as sample, silver nitrate solution as a positive control and empty well as a negative control. The inhibition zone formed in the screening test indicating the antibacterial activity against various human pathogenic bacteria. The silver nanoparticles exhibited more activity then the silver nitrate solution (Fig. 3). For decades silver has been used for the treatment of burns and chronic wounds. Silver has been known to a prominent source of antimicrobial properties from ancient ages. Metallic silver is relatively unreactive however, when exposed to aqueous environments some ionic silver (Ag^+) is released. Certain salts (e.g., silver nitrate) are readily soluble in water and have been exploited as antiseptic agents for many decades. In 1700, silver nitrate was used as antimicrobial agent for various diseases like fistulate from salivary glands, bone and perianal abscesses (Castellano *et al*., 2007; Klasen, 2000). The present research work emphasized human pathogenic bacterial strains, in which *Salmonella typhi, Escherichia coli, Staphylococcus epidermidis* and *Staphylococcus coagulase* positive they are produces the clear zone of inhibition than the other microbial strains used. The order of antibacterial activity of silver nanoparticles follows *Salmonella typhi* > *Escherichia coli* > *Staphylococcus epidermidis* > *Staphylococcus coagulase* positive > *Serratia* spp. (Fig. 4). The present work gives a general idea about the mechanism of silver nanoparticles action against human pathogenic bacterial sources. The antimicrobial mechanisms of biosynthesised silver nanoparticles may differ from species to species of bacteria and size of the nanoparticles. Shahverdi *et al*. (2007) reported that silver nanoparticles have a efficient antimicrobial activity against on *S. aureus* and *E. coli*. The synthesized silver nanoparticles were more effective against gram positive bacterial strains than the gram negative bacteria (Ramgopal *et al*., 2011). Sondi and Salopek-Sondi (2004) documented that the gram negative bacteria of *Escherichia coli* has been used as a model to produce silver nanoparticles and proved that silver nanoparticles have been used as an antimicrobial agent. The prepared silver nanoparticle solution does not interfere with epidermal proliferation and it possesses good antibacterial activity against S*taphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* (Moyer *et al*., 1965; Bellinger and Conway, 1970). In recent studies developed new silver wound dressing, due to the emergence of antibiotic resistant bacteria and use of antibiotics in clinical studies been limited to certain level (Gemmell *et al*., 2006; Chopra, 2007). This study gives an apparent information that the bacterial strains can be used for synthesize of bioactive nanoparticles efficiently with inexpensive substances in an eco-friendly and nontoxic environment.

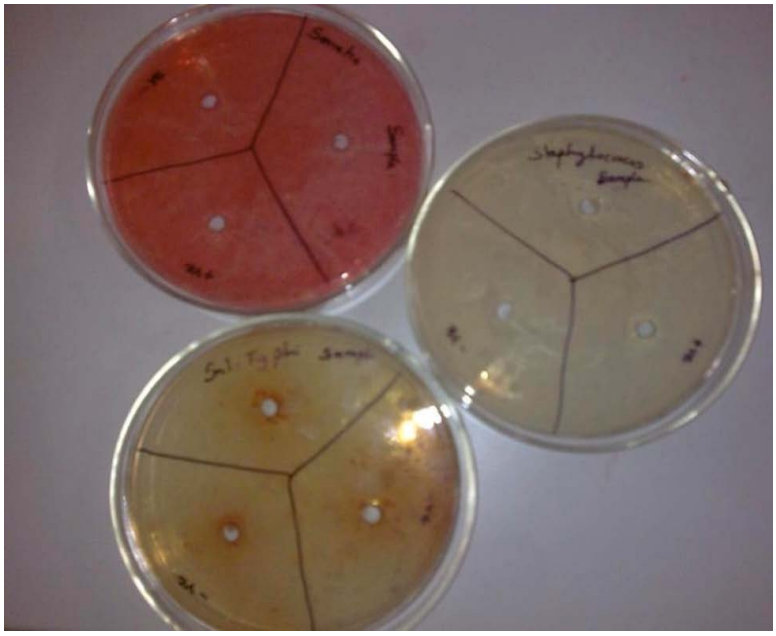

Fig. 3: Antimicrobial activity of silver nanoparticles against various pathogenic bacterial strains shown by well-diffusion method

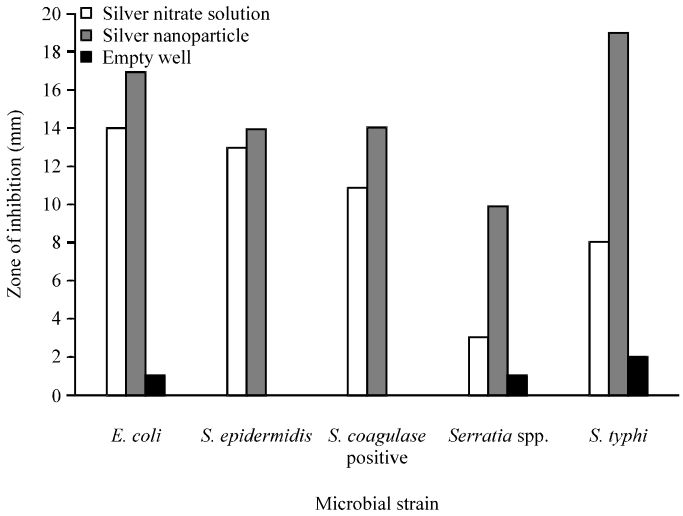

Fig. 4: Zone of inhibition of AgNPs against various pathogenic bacteria

CONCLUSIONS

The development of reliable processes for the synthesis of silver nanomaterials is an important aspect of current nanotechnology research. The biologically inspired experimental process for the synthesis of nanoparticles is emerging and attractive techniques. Herein, our experiments describe the environmentally accepted simple green synthesis process for production of extracellular nanoparticles. Understanding of biological processes on the nanoscale level is a strong driving force

behind development of nanotechnology. The synthesised nanoparticles can be used as a bioactive nanodrug for antimicrobial activity and characterization of antibacterial properties of human pathogenic bacterial strain. In an addition, the application of silver in combination with microbial system would be effective in enhancing its antimicrobial activity. In this study, revealed that the produced nanoparticles were found to be most active against the clinically isolated human pathogenic strains. But, further studies about the characteristics of antimicrobial activities and mechanisms of new silver compounds and combination system of silver and other antimicrobials are required to obtain quantitative evaluation of potentials of silver as a representative antimicrobial material.

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