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RESEARCH ARTICLE

FUNGUS-MEDIATED SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES AND ITS ANTIBACTERIAL ACTIVITY AGAINST CLINICALLY ISOLATED PATHOGENS

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ABSTRACT

Today, nano metal particles have drawn the attention of scientists because of their extensive application to new technologies in chemistry, electronics, medicine, and biotechnology. Beside many physical and chemical methods which have been developed for preparing metal nanoparticles, nanobiotechnology also serves as an important method in the development of clean, nontoxic, and environmentally friendly procedures for the synthesis and assembly of metal nanoparticles. To be utilized in different scientific fields, biological synthesis still requires the optimization of reaction conditions, and an understanding of the biochemical and molecular mechanisms of the reaction for obtaining better chemical composition, shape, size, and monodispersity. By noticing the potential of the natural world to produce bio-nanomaterials under normal conditions, we are trying to explain simple practical methods that can be used for bacterial synthesis of metal or metalloid nanoparticles. In conclusion, the filamentous fungus has shown potential for extracellular synthesis of fairly monodispersed, silver nanoparticles in the range of 5–20 nm. The kinetics of silver nanoparticles synthesis using the cell filtrate indicates that the synthesis of nanoparticles would be suitable for developing a biological process. Furthermore, the extracellular synthesis would make the process simpler and easier for downstream processing. In future, it would be important to understand the biochemical and molecular mechanism of the synthesis of the nanoparticles by the cell filtrate in order to achieve better control over size and polydispersity of the nanoparticles.

Key words: Nanoparticles, X- ray diffraction analysis, antimicrobial activity, Nanobiotechnology.

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INTRODUCTION

Nanotechnology is the application of science and technology to control matter at the molecular level. At the nanoscale level, the properties of matter are significantly different from their macroscopic bulk properties. The term nano is adapted from the Greek word meaning "dwarf." When used as a prefix, it implies 10^{-9}. A nanometer (nm) is one billionth of a meter, or roughly the length of three atoms side by side. The field of nanotechnology is an immensely developing field as a result of its wide-ranging applications in different areas of science and technology. The concept of nanotechnology was given by physicist Professor Richard Feynman in 1959. Research on synthesis of nanoparticles is the current area of interest due to the unique visible properties (chemical, physical, optical, etc.) of nanoparticles compared with the bulk material. The nanoparticles of a wide range of materials can be prepared by a number of methods. In synthesis and assembly strategies of nanoparticles or nanomaterials, precursors from liquids, solid or gas phase are used (Mansoori, 2005). Currently, there is a growing need to using environmental friendly nanoparticles that do not produce toxic wastes in their process synthesis protocol. It is well known that biological entities like microorganisms and living cells are the best examples of machines that possess operating parts at the nanoscale level and perform a number of jobs ranging from generation of energy to extraction of targeted materials at a very high efficiency (Goodsell, 2004). The utilization of such microorganisms like bacteria, fungi, herbal extracts and yeasts in the synthesis of nanoparticles is a relatively recent activity. It is known that certain bacteria, yeasts and now fungi play an important role in remediation of toxic metals through reduction of the metal ions so long as they are not toxic in other ways. In synthesis and assembly strategies of nanoparticles or nanomaterials, precursors from liquids, solid or gas phase are used. Silver has known to be a metal that came into use even before Neolithic revolution. Even the Greeks used it for

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cooking and to keep water safe. The first recorded medicinal use of silver was reported during 8th century (Moyer, 1965a). Silver nanoparticles (AgNPs) have emerged as an arch product from the field of nanotechnology. Silver has gained interest over the years because of distinctive properties, such as good conductivity, chemical stability, catalytic and antibacterial activity (Chen and Schluesener, 2008). Because of their tolerance and metal bioaccumulation ability, fungi are taking the center stage of studies on biological generation of metallic nanoparticles. Mukherjee *et al* studied the synthesis of intracellular Ag NPs using the fungus Verticillium. (Mukherjee P *et al.,* 2001). The cell wall of the microorganisms plays a major role in the intracellular synthesis of nanoparticles. The cell wall being negatively charged interacts electrostatically with the positively charged metal ions. Mukherjee *et al.,* (2001) reported stepwise mechanism for intracellular synthesis of nanoparticles using *Verticillium* sp. The recent emergence of nanotechnology has provided a new therapeutic modality in silver nanoparticles for use in medicine. Silver nanoparticles have been known to have inhibitory and bactericidal effects and thus extend its application as an antibacterial agent (Law *et al.,* 2008). Nanocrystalline silver dressings were introduced commercially as antimicrobial dressings in 1998 and these have found to improve wound healing (Wright *et al.,* 2002), which may result from potent anti-inflammatory activity.

The treatment of murine infected burns with silver nanoparticles was found to increase the rate of healing and decrease the scarring in comparison with silver sulfadiazine (Tian *et al.,* 2007). Integration of nanotechnology with biotechnology and medicine means the ability to uncover the structure and function of biosystems, which intrinsically have an organizational level at the nanoscale (Kairemo *et al.,* 2008). Chen X *et al.,* 2008 reported that nanotechnology is a most promising field for generating new applications in medicine. However, only few nanoproducts are currently in use for medical purposes. A most prominent nano product is nanosilver. Allahverdyev AM *et al.,* 2011 reported that nanobiotechnology is the creation of functional materials, devices and systems at atomic and molecular scales (1-100nm), where properties differ significantly from those at a larger scale. Chekman IS et al 2011 reported that current studies, dedicated to metallic (gold, silver, iron and copper) nanomaterials, this metals own unique physical and chemical properties which determine their application. The medical applications of metallic nanomaterials includes therapy and prophylaxis of diseases, development of new drugs and improvement of conventional ones, nanodiagnostics.

Musee N *et al.,* 2011 reported that nanotechnology is currently at the forefront of scientific research and technological developments that have resulted in the manufacture of novel consumer products and numerous industrial applications using engineered nanomaterials (ENMs). Stenberg MC *et al.,* 2011 reported that silver nanoparticles (AgNPs) are becoming increasingly prevalent in consumer products as antibacterial agents. Thirumurugan G *et al* 2011 reported that biosynthesis of silver nanoparticles (NPs) using microorganisms has been reported but methodologies of synthesis are slow and the silver nanoparticles are not stable. Srivastava M et al 2012 reported that silver nanoparticles (Ag NPs) and silver (Ag) based materials are increasingly being incorporated into consumer products, and although humans have been exposed to colloidal Ag in many forms for decades, this rise in the use of Ag materials has spurred interest into their toxicology. Silver nitrate was combined with sulfonamide to form silver sulfadazine cream, which served as a broad-spectrum antibacterial agent and was used for the treatment 0f burns. Silver sulfadazine is effective against bacteria like *E. coli, S. aureus, Klebsiella* sp, *Pseudomonas* sp. It also possesses some antifungal and antiviral activities (Fox and Modak., 1974. Commendable efforts have been made to explore this property using electron microscopy, which has revealed size dependent interaction of silver nanoparticles with bacteria. Nanoparticles of silver have thus been studied as a medium for antibiotic delivery and to synthesize composites for use as disinfecting filters and coating materials.

MATERIALS AND METHODS

Collection of plant materials

The samples from healthy living plants used for isolation of endophytic fungi were collected in different places.

Isolation of endophytic fungi

Different samples of plant leaves were collected and washed with running water. The leaves were cut into segments (5×5 mm) aseptically and isolation of endophytic fungi followed a modified procedure as described by Huang *et al.* (2009).

Preparation of fungal mat

The isolated endophytic fungi strains from the plant used for biosynthetic experiments was grown aerobically in 100 ml of the PDA broth medium The Erlenmeyer flasks were inoculated with spores and incubated at 28° C for 3 to 4 days. After the incubation, the biomass was extensively washed with sterile distilled water to remove any medium component. Fresh and clean biomass was taken into the fresh Erlenmeyer flask, containing 25 ml of sterile water. The flasks were agitated at 120 rpm at 28° C for 48 to 72 hours, then the biomass was filtered (Whatman filter paper No. 1) and cell-free filtrate was used for the biological synthesis of silver nanoparticles experiment.

Fungal genomic DNA isolation

The fungal genomic DNA was isolated by standard isolation procedure.The PCR reactions for sequencing were carried out based on the methodology of White *et al.,* (1990) in Thermocycler.

Primers

Forward: 5′-GGAAGTAAAAGTCGTAACAAGG-3′
Reverse: 5′-TCCTCCGCTTATTGATATGC-3′

The amplified fragment includes ITS1, 5.8S and the ITS2 of rDNA. Amplification was performed in a 50 µl reaction mixture containing the following ingredients.

Agarose gel electrophoresis

Electrophoresis was performed in a horizontal sub-marine apparatus. Agarose (2%) was melted with TE buffer and 2 µl of ethidium bromide was at the concentration of 4 mg/ml. TE buffer was used as the tank buffer and electrophoresis was carried out for 30 minutes at constant voltage. The gel was visualized under UV transilluminator.

Gene sequencing

The gene sequencing was carried out using Beckman Coulter CEQ 8000 autoanalyzer. The amplified products were cleaned up using QIAQuick (Qiagen) Spin column. The cycle sequencing was carried out using DTCS quick start Dye terminator kit (Beckman Coulter).

Reaction conditions: (30 cycles)

90°C for 20 seconds for denaturation.
48°C for 20 seconds for primer annealing
60°C for 60 seconds for polymerisation.

The removal unbound dye and nucleotide from cycle sequenced product was carried out using DyeEx spin columns (Qiagen). The purified samples were sequenced by Beckman Coulter CEQ8000 sequencer.

Biosynthesis of silver nanoparticles

To the cell filtrate, 1mM aqueous solution of silver nitrate (AgNO3) was mixed in an Erlenmeyer flask and agitated at 25°C in dark condition. The control (without the silver ions) was maintained along with the experimental flasks. The formation of silver nanoparticles by the cell-free filtrate of the microorganisms studied was investigated by the observation of the change in the color of the solution.

Characterization of biologically synthesized nanoparticles

An important stage in biosynthesis of nanoparticles is physico-chemical characterization of generated nanoparticles. Knowing about size, shape, surface area, homogeneity, and other features will provide valuable information of nanoscale systems and insight into synthesis control of nanoparticles for commercial applications. Some common techniques of characterization are UV–visible spectrometry, X-ray diffraction (XRD), energy dispersive spectroscopy (EDS) and electron microscopy (TEM).

Application studies

Antimicrobial activity of biologically synthesized nanoparticles

Antibacterial activity was analyzed with synthesized silver nanoparticles by well diffusion method against clinically isolated Gram negative (*Pseudomonas aeruginosa, Proteus mirabilis*) and Gram positive (*Staphylococcus aureus Bacillus substlis,*) microorganisms by Kirby Boyer method.

Antimicrobial effect of siver naanoparticles coated textile fabrics

Cotton fabrics with dimensions of 5cm × 5cm was made and washed with distilled water. The cotton fabrics pieces were then sterilized and were allowed to dry in a hot air oven. After drying, the cotton fabrics were transferred into a conical flask containing biologically synthesized silver nanoparticles and kept in orbital shaker for 24 hours in a dark environment. After 24 hours, the fabrics was removed from the conical flask and dried in a hot air oven again. The silver nanoparticles coated fabrics was cut into small pieces (1cm*1cm) using sterile scalpel aseptically.

Simultaneously, 300ml of Muller Hinton Agar (MHA) medium was prepared and plated aseptically into the sterile plates. An overnight nutrient broth culture of clinically isolated pathogens were prepared and made a lawn culture using sterile swab over the MHA plates. After the lawn preparation, silver nanoparticles coated fabrics were placed over the different lawn prepared using different clinically isolated pathogens. The Petri plates were incubated for 24 to 48 hours and observed for clear the zone formation which indicates the antibacterial activity of the silver nanoparticles coated fabrics.

RESULTS

Visual inspection

The detailed study on extracellular biosynthesis of silver nanoparticle (AgNP) by employing fungus *Guignardia sp.* was carried out in this work. Plate. 1(a) and (b) shows two conical flasks containing the filtrate of the *Guignardia sp.*supernatant in Plate 1(a) and supernatant with Ag^+ ions in (Plate 1 (b), after 2 days of the reaction. It is observed that the colour of the solution in flask (Plate 1 (a) does not change but the colour of the solution in the conical flask Plate 1 (b) turned from colourless to brown after 48 hours of the reaction. It confirmed that the formation of AgNP in flask Plate 1(b) only. It is inferred that AgNP exhibit yellowish brown colour in aqueous solution, this colour arises due to excitation of surface plasmon vibrations in the metal nanoparticles (Basavaraja *et al.,* 2008, Ahmad *et al.,* 2003a). This important observation indicates the reduction of the Ag^+ ions takes place extracellularly. The appearance of a yellowish-brown color in solution containing the biomass is a clear indication of the formation of silver nanoparticles in the reaction mixture.

DNA sequencing

From the DNA sequencing result it has been confirmed that the fungi isolated is 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (564 bps) for *Guignardia sp.* strain

ITS1,ITS2 primer sequence analysis

Sequence alignments provide a powerful way to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be inferred from well designed queries and alignments. BLAST – Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/) provides a method for rapid searching of nucleotide and protein databases. Since the BLAST algorithm detects local as well as global alignments, regions of similarity may provide important clues to the function of uncharacterized nucleotides and proteins.

UV–visible spectrometry

The formation and stability of the reduced AgNP in the fungal filtrate was monitored by using UV–vis spectral analysis. This technique outlined above has proved to be very useful for the analysis of nanoparticles (Henglein, 1993, Sastry *et al.,* 1997, Sastry *et al.,* 1998). As illustrated in Figure- 1, a strong, broad peak located between 420 and 430 nm was observed for the silver nanoparticles prepared using the fungus. Observation of

this peak, assigned to a surface plasmon, is well-documented for various metal nanoparticles with sizes ranging from 2 to 100 nm (Sastry *et al.,* 1998).

X-ray diffraction

The extracellular formation of silver nanoparticles is provided by X-ray diffraction (XRD) analysis of the biologically synthesized silver nano solution and is shown in Figure–2 . The sample of nanoparticles was solution casted in the form of a thin film onto an aluminum foil. The presence of sharp reflections due to (111), (200), (220) and (311) agree well with those reported for fcc silver. The XRD pattern of the silver nitrate-treated sample (Figure-2) corresponds to that of silver nanoparticles. The XRD pattern shows four intense peaks in the whole spectrum of 2θ values ranging from 30 to 80. It is important to know the exact nature of the silver particles formed and this can be deduced from the XRD spectrum of the sample. XRD spectra of pure crystalline silver structures and pure silver nitrate have been published by the Joint Committee on Powder Diffraction Standards (file nos. 04-0783 and 84-0713). A comparison of our XRD spectrum with the standard confirmed that the silver particles formed in our experiments were in the form of nanocrystals, as evidenced by the peaks at 2θ values of 38.25°,46.37°, 64.60° and 77.62◦, corresponding to 111, 200, 220 and 311 planes for silver, respectively.

Transmission electron microscopy

A representative TEM micrograph of silver nanoparticles obtained after 48 hours of incubation is presented Plate – 2. The biologically synthesized silver nanoparticle was deposited on a carbon coated copper TEM grid and was processed. The micrograph showed nanoparticles with variable shape, most of them present in spherical in nature with some others having occasionally triangular shape. The size of the particle ranged from 5 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.

Energy dispersive X-ray spectroscopy

EDX analysis confirmed the biologically synthesized nanoparticles have been of silver. As shown in Figure - 3, well-dispersed nanoparticles could be seen in the samples treated with silver nitrate. EDX analysis also showed a peak in the silver region and a wide range peak indicating Carbon, which is responsible due the carbon grid where the sample was loaded for EDX processing.

Antimicrobial activity of biologically synthesized silver nanoparticles

The antibacterial effects of biologically synthesized silver nanoparticles have been investigated against *Staphylococcus aureus, Proteus mirabilis, Pseudomonas aeruginosa* and *Bacillus subtilis*. The test was performed with loading the biologically synthesized nanoparticles into the well 'A', followed by fungal filtrate in 'B', then sterile distilled water in the well 'C' and silver nitrate (1mM) solution in the well 'D'. Plate 3 (a-d) demonstrates zones of growth inhibition around the well loaded with silver nanparticles. It was observed that a clear zone of growth inhibition was against in *Staphylococcus aureus, Proteus mirabilis, Pseudomonas aeruginosa* and *Bacillus subtilis* confirms the antibacterial property of biologically synthesized nanoparticles and lesser zones was observed in the well loaded with silver nitrate solution. Further there is no clear zone of inhibition was observed in the well loaded with fungal filtrate and sterile distilled water. Furthermore, the biologically synthesized silver nanoparticles has been clearly demonstrated about their antibacterial activity against various bacteria, these particles have been applied to cotton fabrics in order to study their nature of activity to produce sterile materials.

The biologically synthesized silver nanoparticles were incorporated into the cotton and their antibacterial activity was observed. Plate 4 (a-d) demonstrates zones of growth inhibition around the cotton fabrics loaded with silver nanparticles and no zone was observed in the control fabrics which was treated with sterile distilled water. It was observed that a clear zone of growth inhibition was against in *Staphylococcus aureus, Proteus mirabilis, Pseudomonas aeruginosa* and *Bacillus subtilis* confirms their antibacterial property.

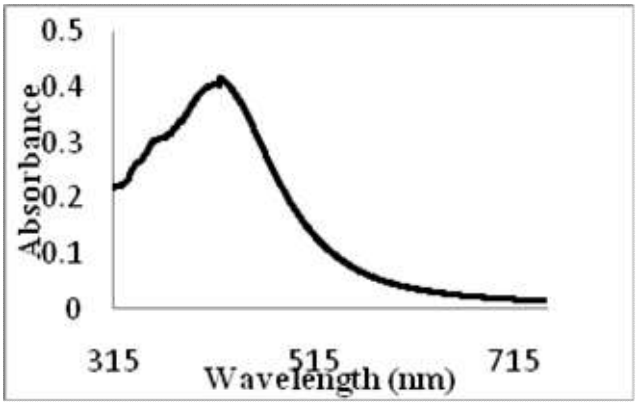


Figure 1. Xrd Analysis of Silver Nanoparticles

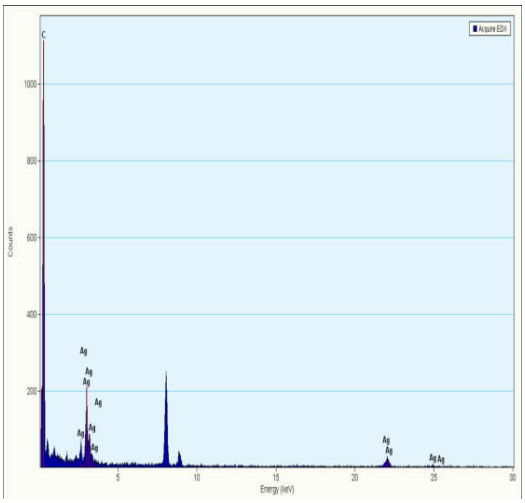


Figure 3. Edx Analysis of silver Nanoparticles

DISCUSSION

The probable mechanism for the nanoparticles biosynthesis and role of proteins during the same has been reported by Kalimuthu *et al.,* (2008) and Ahmad *et al.,* (2003b). These reports suggested the probable role of NADH-dependent

Plate – 1 (a, b) Screening of Silver nanopartides

Plate – 2 - TEM Analysis Of Silver Nanoparticles

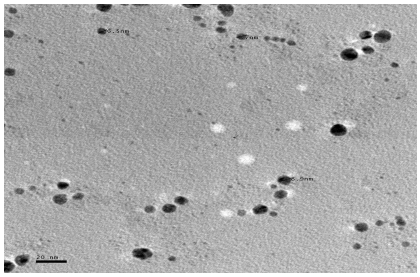
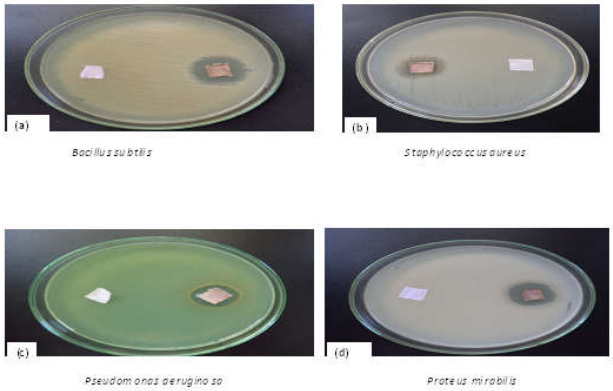


Plate – 4 (a-d) - Antimicrobial activity of silver nanoparticles coated cotton fabrics



Bacillus subtilis Staphylococcus aureus

Pseudomonas aeruginosa Proteus mirabilis

nitrate reductase in the reduction of silver ion to metallic silver. Also, the resistance conferred by bacteria to silver is determined by the 'sil' gene in plasmids (Silver, 2003). Independently, genetic analyses have identified proteins (Brown, 1992 and Brown, 1997) and repeating polypeptides (Barbas *et al.,* 1993) that adhere specifically to inorganic surfaces. Thus, it would not be surprising if repeating polypeptides could regulate the morphology of silver and gold inorganic crystals. The lack of fundamental understanding of the underlying mechanisms of biologically synthesis of NPs is currently an issue. It is both desirable and foreseeable that a great deal of effort will be put towards the elucidation of the synthesis mechanism. To this extent, a systematic investigation of the role that each component of the biological synthesis (DNA sequence, cloning and expression of protein molecules and so on) has in NP synthesis would be an important undertaking, in order to provide a set of rules that will assist in programming the synthesis of NPs. In today's nanobiotechnology one of the most exciting research topics is the formation of nanoparticles by biological systems. Fungi, bacteria, yeasts, actinomycetes and plants have inherent capacity to reduce metals through their specific metabolic pathways (Ahmad *et al*. 2003; Kowshik *et al.* 2003, Sintubin *et al.* 2009). Different types of nanomaterials like copper, zinc, titanium, magnesium, gold and silver have arising as antimicrobial materials, especially silver nanoparticles that are more efficient. These materials exhibit antimicrobial activity against bacteria, viruses and other eukaryotic microorganisms (Rai *et al*. 2009).

Ilic *et al.* (2009) verified that cotton with 10 mg/mL and 50 mg/mL of silver nanoparticles exhibited antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. However, after 5 wash cycles the fabrics with 10 mg/mL had released their silver nanoparticles while the fabrics with 50 mg/mL had released 98.4% of the nanoparticles. To increase the adhesion of silver nanoparticles in the fabrics several strategies have been studied. These results showed that the amount of silver nanoparticles impregnated in the fabrics influenced its laundering durability. A further possibility was applied using biogenic silver nanoparticles to treat fabrics. Due to the protein around the biogenic particles, these particles have stronger affinity and adhesion in fabric fibers (Marcato *et al.* 2010; Duran *et al.* 2010a, b). Other applications of silver nanoparticles are in association with antibiotics to improve their effects (Brocchi *et al.* 2010) and in wound healing (Tian *et al.* 2007; Maneerung *et al.* 2008). In the next item different aspects related to the biogenesis of silver nanoparticles produced by fungi, and multiple applications of biogenic metal nanoparticles, synergistic antibiotic effects, their applications on textile fabrics and their potential importance in some neglected diseases will be discussed. Ingle *et al.* (2008) have evaluated the antibacterial activity of biosynthesized silver nanoparticles produced by *Fusarium acuminatum* on different human pathogens. These authors reported efficient antibacterial activity of silver nanoparticles against multidrug resistant and highly pathogenic bacteria, such as*, S. aureus, S. epidermidis, Salmonella typhi* and *E. coli.* Silver nanoparticles showed remarkable antimicrobial effects than silver ions (1.4–1.9 folds). The maximum antibacterial activity of silver nanoparticles was against *S. aureus*, followed by *S. epidermidis, S. typhi* and the minimum was for *E. coli.* This result demonstrated that specific efficiency of silver nanoparticles can be related with differences due to the strain, which can be related to the bacterial membrane structure.

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