Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria

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Abstract

The development of a reliable green chemistry process for the biogenic synthesis of nanomaterials is an important aspect of current nanotechnology research. Silver nanoparticles (AgNPs) have been known for their antibacterial and bactericidal effect. Resistance to antimicrobial agents by pathogenic bacteria has emerged in recent years and is a major challenge for the health care industry. In the present investigation the use of the fungus Trichoderma viride for the extracellular biosynthesis of AgNPs from silver nitrate solution is reported. It was observed that the aqueous silver (Ag⁺) ions, when exposed to a filtrate of T. viride, were reduced in solution, thereby leading to formation of extremely stable AgNPs. These AgNPs were characterized by means of several techniques. The nanoparticles show maximum absorbance at 420 nm on ultraviolet-visible spectra. The presence of proteins was identified by Fourier transform–infrared spectroscopy. The reduction of Ag⁺ ions to elemental silver was characterized by x-ray photoelectron spectroscopy. Electrokinetic measurement (zeta potential) of AgNPs as a function of pH in 1 × 10⁻³ mol dm⁻³ aqueous solution were evaluated. The transmission electron micrograph revealed the formation of polydispersed nanoparticles of 5–40 nm, and the presence of elemental silver was confirmed by energy-dispersed spectroscopy analysis. The nanoparticles were also evaluated for their increased antimicrobial activities with various antibiotics against gram-positive and gram-negative bacteria. The antibacterial activities of ampicillin, kanamycin, erythromycin, and chloramphenicol were increased in the presence of AgNPs against test strains. The highest enhancing effect was observed for ampicillin against test strains. The result showed that the combination of antibiotics with AgNPs have better antimicrobial effects. A mechanism was also proposed to explain this phenomenon.

From the Clinical Editor: Silver nanoparticles (Ag NPs) represent an important nanomedicine-based advance in the fight against polyresistant bacteria. In this study, the fungus Trichoderma viride was utilized for extracellular biosynthesis of extremely stable Ag NPs. The antibacterial activities of kanamycin, erythromycin, chloramphenicol and especially of ampicillin were increased in the presence of Ag NPs against test strains. © 2010 Elsevier Inc. All rights reserved.

Nanostructured materials have been receiving considerable attention as a result of their unique physical and chemical properties and their important applications in optics, electronics, biomedicine, magnetism, mechanics, catalysis, energy science, and so on. The fabrication of reliable, green chemistry processes for the synthesis of nanomaterials is an important aspect of nanotechnology. Hence, there is a growing need to develop an environmentally benign nanoparticles synthesis process that does not use toxic chemicals in the synthesis protocols. An important aspect of nanotechnology is the development of toxicity-free synthesis of metal nanoparticles, which is a great challenge. The secrets discovered from nature have led to the development of biomimetic approaches to the growth of advanced nanomaterials.

Recently, scientists have endeavored to make use of microorganisms as possible eco-friendly nanofactories for the synthesis of metallic nanoparticles, such as cadmium sulfide, gold, and silver. Beveridge and co-workers have demonstrated that gold particles of nanoscale dimensions may readily be precipitated within bacterial cells by incubation of the cells with Au⁺ ions. Klaus and co-workers have shown that the bacterium Pseudomonas stutzeri AG259, isolated from a silver mine, when placed...
in a concentrated aqueous solution of silver nitrate, played a major role in the reduction of the Ag⁺ ions and the formation of silver nanoparticles (AgNPs) of well-defined size and distinct topography within the periplasmic space of the bacteria.⁸

Whereas intracellular synthesis may on principle accomplish a better control over the size and shape distribution of the nanoparticles, product harvesting and recovery are more cumbersome and expensive. But in the case of extracellular synthesis the reduction of Ag⁺ ions by Fusarium oxysporum suggests the release of fairly strong reducing agents and adds considerably to the range of applicability of fungal-based protocols. The reduction of Ag⁺ ions by the fungus occurs through the release of reduce active enzymes into the solution.⁸ Thus, extracellular synthesis by comparison is more adaptable to the synthesis of a wider range of nanoparticle systems.⁹ Among various metal nanoparticles, AgNPs have several effective applications in the field of biodetection,¹⁰ sensors, antimicrobial filters,¹¹ and bactericidal activity against gram-positive and gram-negative bacteria, including highly multiresistant strains such as methicillin-resistant Staphylococcus aureus.¹² Hence these have been intensively studied using F. oxysporum,⁹ P. stutzeri,¹³ Rhodococcus sp.,¹¹ Thermomonas sp.,¹¹ Phaeosphaeria chrysosporium,¹¹ and others.

With the prevalence and increase of microorganisms resistant to multiple antibiotics and the continuing emphasis on health care costs, many researchers have tried to develop new, effective antimicrobial reagents, free of resistance and cost-effective. Such problems and needs have led to the resurgence in the use of silver-based antiseptics that may be linked to broad-spectrum activity and far lower propensity to induce microbial resistance than antibiotics.¹²

Our aim in the present contribution was to synthesize AgNPs using Trichoderma viride and to investigate the synergistic effect of AgNPs combined with antibiotics against gram-positive and gram-negative bacteria. To our knowledge extracellular synthesis of AgNPs by this fungus has not been reported so far.

Methods

Source of microorganisms

The fungus was obtained from the Culture Collection Center (CAS in Botany, University of Madras, India) and maintained in potato dextrose agar (HiMedia, Mumbai, India) slant at 27°C.

Four bacterial strains—namely, Salmonella typhi (gram-negative rods), Escherichia coli (gram-negative rods), Staphylococcus aureus (gram-positive cocci), and Micrococcus luteus (gram-positive cocci) were obtained from the Culture Collection Center, and the species-level confirmation for all microorganisms was identified using the microbial identification system (BioMerieux, mini API, Roma, Italy).

Production of biomass

To prepare the biomass for biosynthesis studies the fungus was grown aerobically in liquid broth containing (g/L) KH₂PO₄, 7; K₂HPO₄, 2; MgSO₄ 7H₂O, 0.1; (NH₄)₂SO₄, 1; yeast extract, 0.6; glucose, 10. The culture flasks were incubated on an orbital shaker at 27°C and agitated at 150 rpm. The biomass was harvested after 72 hours of growth by sieving through a plastic sieve followed by extensive washing with sterile double-distilled water to remove any medium components from the biomass.

Synthesis of AgNPs

Typically 20 g of biomass (wet weight) were brought into contact with 100 mL sterile double-distilled water for 48 hours at 27°C in an Erlenmeyer flask and agitated as described above. After incubation the cell filtrate was filtered by Whatman filter paper No.1 (Oakland, California, USA). After filtration the observed pH of cell filtrate was 7.2. Into these 100 mL of cell filtrate, a carefully weighed quantity of silver nitrate was added to the Erlenmeyer flask to yield an overall Ag⁺ ions concentration of 10⁻⁷ M, and the reaction was carried out under dark conditions. Characterization of AgNPs

Our investigation included time-dependent formation of AgNPs using ultraviolet-visible (UV-vis) spectrophotometry (Cary 300 Conc UV-Vis spectrophotometer, Varian, Inc., Palo Alto, California, USA). Samples for transmission electron microscopy (TEM) were prepared by drop-coating the AgNPs solution into the carbon-coated copper grid, and their size and morphology were characterized by TEM (JEOL 2000 FX MARK II, Tokyo, Japan). The presence of elemental silver was confirmed through energy-dispersed spectrophotometry (EDS). The sample was prepared by drop-coating AgNPs on Si(111) substrate, and the reduction of silver ions to metallic silver was characterized by x-ray photoelectron spectroscopy (XPS) (VG Microtech ESCA 3000, Sansus, United Kingdom) equipped with a multichannel hemispherical electron energy analyzer at pressure not higher than 1 x 10⁻⁹ torr. The interaction between protein-AgNPs was analyzed by Fourier transform-infrared spectroscopy (FT-IR) (Perkin-Elmer, Shelton, Connecticut). An electrokinetic measurement of AgNPs as a function of pH in 1 x 10⁻³ mol dm⁻³ aqueous solution was evaluated using Zetasizer (Malvern Instruments, Worcestershire, United Kingdom).

Disk diffusion assay to evaluate combined effects

A disk diffusion method was used to assay the synergistic effect of antibiotics with extracellularly synthesized AgNPs for bactericidal activity against test strains on Muller-Hinton agar plates. The standard antibiotic disks were purchased from.
Figure 2. UV spectra recorded as a function of time of reaction of an aqueous solution of $10^{-5}$ M silver nitrate with the cell filtrate of fungal biomass. The time of reaction is indicated next to the respective curves.

Figure 3. Ag 3d core-level spectra recorded from a drop-coated AgNP solution on Si(111) substrate. A single spin-orbit pair is shown.

Figure 4. FT-IR spectrum recorded by making a KBr pellet with synthesized AgNPs.

Figure 5. Bright-field TEM micrograph of biogenic synthesized AgNPs. Scale bar = 100 nm.

Himedia (Mumbai, India). To determine the synergistic effects, each standard antibiotic disk was further impregnated with 10 μL of freshly prepared AgNPs as the final content of 10 μg of AgNPs per disk. A single colony of each test strain was grown overnight in Muller-Hinton liquid medium on a rotary shaker (200 rpm) at 35°C. The inocula were prepared by diluting the overnight cultures with 0.9% NaCl to a 0.5 McFarland standard and were applied to the plates along with the standard and prepared disks containing differing amounts of AgNPs. After incubation at 35°C for 24–48 hours the zones of inhibition were measured using digimatic calipers (Mitutoyo Rochester, New York). The assays were performed in triplicate.

Results

A detailed study on the extracellular biogenic synthesis of AgNPs by *T. viride* was carried out, and the synergistic antibacterial effect of AgNPs with antibiotics against gram-positive and gram-negative bacteria were reported from this work. Figure 1 shows Erlenmeyer flasks containing the filtrate of *T. viride* biomass with Ag⁺ ions at the initial time point and after 24 hours of the reaction end point, respectively. The change in color of the filtrate of *T. viride* was noted by visual observation. The excitation spectra of the AgNPs samples were characterized by UV-vis spectroscopy. The technique outlined above has
Table 1
Mean zone of inhibition (nm) of different antibiotics (with and without AgNPs) against gram-positive and gram-negative bacteria

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>E. coli</th>
<th>S. typhi</th>
<th>S. aureus</th>
<th>M. luteus</th>
<th>Overall synergistic antibacterial effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin (10 μg/disk)</td>
<td>13</td>
<td>24</td>
<td>9</td>
<td>8</td>
<td>18.96</td>
</tr>
<tr>
<td>Ag-NPs + Erythromycin (b)</td>
<td>16</td>
<td>31</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Fold increase % = (\frac{(b – a)}{a} \times 100)</td>
<td>23.06</td>
<td>29.17</td>
<td>11.11</td>
<td>12.50</td>
<td></td>
</tr>
<tr>
<td>Kanamycin (10 μg/disk)</td>
<td>12</td>
<td>13</td>
<td>9</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Ag-NPs + Kanamycin (b)</td>
<td>16</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Fold increase % = (\frac{(b – a)}{a} \times 100)</td>
<td>33.33</td>
<td>46.35</td>
<td>22.22</td>
<td>10.00</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Chloramphenicol (10 μg/disk)</th>
<th>Ampicillin (10 μg/disk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol (a)</td>
<td>22</td>
<td>29</td>
</tr>
<tr>
<td>AgNPs + chloramphenicol (b)</td>
<td>28</td>
<td>36</td>
</tr>
<tr>
<td>Fold increase % = (\frac{(b – a)}{a} \times 100)</td>
<td>27.27</td>
<td>24.14</td>
</tr>
<tr>
<td>Ampicillin (a)</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>AgNPs + Ampicillin (b)</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>Fold increase % = (\frac{(b – a)}{a} \times 100)</td>
<td>75.00</td>
<td>81.82</td>
</tr>
</tbody>
</table>

* Percentage fold increases of individual antibiotics were calculated using the formula \(\frac{(b – a)}{a} \times 100\). The mean zone of inhibition around disk containing AgNPs alone (10 μg) was 8 mm.

proved to be very useful for the analysis of nanoparticles. The UV-vis spectra of T. viride reaction vessel at different times of reaction are plotted in Figure 2, and the times at which the aliquoted samples removed for analyses are indicated next to the respective curves. The strong surface plasmon resonance centered at ~423 nm clearly showed an increase in intensity with time and stabilized after ~24 hours of reaction. The reduction of Ag+ ions to elemental silver by T. viride is characterized by XPS analysis and is shown in Figure 3. The Ag 3d spectrum could be deconvoluted into a single spin-orbit pair (spin-orbit splitting ~5.9). The Ag 3d5/2 and 3d3/2 peaks occurred at a binding energy of 368.1 eV and 374 eV, respectively. The FT-IR spectrum of extracellular biosynthesized AgNPs is shown in Figure 4. The spectrum shows the presence of bands at 1650, 1540, 1423, and 1060 cm⁻¹. The TEM technique used to visualize size and shape of the extracellular biosynthesized AgNPs is shown in Figure 5. The morphology of nanoparticles is highly variable, with spherical and occasionally rodlike nanoparticles observed on micrographs. The TEM micrograph suggests that particle diameters ranged from 5 to 40 nm.

Synergistic effect of AgNPs with antibiotics

The minimum inhibitory concentrations (MIC) of extracellular biosynthesized AgNPs on gram-positive and gram-negative bacteria were determined by broth dilution method. The observed MIC values for AgNPs were 30, 35, 80, and 65 μg/mL for E. coli, S. typhi, S. aureus, and M. luteus, respectively.

The combination of these AgNPs with different antibiotics was investigated against gram-positive and gram-negative bacteria using the disk diffusion method. The diameter of the inhibition zone (nm) around the different antibiotic disks with and without AgNPs against test strains is shown in Table 1. The antibacterial activity of ampicillin, kanamycin, erythromycin, and chloramphenicol increased in the presence of AgNPs against test strains. The highest percentage of fold increase was found for ampicillin followed by kanamycin, erythromycin, and chloramphenicol against all test strains as shown in Figure 6.

Discussion

The study of biosynthesis of nanomaterials offers a valuable contribution to nanobiotechnology. The biosynthetic methods have been investigated as an alternative to chemical and physical ones. In this regard T. viride proves to be an important biological component for extracellular biosynthesis of stable AgNPs. It was observed that the reduction of the Ag+ ions during the exposure to T. viride filtrate can be easily followed by visual observation and UV-vis spectroscopy. It is well known that AgNPs show a yellowish brown color in aqueous solution; this color arises from excitation of surface plasmon vibrations in the metal nanoparticles. It is observed from the spectra that the surface plasmon resonance band of AgNPs occurs at 420 nm, and this absorption steadily increases in intensity as a function of time of reaction. The sharp drop in reaction time from a few days to 24 hours observed for the cell filtrate of T. viride is a highly significant advance toward achieving the goal of developing a rapid method for AgNPs synthesis. An absorption band at 260 nm is clearly visible and is attributed
to electronic excitation in tryptophan and tyrosine residues in proteins.\textsuperscript{[22]} This observation indicates the release of extracellular proteins in the colloidal solution; thus it is interesting to note from our study that not only is the NADPH (reduced form of nicotinamide adenine dinucleotide phosphate)-dependent reductase enzyme specific to \textit{F. oxysporum} as suggested by others,\textsuperscript{[1]} but it is also involved in the reduction of $\text{Ag}^{+}$ to $\text{Ag}^{0}$ in the case of the fungus \textit{T. viride} under similar experimental conditions. The possible mechanisms suggested—reduction of $\text{Ag}^{+}$ to $\text{Ag}^{0}$ is due mainly to conjugation between the electron shuttles with NADPH-dependent reductase participation.\textsuperscript{[6]} The reduction of $\text{Ag}^{+}$ ions to elemental silver by \textit{T. viride} is provided by XPS. The Ag $3d_{x^2}$ and $3d_{y^2}$ peaks occurring at a binding energy of 368.1 eV and 374 eV, respectively, are assigned to the metallic silver. The absence of a higher binding energy Ag 3d component clearly indicates that all the silver ions are fully reduced by the fungus \textit{T. viride}. It was observed from the FT-IR spectrum of AgNPs that the bands at 1650 correspond to a primary amine NH band; similarly, 1540 and 1060 correspond to a secondary amine NH band and primary amine CN stretch vibrations of the proteins, respectively.\textsuperscript{[21]} The positions of these bands were close to that reported for native proteins.\textsuperscript{[22]} The FT-IR results indicate that the secondary structures of proteins were not affected as a consequence of reaction with $\text{Ag}^{+}$ ions or binding with AgNPs. The band at 1425 cm$^{-1}$ is assigned to methylene scissoring vibration from the protein in the solution. This evidence suggests that the release of extracellular protein molecules could possibly perform the function of the formation and stabilization of AgNPs in aqueous medium. It is observed from TEM micrographs that most of the AgNPs are spherical and are in the range of 5–40 nm in size. The particle size distribution

Figure 6. Percentage fold increase in antibacterial effect of antibiotics with AgNPs against test strains.

Figure 7. Particle size histogram of AgNPs.

Figure 8. EDS spectrum of AgNPs. Different x-ray emission peaks are labeled. Strong signals from the atoms in the nanoparticles are observed, whereas weaker signals from copper atoms are also visible.

Figure 9. Zeta potential of AgNPs as a function of pH.
histogram determined from TEM is shown in Figure 7. From this histogram it is observed that there is variation in the particle size, almost 85% of the particles are in the 5- to 20-nm range.

The TEM micrograph shows that the particles are polydispersed and are mostly spherical. Hence, it can be understood that optimization of experimental conditions such as pH, temperature, and concentration of Ag ions, etc., will achieve monodispersity and uniform shape. The additional support of reduction of Ag ions to elemental silver, as confirmed by EDS analysis, shows a peak in the silver region, which confirms the presence of elemental silver in Figure 8. The optical absorption peak is observed at approximately 3 keV, which is typical for the absorption of metallic silver nanocrystals due to surface plasmon resonance.

The MIC of biogenic AgNPs against test strains shows that AgNPs have a less significant effect on growth of gram-positive bacteria than on gram-negative bacteria. This is due to the structural difference in cell wall composition of gram-positive and gram-negative bacteria. The gram-negative bacteria have a layer of lipopolysaccharides at the exterior, followed underneath by a thin (~7-8 nm) layer of peptidoglycan. Although the lipopolysaccharides are composed of covalently linked lipids and polysaccharides, there is a lack of strength and rigidity. The negative charges on lipopolysaccharides are attracted toward the weak positive charge available on AgNPs. However, in the current studies the presence of negatively charged AgNPs was confirmed by zeta potential measurement as shown in Figure 9. These negatively charged AgNPs can attack the gram-negative bacteria by metal depletion, as suggested by others. On the other hand, the cell wall in gram-positive bacteria is principally composed of a thick layer (~20–80 nm) of peptidoglycan consisting of linear polysaccharide chains cross-linked by short peptides to form a three-dimensional rigid structure. The rigidity and extended cross-linking not only endow the cell walls with fewer anchoring sites for the AgNPs but also make them difficult to penetrate. The antibacterial activity of ampicillin, kanamycin, erythromycin, and chloramphenicol increase in the presence of AgNPs against test strains. The increase in synergistic effect may be caused by the bonding reaction between antibiotic and nanosilver. The antibiotic molecules contain many active groups such as hydroxyl and amido groups, which react easily with nanosilver by chelation. More recently, Batach's research showed that the bactericidal effect was caused by silver (I) chelating, which prevents DNA from unwinding.

The percentage of fold increase in ampicillin with AgNPs against Gram positive and Gram negative bacteria are almost the same, where as this type of pattern (results) is not observed with other antibiotics, even though inhibition of Gram positive bacteria is very difficult with AgNPs alone. The mode of action of ampicillin is cell wall lysis, and ampicillin molecules themselves can bind each other through van der Waals interaction and other weak bonds. Ultimately the antimicrobial groups come into contact with AgNPs, wherein the nanosilver core is surrounded by ampicillin molecules as shown in Figure 10. The ampicillin molecules acts on the cell wall, which leads to cell wall lysis and thus increases the penetration of AgNPs into the bacterium. The AgNP-ampicillin complex reacts with DNA and prevents DNA unwinding, which results in more serious damage to bacterial cells. Hence, in this study the extracellular biosynthesis of AgNPs facilitates the process for downstream processing and is known to be economical.
efficient, ecofriendly, and simple in process. The synergistic antibacterial effect with the combination of nanosilver and ampicillin has more potential, when compared with other antibiotics. Here we present a possible explanation for the enhancement of the synergistic antibacterial mechanism. This research provides helpful insight into the development of new antimicrobial agents. To elucidate the mechanism of this synergistic effect, more elaborate experimental evidence will be needed, and we are currently working toward this end.

References