RESEARCH PAPER

Bacteria-mediated synthesis of silver nanoparticles under photo-irradiation and their efficacy against methicillin-resistant \textit{Staphylococcus aureus}

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ABSTRACT

Over the last few decades, morbidity and mortality rates from infectious diseases have increased due to antibiotic resistance. Community-acquired and nosocomial-related methicillin-resistant bacterial strains are proliferating, which makes the discovery of new antibiotics and the search for new approaches to fight pathogenic bacteria an urgent task. One of the new promising classes of antimicrobials is silver nanoparticles (AgNPs). The goal of this study was to investigate the bacteria-mediated synthesis of AgNPs and evaluate their antibacterial activity against methicillin-resistant \textit{Staphylococcus aureus} (MRSA). Indigenous bacteria from soil samples from Multan (Pakistan) were isolated and three strains – \textit{Klebsiella} spp., \textit{Citrobacter freundii}, and \textit{Bacillus cereus} – were selected for the synthesis of AgNPs under photo-irradiation. The biosynthesis of AgNPs was confirmed by UV-visible spectroscopy. The AgNPs obtained in our experiments showed strong antibacterial activity against MRSA isolated from patients with community-acquired skin infections (from the dermatology ward of Jinnah Hospital Lahore) with 15 mm to 25 mm zones of inhibition (ZOI) according to the well diffusion method. The results of this project showed that microbial reduction of AgNO$_3$ under photo-irradiation produces AgNPs that are highly active against MRSA.

Keywords: Nanoparticles, silver nanoparticles (AgNPs), antibacterial activity, green chemistry, methicillin-resistant \textit{Staphylococcus aureus} (MRSA), nanotechnology, nanomedicine, soil samples, antibiotic resistance

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INTRODUCTION

Nanotechnology is an emerging field in which nanoscale materials are synthesized by combining science and engineering. It is predicted to become the next technological revolution because nanoparticles have already found a large range of applications in areas like drug delivery, nanomedicine, biomedical devices, environmental remediation, systems for renewable energy and catalysis, cosmetics, and electronics.

A nanoparticle is a small particle with a size ranging from 1 nm to 100 nm. Among different metal nanoparticles, silver nanoparticles (AgNPs) have drawn the attention of researchers because of their novel physicochemical and biomedical properties such as high thermal and electrical conductivity, chemical stability, nonlinear optical effect, and catalytic activity as compared to other metal nanoparticles [1]. AgNPs have a strong cytotoxic effect against a
Synthesis of silver nanoparticles

Wide range of microorganisms from bacteria to fungi, particularly because of their high surface-to-volume ratio [2].

Different physical, chemical, and biological methods are used for the synthesis of nanoparticles; however, biological methods are more promising because they enable cost-effective nanoparticle production in environmentally friendly conditions. Microbial synthesis of AgNPs is a more useful approach due to its short production time compared to other methods. According to this method, bacterial intracellular enzymes react with the metal salt to produce metal nanoparticles [3]. The chemical synthesis of AgNPs can be conducted by several methods, e.g., chemical reduction, electrochemical reduction, reduction in micro-emulsion systems, or ultrasonic-assisted reduction. However, these methods have proven to be precarious for the environment and dangerous for humans [4]. Thus, the biological synthesis of AgNPs is a more useful and safer method, and, therefore, it represents a suitable alternative to chemical methods that use toxic reagents. In the extracellular synthesis of AgNPs, the synthesis of nanoparticles occurs outside the cell by using the components of culture supernatant, aqueous cell-free extract, or biomass. In intracellular synthesis, bacterial cells synthesize AgNPs in the culture medium containing silver salt. For the biogenic synthesis of AgNPs, the extracellular method has advantages over the intracellular method as it is more economical and less laborious due to the easy recovery of nanoparticles from the solution. Meanwhile, intracellular synthesis requires ultrasonic treatment and the use of extra detergents in order to break the bacterial cell wall and bacterial membrane in recovery steps [5].

Antibiotic resistance is one of the main healthcare challenges faced by humans and animals nowadays. Many human pathogenic bacteria are resistant to conventional antibiotics and among them, MRSA is a prominent one. Due to continuously emerging bacterial resistance, the search for new antimicrobials and the development of new antimicrobial strategies are extremely important. The application of AgNPs can be a remarkably effective approach to tackling the infections caused by MRSA [6] since the antimicrobial and antiviral activities of AgNPs are well-documented. The soil known to harbor a vast variety of microorganisms [7] was selected as a source of bacteria in this project. The present study aimed to scrutinize the potential of bacteria isolated from the soil of Multan (Pakistan) for the microbial synthesis of AgNPs as well as to show that these AgNPs potentially can be used to control nosocomial infection caused by MRSA all over the world.

**MATERIALS AND METHODS**

**Isolation of AgNP-synthesizing bacteria**

Soil samples were collected from different areas of Multan (Pakistan) in sterilized zip-lock bags, stored in an icebox, and transported to the laboratory. Fifty-one bacterial species were isolated from the soil samples by two-fold serial dilution at 42°C and were labeled S-1 to S-51.

**Bacterial identification**

The isolates were identified based on physiological, morphological, and biochemical characteristics such as Gram staining; spore formation; lactose, mannitol, and glucose fermentation; urease production; citrate utilization; indole and catalase production; acetylmethylcarbinol formation; glucose oxidation; H₂S production. Bergey’s Manual of Determinative Bacteriology was followed to identify bacterial isolates [8].

**Extracellular biosynthesis of AgNPs**

Three bacterial strains – *Klebsiella* spp., *Citrobacter freundii*, and *Bacillus cereus* – were selected from the fifty-one isolates studied (Table 1) based on their ability to form good-quality AgNPs in the course of extracellular biosynthesis. For this purpose, each strain was inoculated under aseptic conditions in 50 ml of sterile N-broth and incubated in an Orbitek rotating shaker at 120 rpm and 37°C for 24 h. After incubation, the culture of each strain was centrifuged at 6000 rpm for 12 min and the supernatant was separated. Silver nitrate (AgNO₃) solution was prepared and added at 1 mM final concentration to the supernatant of each strain. The obtained reaction mixture was incubated in a rotating shaker at 120 rpm and room temperature for 72 h using the sunlight (or the light of a tungsten lamp in the absence of sunlight) for photo-irradiation. The supernatant of the corresponding strain without AgNO₃ was incubated in the dark as a control. All the flasks underwent periodic visual inspection for color change. During the reaction, the color of the reaction mixture changed from pale yellow to brown as an indication of the extracellular formation of AgNPs [9].

Table 1. Isolation of bacterial strains for the biosynthesis of AgNPs from different soil samples

<table>
<thead>
<tr>
<th>Soil location</th>
<th>Soil source</th>
<th>Isolate number</th>
<th>Bacterial species</th>
<th>AgNPs production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shahrurkne alam</td>
<td>Agriculture field</td>
<td>S-3</td>
<td><em>Klebsiella</em> spp.</td>
<td>Significant positive result</td>
</tr>
<tr>
<td>Gulgasht colony</td>
<td>Garden soil</td>
<td>S-13</td>
<td><em>Citrobacter freundii</em></td>
<td>Significant positive result</td>
</tr>
<tr>
<td>Rasheedabad</td>
<td>Agriculture field</td>
<td>S-44</td>
<td><em>Bacillus cereus</em></td>
<td>Significant positive result</td>
</tr>
</tbody>
</table>
Characterization of AgNPs

UV-visible spectroscopy was performed using 1ml aliquots of the final reaction mixture to confirm the synthesis of AgNPs and to measure their concentration. Utilizing this technique, the surface plasmon resonance was observed and the production of AgNPs was verified [10].

Isolation and identification of MRSA

Twenty-one Staphylococcus aureus strains (SA30 – SA50) were isolated from the pus of patients with community-acquired skin infections from the dermatology ward of Jinnah Hospital Lahore. The studied bacteria were isolated on mannitol salt agar (MSA) and identified by microscopic, morphological, and biochemical methods (Table 2) [10]. Antibiotic susceptibility testing of all S. aureus isolates was performed using the Kirby-Bauer disk diffusion susceptibility method – as per the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI-2017) against the following antibiotics: cefoxitin (30 µg), amoxicillin-clavulanic acid (30 µg), tetracycline (30 µg), ceftazidime (30 µg), erythromycin (15 µg), oxacillin (1 µg), methicillin (5 µg), minocycline (30 µg), and gentamicin (10 µg). The inoculum size was equal to the McFarland 0.5 M standard in order to achieve a comparable ZOI against the selected antibiotics with a specific concentration. For quality control, Staphylococcus aureus (ATCC 25923) was used [12].

Analysis of the antibacterial activity of AgNPs against MRSA

Antibacterial activity testing of AgNPs was carried out against ten community-acquired MRSA isolates by the well diffusion method [13]. These isolates were cultured, and then the optical density (OD) of each sample was measured spectrophotometrically at 590 nm and consequently adjusted to 0.1 OD with sterile N-broth. A 40 µl aliquot of the AgNP suspension obtained by microbial synthesis was added into a well on each Petri dish with Muller Hinton Agar (MHA). Then plates were incubated at 37°C for 18-24 h. After incubation, ZOIs were detected and measured [14].

RESULTS

Isolation and identification of bacterial strains for the AgNPs biosynthesis

Fifty-one bacterial strains were isolated from soil samples and then three of them (Klebsiella spp., Citrobacter freundii, and Bacillus cereus) were selected for the AgNPs biosynthesis (Table 1).

Isolation and identification of MRSA bacteria

During MRSA selection, twenty-one bacterial species (SA30 – SA50) isolated from the pus samples were identified as S. aureus (Fig. 1). Ten isolates among them showed resistance against methicillin and oxacillin according to the antibiotic susceptibility test (AST). Most S. aureus species were resistant to tetracycline, cefoxitin, amoxicillin + clavulanic acid, erythromycin, and ceftazidime (Table 3).

Extracellular biosynthesis of AgNPs

After the centrifugation of the inoculum of the three bacterial strains (Klebsiella spp., Citrobacter freundii, and Bacillus cereus), the supernatants containing 1 mM AgNO₃ Table 2. Characteristics of Staphylococcus aureus strains

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony shape</td>
<td>Round</td>
</tr>
<tr>
<td>Colony size</td>
<td>Punctiform</td>
</tr>
<tr>
<td>Colony color</td>
<td>Yellow to golden yellow</td>
</tr>
<tr>
<td>Morphological characteristics</td>
<td></td>
</tr>
<tr>
<td>Gram staining</td>
<td>Gram+, cocci, grape-like arrangement</td>
</tr>
<tr>
<td>Motility</td>
<td>Non-motile</td>
</tr>
<tr>
<td>Spore staining</td>
<td>Non-spore former</td>
</tr>
<tr>
<td>Microscopic characteristics</td>
<td></td>
</tr>
<tr>
<td>Biochemical tests</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>Coagulase</td>
<td>Positive</td>
</tr>
<tr>
<td>β-hemolysis</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Table 3. AST results of all Staphylococcus aureus isolates obtained from clinical samples

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Symbol</th>
<th>Resistant, n (%)</th>
<th>Sensitive, n (%)</th>
<th>Intermediate, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin + clavulanic acid</td>
<td>AUG</td>
<td>17 (80.95%)</td>
<td>5 (14.29%)</td>
<td>1 (4.76%)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>FOX</td>
<td>18 (85.72%)</td>
<td>2 (9.52%)</td>
<td>1 (4.76%)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>TE</td>
<td>17 (80.96%)</td>
<td>2 (9.52%)</td>
<td>2 (9.52%)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>CN</td>
<td>6 (28.57%)</td>
<td>13 (61.91%)</td>
<td>2 (9.52%)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>MET</td>
<td>10 (47.62%)</td>
<td>11 (52.38%)</td>
<td>0</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>OXA</td>
<td>5 (23.81%)</td>
<td>15 (71.43%)</td>
<td>1 (4.76%)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>E</td>
<td>18 (85.72%)</td>
<td>3 (14.28%)</td>
<td>0</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>CAZ</td>
<td>15 (71.44%)</td>
<td>5 (14.28%)</td>
<td>3 (14.28%)</td>
</tr>
<tr>
<td>Minocycline</td>
<td>MH</td>
<td>2 (9.52%)</td>
<td>18 (85.71%)</td>
<td>1 (4.77%)</td>
</tr>
</tbody>
</table>
were used for the biosynthesis of AgNPs. The reaction mixture in all three flasks changed its color from yellow to dark brown after exposure to sunlight, which indicated the formation of AgNPs from AgNO$_3$ (Fig. 2). At the same time, the controls (the supernatants after the centrifugation of bacterial inoculum without AgNO$_3$) showed no color change at room temperature in the dark.

**Proof of AgNPs forming and their characterization**

As the brown color intensity in the reaction mixture increased over time, its UV absorbance increased as well. The peaks in the UV region of the spectra were observed between 410-450 nm due to the surface plasmon resonance [15]. The reaction mixture containing the isolate of *Klebsiella* spp. showed a peak at 420 nm, while the solutions with *Citrobacter freundii* and *Bacillus cereus* showed peaks at 440 nm. Since UV absorption at 400-470 nm is a well-documented property of AgNPs in aqueous solutions [15], our spectral data confirm the effective formation of AgNPs in all three experiments (Fig. 3).

**Antimicrobial activity of AgNPs against MRSA**

Our results proved that AgNPs are effective against all 10 MRSA strains (Table 4, Fig. 4). In our experiments, AgNPs showed the maximum ZOI (25 mm) against MRSA38 and the minimum ZOI (15 mm) against MRSA37 (Table 4).
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Fig. 3. UV-visible spectra of AgNPs synthesized by *Klebsiella* spp (A), *Citrobacter freundii* (B), and *Bacillus cereus* (C).

Fig. 4. Antibacterial activity of AgNPs. A. Against MRSA33. B. Against MRSA38.
Control A – supernatant of bacterial strains.
Control B – 1 mM AgNO₃ solution in N-broth.
**DISCUSSION**

There are several approaches to the synthesis of metal nanoparticles, however, the biological approach is more feasible and environmentally friendly. Noble metal nanoparticles have found applications in many areas of science and technology, particularly as antimicrobial agents. Among the various noble metals, AgNPs showed the highest antimicrobial activity and, therefore, are considered the most suitable choice to be used as antibacterials [16]. Very few bacterial strains, such as *P. aeruginosa*, *E. coli*, and *Bacillus* spp., are able to synthesize AgNPs since they can withstand the high concentration of Ag⁺, which is necessary to accomplish this process [17, 18]. Thus, Priyadarshini et al. [19] reported that only one strain out of 127 strains of bacteria was able to synthesize AgNPs. Only three isolates studied in our project (*Bacillus cereus*, *Klebsiella* spp., and *Citrobacter ferrundii*) were able to synthesize high-quality AgNPs, which corresponds to the results published by Salunke et al. [20] and Shakhatreh et al. [16]. The AgNPs synthesized by *Bacillus* spp. and *E. coli* are spherical and quite stable. The size of AgNPs can differ significantly depending on the bacterial strain and conditions used for their synthesis [21]. For example, the AgNPs synthesized using the culture supernatant of *P. stutzeri* ranged from 15 nm to 20 nm, however, under optimized conditions, their average size was 8 nm [22].

It was demonstrated in several studies that bacterial AgNP synthesis can be accomplished both inside and outside of the bacterial cell. The formation of AgNPs outside of the cell, on the surface of the cellular membrane, and in the cytoplasm was confirmed by electron microscopy [23]. Although the number of publications on the bacterial synthesis of metal nanoparticles is quickly growing, the mechanism of this process is still poorly understood. Enzymes and proteins present on the cell wall and secreted by bacteria are believed to participate in the extracellular synthesis of AgNPs by reducing silver ions to Ag⁺ [24]. Thus, the study by Wang et al. [24], which focused on the extracellular synthesis of AgNPs by *Bacillus methylotrophicus*, demonstrated that the nitrate reductase released by the bacteria to the medium was responsible for producing AgNPs. Therefore, we believe that in our study, bacterial enzymes and proteins present in the supernatant after the centrifugation were involved in the Ag⁺ reduction to Ag⁰. The results of several studies suggest that the enzyme nitrate reductase is responsible for silver ions reduction in the presence of NADPH [21, 26], although the exact mechanism of this reaction is still unknown. The data recently published by Hietzschold et al. [27] suggest that silver nitrate can be successfully reduced to AgNPs by NADPH in the absence of nitrate reductase. However, that does not prove that this enzyme is not involved in the reduction process in the systems containing nitrate reductase. Some scientists consider that the electrons released in the process of nitrate-to-nitrite conversion mediated by nitrate reductase are involved in the reduction of Ag⁺ to Ag⁰. This assumption is incorrect because electrons are consumed and not released when nitrate is converted to nitrite [28, 29]. On the other hand, being a fairly strong oxidant, Ag⁺ (Ag⁺ → Ag⁰, E°⁺ = 0.799 V) would actively compete for electrons with nitrate ions (NO₃⁻ → NO₂⁻, E° = 0.450 V), that would lead to the formation of Ag⁰ (AgNPs) in these systems.

A number of methods require elevated temperature (40-80°C) for the silver nitrate bio-reduction. Other methods involve photo-irradiation to accelerate the reduction process [16]. The mechanism of photoreduction of silver nitrate to AgNPs in the presence of nitrate reductase is poorly understood, and it is quite clear that more studies are necessary to investigate this process. It should be mentioned that since the enzyme absorbs light in the visible area of the spectrum [30, 31], sunlight irradiation of the reaction mixture should significantly influence the silver nitrate reduction process [32]. The quick synthesis of nanoparticles for mass production utilizing cell filtrates under photo-irradiation showed better kinetics of the AgNP synthesis than without irradiation [33]. The results of our study support this conclusion since we have described here a highly efficient method of AgNP synthesis via photo-irradiation. According to Barraza-Vergara et al. [34], the concentration of AgNO₃ (1-100 mM) used for the synthesis of AgNPs at elevated temperature (55°C) had a significant impact on the antimicrobial activity of the AgNPs produced. However, in

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**Table 4. ZOI of AgNPs against MRSA obtained by the well diffusion method**

<table>
<thead>
<tr>
<th>Strain IDs</th>
<th>Size of the ZOI against MRSA strains (mm)</th>
<th>by AgNPs</th>
<th>Control A</th>
<th>Control B</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA31</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA32</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA33</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA34</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA35</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA36</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA37</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA38</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA39</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRSA40</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Control A – supernatant of bacterial strains.  
Control B – 1 mM AgNO₃ solution in N-broth.*
our study, the AgNPs synthesized using the lowest concentration of AgNO₃ (1 mM) at room temperature demonstrated excellent antimicrobial activity. The stability of AgNPs synthesized by bacteria varies depending on the method of the synthesis and storage conditions. The AgNPs synthesized in our project were quite stable and, therefore, no stabilizing agent was added to the nanoparticle suspension. To maintain their stability over time, AgNPs were kept in aluminum foil and stored in the fridge [35].

MRSA poses a great threat to human health because of its multidrug resistance and a high nosocomial infection rate. Therefore, we evaluated the antibacterial activity of AgNPs against MRSA, proving the AgNPs to be highly effective against these bacteria [36]. The mode of AgNPs antibacterial action remains to be elucidated, but there are several mechanisms proposed. Some scientists suggest that these particles adhere to the bacterial cell membrane and degrade the lipopolysaccharide molecules in the outer membrane of gram-negative bacteria which results in the formation of pores in the membrane, altering the membrane permeability and respiratory functions of the cells [37, 38]. There is another plausible mechanism of their action: AgNPs could release toxic Ag⁺ ions which react with thiol groups of proteins present in the cell, leading to the activation of reactive oxygen species (ROS) that cause oxidative damage to the microbial cell. Finally, it was suggested that AgNPs enter the cell and inactivate DNA replication, leading to cell death [39, 40].

Previous research shows that AgNPs are more effective against gram-negative than against gram-positive bacteria. It is believed that the reason behind this is that AgNPs carry a positive charge and they efficiently interact with negatively charged lipopolysaccharides in the outer membrane of gram-negative bacteria. This leads to AgNPs penetrating more effectively into gram-negative microbial cells as compared to gram-positive bacteria [41]. However, in our study, all 10 strains of MRSA, which are gram-positive bacteria, were susceptible to AgNPs. Thus, it could be suggested that the interaction with lipopolysaccharides is not the main process that defines the susceptibility of bacterial cells to AgNPs.

We have found that AgNPs synthesized in our study exhibit remarkable activity against MRSA as shown by the ZOI sizes ranging from 15 mm to 25 mm (Table 4, Fig. 4). This level of activity against MRSA is significantly higher as compared to the results of previous studies, e.g., by Paredes et al. [42]. The AgNPs synthesized by Saleem et al. showed only an 18 mm ZOI [15]. Overall, our findings prove that the reduction of AgNO₃ under photo-irradiation is a very effective approach to synthesizing AgNPs that are highly active against MRSA, although this method needs more investigation. It should be also mentioned that AgNPs functionalized with ampicillin show a potent bactericidal effect and can be used against multidrug-resistant microorganisms [43].

**CONCLUSION**

The present study emphasizes that indigenous bacteria from Multan (Pakistan) can be successfully used to produce metal nanoparticles. AgNPs can be applied not only in biomedicine and cancer therapy but in targeted drug delivery as well. They can be used in combination with antibiotics to broaden their spectrum of antimicrobial activity.

**REFERENCES**


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