Biosynthesis, characterization and antibacterial activity of silver nanoparticles against ESBL producing water-borne pathogens

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Abstract

In the present work, biosynthesis of silver nanoparticles (AgNPs) using isolate of Shigella sp. AS8 culture supernatant as a reducing agent has been demonstrated. Synthesis of AgNPs was completed within 180 min of incubation at 35 °C under bright light condition. The biosynthesized nanoparticles were characterized by UV-vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), Dynamic light scattering (DLS), Transmission electron microscopy (TEM), Field emission scanning electron microscopy (FE-SEM) equipped with energy dispersive X-ray (EDX) and Atomic force microscopy (AFM). The formation of brown color reaction product with strong UV-vis metal absorption maxima at 411 nm due to surface plasmon resonance (SPR) indicated the synthesis of AgNPs. FTIR spectrum confirmed association of supernatant protein molecules with synthesized nanoparticles. DLS, TEM, FE-SEM and AFM showed biosynthesized nanoparticles were spherical in shape with an average size of 20 nm. EDX data analysis reveals presence of metallic silver. While, XRD analysis revealed that synthesized particles were pure and crystalline in nature. Further, AgNPs were evaluated as an antibacterial agent against extended spectrum β-lactamase (ESBL) positive water-borne pathogens. The results of present study suggest that biosynthesized AgNPs can be used to combat ESBL producing multidrug resistant bacteria. Copyright © 2016 VBRI Press

Keywords Biosynthesis, silver nanoparticles, shigella sp. AS8, ESBL, antibacterial activity.

Introduction

Nanotechnology deals with the design, manipulation and synthesis of particles of below 100 nm. Metal nanoparticles have gained interest in research during the past few decades due to their unique size and shape dependent physical, chemical and biological properties [1, 2]. Noble metal nanoparticles such as silver, gold, titanium and copper etc. showed various applications in different area of sciences and technology as compared to their bulk counterparts. Among them, Silver nanoparticles (AgNPs) in particular have attracted attention in various fields, such as antimicrobials, anti-cancer, drug delivery, bio-molecular detection and catalysis [3-9]. Synthesis of well-defined nanoparticles is an important aspect of nanotechnology. Nanoparticles are mostly synthesized by using physical, chemical and biological methods. Due to high energy demand and generation of hazardous by-products, physical and chemical methods used for synthesis of nanoparticles are being taken over by eco-friendly green chemistry process [10-13]. In green chemistry route usage of bacteria, fungi and plants have offered a promising source to avoid chemical toxicity [14-17]. Among them, bacteria are mostly used for biosynthesis due to their high growth rate, easy handling and achievable genetic modification [18]. Bacteria can synthesize nanoparticles by both intracellular and extracellular approaches [19]. Intracellular production accomplishes a better control over uniformity of size and shape as well as polydispersity than extracellular synthesis [20]. However, extracellular approach is mostly used in order to understand the mechanism of synthesis, easy downstream, rapid scale up and simple isolation of nanoparticles [21]. Silver mine bacterial isolate Pseudomonas stutzeri AG259 was the first ever microbe to be used for synthesis of AgNPs [22]. Recently, various studies reported the synthesis of AgNPs using different bacteria such as Bacillus subtilis, Klebsiella pneumoniae, Brevibacterium frigoritolerans, Pseudomonas aeruginosa, Bacillus spp. and Bhargavaea indica [23-28]. Although, bacterial mediated synthesis process is much slower than plant extract or other chemical methods, which limit its use in industry for
large scale production [29]. Now, it has been reported that bacterial mediated AgNPs synthesis significantly increases in the presence of visible light [30, 31].

The emergence of extended spectrum β-lactamases (ESBLs) producing multidrug resistant bacteria has created major human health problem throughout the world, including India [32, 33]. The ESBLs are β-lactamases imparting resistance to bacteria through hydrolysis of β-lactum antibiotics up to third generation cephalosporin and are inhibited by clavulanic acid [34]. Most prevalent ESBLs types have evolved through point mutations in their parental TEM and SHV enzymes [35, 36] and now more than 400 variants have been reported from all over the world (www.lahey.org). The ESBL positive bacteria are not only present in clinical setting but also occur in natural environment [37]. Generally, river aquatic ecosystem encourages bacteria to exchange their resistant genes and genetic platforms to clinically important bacteria to create human pathogens with new resistant mechanisms [38, 39]. The worldwide prevalence and high mortality rates due to ESBLs producing multidrug resistant bacteria obliges scientific community to develop novel compound with new target sites to prevent them. In order to promote alternative strategies towards combating drug resistant microorganisms such as ESBLs, AgNPs could be a potent antibacterial agent. While, previous studies claimed effectiveness of AgNPs against ESBL producing bacteria [40, 41].

In this study, we described an eco-friendly and single step process for biosynthesis of AgNPs using the culture supernatant of Shigella sp. AS8. Based on our literature survey, this is the first report on biosynthesis of AgNPs using this isolate. Synthesized nanoparticles were characterized by various standard techniques such as UV-vis, FTIR, XRD, DLS, TEM, FE-SEM, EDX and AFM. Further, antibacterial activity of AgNPs was investigated against ESBL producing water-borne bacteria.

Experimental

Materials

All the culture media (Luria Agar, Luria Broth and Mueller Hinton Agar) were purchased from Himedia, Mumbai, India and used after sterilization. All the antibiotics (Cefotaxime, Ceftazidime, Ceftriaxone, Amikacin, Ertapenem, Imipenem, Cefazolin, Ampicillin, Colistin, Polymyxin B, Rifampicin, Trimethoprim, Chloramphenicol) and kit for ESBL identification were purchased from Himedia, Mumbai, India. Silver nitrate (AgNO₃) with 99 % purity was obtained from Merck Ltd., India. All the reagents for molecular work (PCR buffer, deoxynucleotides (dNTPs) and Taq DNA Polymerase) were taken from Bangalore Genei Pvt. Ltd., India and used without further purification. All glassware and Milli-Q water were sterilized before use in entire experiments.

Material synthesis

Isolation and identification of Shigella sp. AS8

Water samples from industrial effluent were collected from Sahibabad Site-IV industrial area, Sahibabad, Uttar Pradesh, India. The collected samples were serially diluted and spread on Luria agar (LA) plates and incubated at 37 °C for overnight. After incubation, morphologically different colonies were further subcultured on LA to obtain pure colonies. All the bacterial isolates were screened for extracellular synthesis of AgNPs and based on their ability to reduction of silver metal ions, Shigella sp. AS8 was selected and identified by 16S rRNA sequence analysis. The partial 16S ribosomal gene sequence of the isolate has been submitted to NCBI with an accession no. KU755550.

Biosynthesis of AgNPs

For biosynthesis of AgNPs, the bacterial isolate AS8 was inoculated into 100 ml of Luria Broth (LB) in a 250 ml Erlenmeyer flask and incubated at 37 °C for 24 h with continuous shaking at 120 rpm. Supernatant from stationary phase culture was collected by centrifugation at 9000 rpm at 4 °C for 10 min. In a typical reaction procedure, 1 ml of cell free culture supernatant was mixed into 99 ml of freshly prepared 1mM aqueous AgNO₃ solution (1% v/v) in a 250 ml reaction vessel and incubated at 35 °C under bright light condition. Simultaneously culture supernatant and AgNO₃ (1mM) solution was maintained as a control. Synthesis of AgNPs was preliminarily determined by visual observation of color change in the reaction mixture during incubation. Further, formation of nanoparticles with function of reaction time was monitored by double beam UV-visible Spectrophotometer (Labtronics LT-2800). The synthesized AgNPs was isolated from solution by simple centrifugation at 10000 rpm at 4 °C for 30 min as described earlier [40]. The clear supernatant was discarded and pellet was washed four times with sterile Milli-Q water to remove the impurities. The isolated AgNPs was dried in hot air oven at 40 °C to obtain powdered AgNPs which was used for further characterizations.

Characterizations

The powdered AgNPs was mixed uniformly with potassium bromide (KBr) by a mortar and pestle. The mixture was highly pressed in a French press to make a plate and FTIR spectrum was recorded by Bruker Tensor 37 instrument within spectral range between 4000-400 cm⁻¹ at a resolution of 4 cm⁻¹ against a blank KBr pellet. The XRD pattern of powdered nanoparticles was investigated by X pertPro PANalytical X-ray diffractometer using Cu-Kα radiation (k=1.54 Å) operating at 45 kV with 40 mA. The powdered AgNPs was diluted with sterile Milli-Q water and distribution of particle size in aqueous medium was measured by DLS using spectrscatter RıNA, at 20 °C for 10 cycles. The exact size and shape of synthesized particles were determined by
TEM (Tecnai G2 200kV) instrument. Samples for TEM analysis were prepared by placing a drop of AgNPs solution on carbon coated copper grids and examined after drying at room temperature. The surface morphology of synthesized AgNPs was observed by FE-SEM (Zeiss Sigma). The metallic nature of nanoparticles was analyzed by EDX combined with FE-SEM. Synthesized AgNPs was spin coated on glass substrate and their topological features were studied by AFM (Bruker nanoscope V) instrument.

Isolation and identification of ESBL producing bacteria

Water samples were collected from Delhi stretch of river Yamuna, India to isolate ESBL producing bacteria. For this, serially diluted samples were spread on LA plates containing cefotaxime (1mg/L) to isolate resistant bacteria. After incubation, morphologically distinct colonies were picked from each plate and streaked on LA plates to obtain pure culture. All isolates were screened for ESBL production by preliminary and Phenotypic Disc Confirmatory test (PDCT), as per guidelines of Clinical Laboratory Standards Institute (CLSI-2014). Preliminary test was done with antibiotics cefotaxime (CTX), ceftazidime (CAZ) and Ceftriaxone (CTR) by disk diffusion method. Those isolates showing inhibition zone of ≤ 27 mm for CTX, ≤ 22 mm for CAZ and ≤ 25 mm for CTR were suspected as ESBL positive and further confirmed by PDCT. Antibiotics disc cefotaxime and ceftazidime (30 µg) alone and in combination with clavulanic acid (20+10 µg) were used for PDCT. A ≥ 5 mm zone of inhibition for antibiotic combined with clavulanic acid compared to antibiotic alone was considered as positive for ESBL production. For quality control, K. pneumoniae ATCC 700603 and E. coli 25922 were used as ESBL positive and negative control respectively. The presence of blaTEM and blaCTX-M types of ESBLs genes in bacteria were verified by PCR amplification with specific primers. Finally, ESBL positive isolates were identified by 16s rRNA gene sequence analysis and nucleotide sequence data have been submitted to NCBI GenBank.

Antibiotic profiling of ESBL producing bacteria

Antibiotic sensitivity test for ESBL positive isolates were performed by Kirby Bauer’s disc diffusion method. For this, bacterial suspension was spread on Muller Hinton Agar (MHA) plates and antibiotic discs were placed and the plates were incubated at 37 °C overnight. The obtained results were interpreted as per guidelines of Clinical Laboratory Standards Institute (CLSI-2014). Antibiotics used in this study, were Amikacin (AK), Ertapenem (ETP), Imipenem (IMP), Cefazolin (CZ), Ampicillin (AMP), Colistin (CL), Polymyxin B (PB), Rifampicin (RIF), Trimethoprim (TR) and Chloramphenicol (C).

Antibacterial activity of AgNPs against ESBL producing bacteria

Antibacterial activity of biosynthesized AgNPs was studied against ESBL producing isolates by well diffusion method. For this, AgNPs solution (1 mg/ml) of different volumes (10 µl, 20 µl, and 30 µl) were poured in agar well and incubated at 37 °C for overnight. Antibiotic cefotaxime and cell free culture supernatant were used as positive and negative control respectively. After incubation the clear zone of inhibition (ZOI) across the well was measured in millimeters scale. The minimum inhibitory concentration (MIC) was determined by broth micro dilution method using 96-well microtiter plates. Further, viability of treated bacterial cells was investigated by spread plate method. The log phase bacterial cells were treated with synthesized AgNPs at their MIC concentration. After that, treated and untreated cells were centrifuged and their cell number was adjusted approximately to 1×10⁶ CFU/ml with sterile Milli-Q water. Same volume of both bacterial suspensions were separately spread on LA plates and incubated at 37 °C for overnight. After incubation colony was counted from both plates and toxicity of AgNPs was calculated using following formula.

Toxicity (%) = (N₀-Nᵣ/N₀) × 100,

where N₀ is the initial colony number and Nᵣ is the colony number after treatment.

Results and discussion

Biosynthesis of AgNPs

The culture supernatant of bacterial isolate AS8 incubated with (1mM) AgNO₃ solution at 35 °C for synthesis of silver nanoparticles. Appearance of light brown color within 30 min and deep brown at 180 min of incubation indicates the formation of AgNPs.

Fig. 1 UV-vis spectra of AgNPs synthesized by Shigella sp. AS8 culture supernatant with different time intervals.
At the same time there was no such change observed in control setup. It is well known that AgNPs exhibit brown color in aqueous medium due to SPR [42]. A strong UV-vis absorbance maxima located at 411 nm due to excitation of surface plasmon resonance (SPR) confirmed successful synthesis of AgNPs Fig. 1 [10, 24]. The UV-vis spectroscopy analysis also revealed that AgNPs formation gradually increased with function of reaction time and maximum synthesis occurred at the end of 180 min Fig. 1. The biosynthesis of AgNPs was faster under bright condition which may be due to the activation of reducing agents present in culture supernatant, releasing electron for the reduction of silver ions to silver nanoparticles [29,30]. This may be speculated that Shigella sp. AS8 supernatant biomolecules in combination with visible light are capable to synthesis AgNPs within short time period.

Characterizations of AgNPs

In order to determine the possible role of supernatant biomolecules for synthesis and stabilizing AgNPs, FTIR spectroscopy analysis was performed. The FTIR spectrum showed intense absorbance peaks at 3352 cm$^{-1}$, 1610 cm$^{-1}$, 1550 cm$^{-1}$, 1384 cm$^{-1}$ and 1076 cm$^{-1}$ Fig. 2(a). In this spectrum presence of a strong band at 3352 cm$^{-1}$ due to N-H stretching vibrations of primary and secondary amide linkage [43]. The peaks observed at 1610 cm$^{-1}$ and 1550 cm$^{-1}$ due to C-C and N-H stretching vibration of amide-II in protein linkage [44, 45]. The band revealed at 1384 cm$^{-1}$ and 1076 cm$^{-1}$ due to C-N stretching vibration of aliphatic amines [46, 47, 2]. Based on FTIR data corroborated with previous reports we suggest that isolate AS8 supernatant protein molecules play major role for capping as well as synthesis of AgNPs [13, 19, 21].

The crystalline nature of synthesized AgNPs was determined by XRD, which showed distinct peaks at 20 values 38.49, 46.49, 64.63 and 77 corresponding to the intensity of (111), (200), (220) and (311) planes for face centered cubic metallic silver Fig. 2(b). Our obtained values are in line with the database of joint committee on powder diffraction standards (JCPDS) file no: 04-0783. The appearance of some additional peaks in this XRD spectrum may be due to the association of supernatant protein molecules with synthesized AgNPs [24, 48].

The distribution of particle size in aqueous solution was determined by DLS shown in Fig. 3(a). The DLS histogram shows maximum particles having the size of 20 nm, is in good agreement with TEM studies. The TEM image of synthesized AgNPs shown in Fig. 3(b) revealed that particles are spherical in shape and size range from 12-25 nm.

![Fig. 2](a) FTIR spectrum of AgNPs and (b) XRD pattern of biosynthesized AgNPs, asterisks indicate some unassigned peaks may be due to the association of supernatant protein molecules with AgNPs.

![Fig. 3](a) DLS result showing mean particles size in aqueous medium and (b) TEM micrograph of purified AgNPs.
Fig. 4(a) depicts the FE-SEM image of AgNPs, is in close agreement with TEM data. The elemental analyses of nanoparticles were carried out by EDX spectroscopy along with FE-SEM. The EDX spectrum showed a strong signal at 3 keV Fig. 4(b), which is typically for the absorption of metallic silver nanocrystals due to SPR [49]. Synthesized particles were further studied by AFM with intermittent mode to understand their exact configuration. Fig. 5(a-b) depicts the 2D and 3D image of the sample spin coated on the glass substrate. The particles are spherical in shape with almost uniform distribution. The agglomerations of the particles are observed on the surface due to multilayer coating of the particle leading to formation of a few huge clusters on the surface.

Isolation and identification of ESBL producing bacteria

The ESBL positive isolates of *E. coli* MK26, *Enterobacter* sp. NK8 and *Klebsiella pneumoniae* OK4 were isolated from river Yamuna, India. Above bacterial isolates were identified by 16S rRNA gene sequences analysis and sequences data have been submitted to NCBI with accession nos. KU958497, KU985168 and KU985496. All the isolates were found positive for preliminary and PDCT test as per CLSI guidelines. Further, PCR amplification was carried out to find out the presence of ESBL genes in the isolates. The successful PCR amplification confirmed that isolate *Enterobacter* sp. NK8 harbored both blaTEM and blaCTX-M genes, whereas *E. coli* MK26 and *Klebsiella pneumoniae* OK4 carrying only blaTEM and blaCTX-M type of resistant determinant respectively. Presence of ESBL producing bacteria in natural habitats of river Yamuna has been also reported by Azam et al., 2016 [50]. The increase prevalence of ESBL producing bacterial in aquatic resource suggests the impact of human activity in spread of resistance from industrial, agricultural and waste water discharged from municipal and clinical environments.

Antibiotic profiling and antibacterial activity of AgNPs

Antibiotic profiling data of ESBL positive isolates were analyzed as per CLSI guidelines. All the isolates showed resistance toward tested antibiotics AMP, RIF, TR and first generation Cephalosporins (CZ) and considered as multidrug resistant (MDR). The highest level of resistance was observed in NK8 isolates for six different antibiotics. All the isolates showed susceptibility towards AK, C, CL and Carbapenem (IPM). The high level of resistance against commonly used antibiotics among ESBLs producing bacteria due to the presence of blaTEM and blaCTX-M type of resistant determinant. The acquisition and transmission of resistance genes from aquatic microflora to clinically important pathogens create human pathogens with novel drug resistant mechanisms.
Antibacterial activity of synthesized AgNPs was tested against ESBL producing isolates by well diffusion method Table 1. The clear zone of inhibition (ZOI) across the well was observed in all ESBL producing isolates Fig. 6. There was no antibacterial activity observed for culture supernatant used as negative control. Minimum inhibitory concentration (MIC) was determined as lowest concentration of nanoparticles responsible for inhibiting the growth of tested isolates, varied from 5.6-6.3 µg/ml Table 1. Synthesized AgNPs showed approximately 70 % toxicity against tested isolates after 5 h of treatment. Hence, this antibacterial report demonstrated that biosynthesized AgNPs have promising antibacterial efficacy against ESBL producing bacteria. The antimicrobial property of silver nanoparticles has been well known but their exact modes of action are yet to be established. The proposed hypothesis state that AgNPs could get attached to bacterial cell wall, cell membrane and disrupt them. Silver nanoparticles also penetrate inside the bacteria and bind with sulfur containing proteins, DNA and leading to cell death [3].

Table 1 Diameter zone of inhibition by AgNPs against ESBL producing bacterial isolates.

<table>
<thead>
<tr>
<th>ESBL producing bacteria</th>
<th>ZOI (mm) for different concentration of AgNPs</th>
<th>MIC (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>(-ve) control (+ve) control</td>
<td>10</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>KP 700603</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>E. coli MK26</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Enterobacter sp. NK8</td>
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<td>11</td>
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<tr>
<td>Klebsiella pneumoniae</td>
<td>0</td>
<td>10</td>
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<tr>
<td>OK4</td>
<td>0</td>
<td>12</td>
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Conclusion

In this paper we represent a clean, efficient and eco-friendly approach for biosynthesis of AgNPs using Shigella sp. AS8 isolate. To the best of our knowledge, biosynthesis of AgNPs using this isolate has not been reported so far. Bacterial culture supernatant biomolecules play major role for reduction of Ag⁺ to Ag⁰ and formation of AgNPs. This method produced metallic, crystalline and spherical nanoparticles with an average size of 20 nm within 180 min. Further, synthesized nanoparticles exhibit excellent antibacterial activity against all tested isolates. This result suggests that biosynthesized AgNPs may be used as a next lot of candidate to combat ESBL producing multidrug resistant pathogens.

References