Biosynthesis and characterization of silver nanoparticles by *Pseudomonas pseudoalcaligenes*

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Abstract

Due to toxicity of silver to humans, the biosynthesis of non-toxic nano-particles with profound antimicrobial activity is significant. In this study, we have biosynthesized the AgNPs from the left over water after washing ornaments of goldsmith shop and evaluated their antibacterial activity. The water sample collected from goldsmith ship was spread on LBA medium (LB Broth + Nutrient agar + distilled water) containing 3.5mM $AgNO_3$ to isolate the pure culture of AgNPs forming bacteria. The bacterial culture color change from yellow to brown indicates of bio-reduction of Ag ions to AgNPs. The AgNPs forming bacteria pure colony isolation and culture were done using standard microbiological technique. The culture was chemically characterized using UV-Vis and FTIR spectroscopy and molecularly by sequencing and BLAST program. The anti bacterial activity of AgNPs and $AgNO_3$ was observed against Staphylococcus aureus by agar well diffusion method.

On analysis of AgNPs forming brown colored culture, the observation of absorbance peak at 450 nm wavelength by UV-Vis spectra confirms the production of nanoparticles and the observation of interface between bio-organic functional groups and metal nanoparticles by FTIR confirms the AgNPs production. On further molecular analysis, AgNPs producing bacterial culture showed >99.9% similarity with Pseudomonas pseudoalcaligenes. The higher zone of inhibition has been observed with AgNPs in comparison to $AgNO_3$ against Staphylococcus aureus. In conclusion, the AgNPs synthesized by Pseudomonas pseudoalcaligenes have higher antibacterial activity than $AgNO_3$ and it may be due to higher surface area.

Keywords: Silver nitrate, silver nanoparticle, *Pseudomonas pseudoalcaligenes,* anti-bacterial activity.

Introduction

Antimicrobial activity of silver has been utilized extensively in clinical settings. However, it has limited medical application due to the toxic effect of silver salts (e.g. silver nitrate). In recent years, the problem of silver toxicity has been minimized by synthesizing the nano–particles (NP) which are non-toxic to humans and have profound antimicrobial activity[1]. The nanoparticles (1-100 nm) possess unique physicochemical, optical and biological properties which can be utilized for various desired applications. Silver nanoparticles (Ag-NPs) are of diverse shapes, sizes, films which make it suitable for various medical diagnosis and therapeutic applications. These Ag-NPs are being used for coating medical devices for infection-free transplantation and wound healing dressing[1,2].

The integration of nanoparticles with biological molecules was found to be helpful for development of diagnostic devices like contrast agents, quantum fluorescent biomarkers for cells labeling, tools in cancer therapeutics and broad spectrum of antibacterial agent. Moreover, studies have proved that AgNPs impose high antimicrobial activity as compared with bulk silver metal due to high specific surface area.

The various physical, chemical, photochemical and cryochemical methods employed for synthesis of NPs are environmentally hazardous, costly and inefficient[3]. However, the biogenic synthesis of NPs has emerged as a novel approach because of low cost, clean, non-toxic and eco-friendly nature. The biosynthesis of AgNPs can be carried out using bacteria, fungi and plant extract as bio-reducing agents[4,5]. Various studies have reported the biogenic synthesis of nanoparticles for Au, Ag, Pt, Pd, Cu and Ag (Silver) using bacteria[6]. NPs biosynthesis is advantageous because the bio-species itself can act as template, reducing and capping agent for NPs.

To exploit the advantageous antimicrobial properties of NPs, the present study has been planned to develop a novel AgNP which has been synthesized from the bacterial strain isolated from water used for cleaning the silver ornaments. This has allowed us to assess the impact of high surface area on AgNP anti-bacterial activity as compared to silver nitrate ($AgNO_3$) anti-bacterial activity which was evaluated on *Staphylococcus aureus*.

Material and Methods

Sample collection: The water sample left after cleaning silver ornaments was collected from a local goldsmith shop. The water sample was stored at 4°C and used for biosynthesis of AgNPs.

Isolation of bacterial culture: The water sample was spread on LBA medium (LB Broth + Nutrient agar + distilled water) containing 3.5mM $AgNO_3$ on Petri plates and incubated at

30°C for 72 hours. The Petri plates were monitored for the change of color of media from yellow to brown which indicates bio-reduction of Ag ions to AgNPs. A loop full of inoculum of bacterial colony from the brown turned area was streaked on another plate containing LBA medium + $AgNO_3$. Isolated colonies were streaked to isolate pure cultures on LBA medium + $AgNO_3$ plate.

Production of wet biomass and AgNPs: Loops full of pure colonies were inoculated in test tubes containing 3ml of LB broth + $AgNO_3$ solution along with separate control tube (without bacterial inoculum) and incubated at 30°C for 72 hours. Test tubes were monitored for color change of broth from yellow to brown. The culture in these test tubes was inoculated in 50 ml of LB broth + $AgNO_3$ solution along with separate control flask (without bacterial inoculum) and incubated at 30°C for 7 days. The change in color of media from yellow to brown indicates the AgNP production. Then the flasks were stored at 4°C for further use in the investigation.

Molecular Identification: The pure bacterial culture was prepared by transferring a loop of single bacterial colony from plate to nutrient broth and incubated at 37°C for 24 hours in shaker (Orbitek). Routine CTAB method was employed to isolate total genomic DNA. Isolated DNA was analyzed for quantity and quality by agarose gel electrophoresis. The genomic DNA was used as template for PCR amplification of 16S rRNA gene using universal 16S primers for 30 cycles. PCR amplicons were analysed on 1.5% agarose gel and sequenced. The sequencing data obtained was analyzed using BLAST program.

UV-Vis spectral analysis: This media sample containing synthesized AgNPs was analyzed for optical characteristics using UV-Vis spectral analysis to confirm AgNP synthesis. Absorption spectra of seven days incubated culture was recorded using UV-Vis spectrophotometer within a range of 200-700nm and analyzed to check characteristic absorption peaks.

Fourier Transformation Infrared Spectroscopic (FTIR) analysis: The synthesized AgNPs were further characterized by FTIR technique. The FTIR spectrum of sample was recorded on FTIR instrument in the mid IR region of range 400-4000cm^{-1}. The sample was directly absorbed on KBr crystal and transmittance spectrum was recorded**.** Spectra provide information of different chemical bonds present on analysis of absorption peaks in different region.

Antibacterial activity of silver nanoparticles: Antibacterial activity of biogenic AgNPs against *Staphylococcus aureus* was evaluated by agar well diffusion method. 100µl of inoculum of the test bacterial culture of *Staphylococcus aureus* was spread on Luria Bertani (LB) medium solid agar plates and at the same time, two wells were punched in the plates. In the one well, culture having AgNPs was poured and in other LB broth + $AgNO_3$ was poured as control and incubated overnight. The zone of inhibition developed was observed, analyzed and compared against zone in control.

Results

Production of wet biomass and biosynthesis of silver nanoparticles: The left over water sample of goldsmith shop has been found to be positive for presence of AgNPs forming bacteria. The pure culture of AgNP forming bacteria has been isolated and wet biomass was produced using standard microbiological techniques (figure 1, 2). The wet biomass of the AgNP forming bacteria has been taken for characterization and to investigate the antibacterial activity against *Staphylococcus aureus*.

Characterization of AgNPs: Change of media color from yellow to brown primarily affirms the synthesis of AgNPs but UV-Vis spectral analysis provides the solid confirmation. Absorption spectrum of the synthesized AgNPs was recorded in range 200-700nm wavelength and the characteristic peak at wavelength 450nm in this study confirms the synthesis of nano-particles.

Synthesized AgNPs were also subjected for Fourier Transform Infrared (FTIR) spectroscopy for further characterization and the spectrum was recorded in the mid IR region of 400–4000 cm^{-1}.

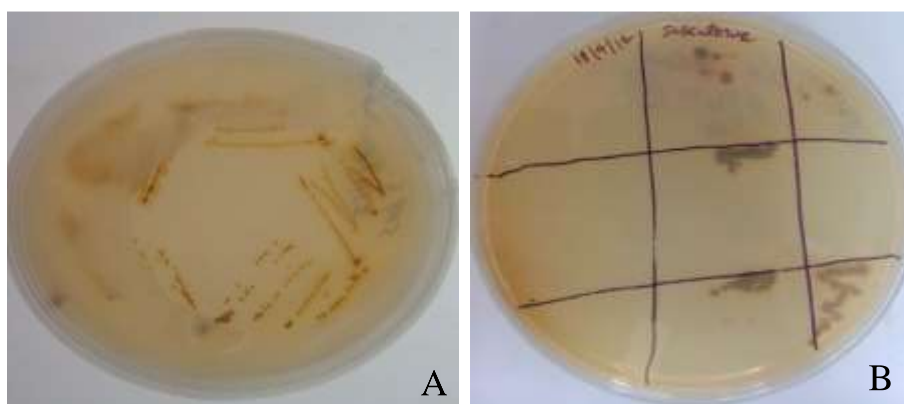

Figure 1: A Streaking to isolate single pure colonies of bacteria, B Isolation of pure cultures of AgNPs forming bacteria

Figure 2: Biomass synthesis in 50ml culture medium after 7 days of incubation. The flask C represent the control without inoculums showing no color change, flask 1-5 containing 50ml culture with inoculum showing change in color occurred from yellow to brown

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Pseudomonas pseudoalcaligenes strain CMBT/MDU/3001 16S ribosomal RNA gene, partial sequence	1439	1439	100%	0.0	100.00%	KM252673.1
☑	Pseudomonas oleovorans JCM 13980 gene for 16S rRNA, partial sequence	1428	1428	100%	0.0	99.74%	LC508006.1
☑	Pseudomonas oleovorans JCM 13974 gene for 16S rRNA, partial sequence	1428	1428	100%	0.0	99.74%	LC508001.1
☑	Pseudomonas oleovorans JCM 13973 gene for 16S rRNA, partial sequence	1428	1428	100%	0.0	99.74%	LC508000.1
☑	Pseudomonas oleovorans subsp. oleovorans JCM 11598 gene for 16S ribosomal RNA, partial sequence	1428	1428	100%	0.0	99.74%	LC507444.1
☑	Pseudomonas sp. phDV1 chromosome, complete genome	1428	5670	100%	0.0	99.74%	CP031606.1
☑	Pseudomonas oleovorans subsp. oleovorans JCM 5968 gene for 16S ribosomal RNA, partial sequence	1428	1428	100%	0.0	99.74%	LC462171.1
☑	Pseudomonas sp. IR46 16S ribosomal RNA gene, partial sequence	1428	1428	100%	0.0	99.74%	KJ396823.1
☑	Pseudomonas pseudoalcaligenes genome assembly Ppseudo_Pac, chromosome : I	1428	5703	100%	0.0	99.74%	LK391695.1
☑	Uncultured bacterium clone nbw117a03c1 16S ribosomal RNA gene, partial sequence	1428	1428	100%	0.0	99.74%	KF064855.1

Figure 3: The blast analysis of the bacterial strain isolated and sequenced and submitted with GenBank accession no. KM252673. Thee sequences on BLAST showed > 99.9 % of similarity with *Pseudomonas pseudoalcaligenes / oleovorans.* The *Pseudomonas oleovorans* has been described first and, later due to almost similar molecular profile and DNA-DNA relatedness the *Pseudomonas oleovorns* united to *Pseudomonas pseudoalcaligenes*

Absorption peaks were recorded approximately at 3451, 2952, 2854, 1637, 1409, 1351, 1272, 1096, 681, 488, 458 and 431 cm^{-1}. The peak at 3451.45 was assigned to the stretching vibrations of hydroxyl group. The peaks at 1637.48, 1409.32, 1272.62 cm^{-1} correspond to C=O, C-N stretching vibrations of aromatic and aliphatic amines respectively.

One peak was observed at 1096.82 cm^{-1} assigned to the (C=O) groups. In addition to this, peak at 681.53 cm^{-1} corresponds to metal binding carboxylic (M↔C≡O) groups.

Molecular identification: The 16S rRNA sequence of bacterial isolates was amplified and sequenced. The sequence was submitted to NCBI (Accession number - KM252673). The bacterial isolate has been found almost similar (> 99%) to *Pseudomonas pseudoalcaligenes* or *oleovorans* on BLAST analysis (Figure 3).

Antibacterial activity of AgNP: The larger diameter of the zone of inhibition developed by AgNP on the agar plates against *Staphylococcus aureus* in comparison to control silver nitrate confirms the better antibacterial activity of AgNP than the silver nitrate (figure 4).

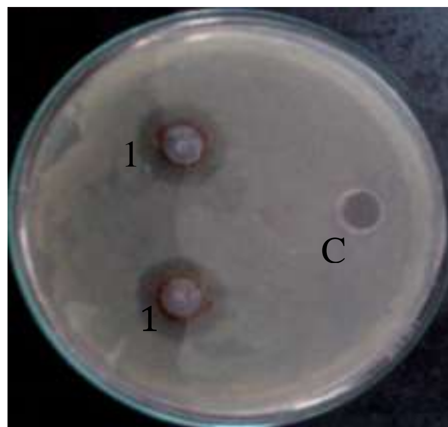

Figure 4: The control has been represented as "C" showing the zone of inhibition of silver nitrate ($AgNO_3$) while the zone of inhibition of AgNPs represented as 1. Higher antibacterial activity of AgNPs has been observed in comparison to $AgNO_3$ against test bacteria *Staphylococcus aureus*

Discussion

Use of microbe as bio-reductant for synthesis of AgNPs is a novel and efficient approach being extensively used these days. In this study, the biosynthesis of AgNPs has been carried out using bacterial isolates as bio-reductant. The bacteria were isolated from water collected from goldsmith shop. Various reports have been published on the biogenic synthesis of the AgNPs by using bacteria. One of reports demonstrated the synthesis of AgNPs by using bacterial isolated from marine water samples collected from Nellore marine area in which the pure cultures of potential bacterial strains were isolated on Zobell marine agar plates[7].

However, in the present study the pure cultures of bacteria were isolated from the water samples on LB-agar plates having silver nitrate as substrate for AgNPs synthesis without any need of special media to isolate pure cultures. The color change of media from yellow to brown provides preliminary signal for synthesis of the AgNPs. The color change is occurring due to the excitation of surface plasmon vibration in the AgNPs. The observation of color change has been widely used for screening microbial isolates for AgNP synthesis[8,9].

UV–Vis spectroscopy is an effective technique for the analysis of nanoparticles [10]. An absorption peak at 450nm observed in this study under UV-Vis spectra provides confirmation for the synthesis of AgNPs as reported elsewhere[7,11]. The AgNPs on FTIR spectroscopy further confirmed that nanoparticles are bound to the functional organic groups (carboxyl and amine) from the bacterial content of protein.These functional groups may act template, reducing and capping of nanocrystals. The interface between bio-organic functional groups and metal nanoparticles indicates the biosynthesis of AgNPs.

In this study, the higher antibacterial activity of synthesized AgNPs has been observed in comparison to $AgNO_3$ as demonstrated by well diffusion method on *Staphylococcus aureus* plate. Some other studies have also reported the higher antimicrobial activity of AgNPs than $AgNO_3$ on pathogens like *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228 and *E. coli* ATCC 8739 [12,13].

Another study demonstrated the antimicrobial activity of AgNPs in combination with various antibiotics in the order AgNPs + antibiotics > AgNPs > antibiotics[14]. Our results are in accordance of the same and showed better antimicrobial activity of AgNPs than silver nitrate. The high antibacterial activity may be due to high surface area which has also been demonstrated earlier[15]. The phylogenetic analysis of 16S rRNA gene sequence using BLAST analysis revealed > 99% similarity of our bacterial isolate with the *Pseudomonas pseudoalcaligenes* (Accession number - KM252673) suggesting that the AgNPs have been synthesized by the *Pseudomonas pseudoalcaligenes*.

Conclusion

In conclusion, the current investigation demonstrates the biosynthesis of AgNPs by *Pseudomonas pseudoalcaligenes* isolated from the leftover water of goldsmith shops used for washing silver ornaments have better antibacterial activity than $AgNO_3$ which may be because of higher surface area.

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