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Lactobacillus Mediated Synthesis of Silver Oxide Nanoparticles

Regular Paper

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Abstract The ability of prokaryotic microorganisms to reduce the inorganic metals has opened up an exciting eco-friendly approach towards the development of natural 'nano-factories'. However, a number of issues have to be addressed from the nanotechnological and microbiological point of view before such a biosynthesis approach can compete with the existing physical and chemical methods. This report investigates the synthesis of silver oxide nanoparticles using *Lactobacillus mindensis*, isolated using fixer solution from an X-ray photographic laboratory. Nanoparticles obtained were characterized by means of UV-vis spectroscopy, transmission electron microscopy (TEM), and X-ray diffraction (XRD). The UV-vis spectrum shows the absorbance maximum at 430 nm, which is a characteristic of surface plasmon resonance of silver. Further, the presence of stable nanoparticles in the range of 2-20 nm was determined using TEM analysis. Silver nanoparticles in the form of silver oxide were confirmed in the XRD study. In conclusion, *Lactobacillus mindensis* serves as a promising candidate in the quest to synthesize silver oxide nanoparticles through green chemistry.

Keywords Silver Oxide Nanoparticles, *Lactobacillus*, Green Chemistry

1. Introduction

Nanotechnology involves the study of structures at 1-100 nanometres (nm) which possess novel properties and functions attributable to their small size [1]. These nanomaterials may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine and water treatment [1-3].

The synthesis of silver nanoparticles involves reduction of silver ions (Ag^+) in aqueous solution, yielding colloidal silver with particle diameter of several nanometres [4]. Initially, the reduction of various complexes with Ag^+ ions leads to the formation of silver atoms Ag(0), which is followed by agglomeration into oligomeric clusters. These clusters eventually lead to the formation of colloidal Ag particles. When the colloidal particles are much smaller than the wavelength of visible light, the solutions have a yellow colour with an intense band in the 380–400 nm range and other less intense or smaller bands at a longer wavelength in the absorption spectrum. This band is attributed to collective excitation of the electron gas in the particles, with a periodic change in electron density at the surface (surface plasmon absorption) [5].

Various physical and chemical methods to synthesize silver nanoparticles include laser ablation [6], photoinduction [7], electrochemical [8], ultrasonic-assisted [9], chemical reduction [10], microwave assisted [11] and solvothermal methods [12]. However, most of the techniques are capital intensive as well as inefficient in material and energy use. Hence, there is a growing need to adopt the principles of green chemistry to develop an environmentally benevolent biological approach.

Recently, the use of biologically mediated silver nanoparticles using green algae, fungi [13-15], bacteria [16], actinomycetes [17], and plant extracts [17,18] is gaining impetus due to their diverse properties like catalysis, optical polarizability, electrical conductivity, antimicrobial activity and Surface Enhanced Raman Scattering (SERS) [18-20]. These intrinsic properties of a metal nanoparticle are mainly determined by its size, shape, composition, crystallinity and structure. Silver oxide nanoparticles are a very interesting class of metal oxides; silver being a multivalent, it forms various phases like Ag_2O, AgO, Ag_3O_4 and Ag_2O_3 by interacting with oxygen [21]. Experimentally it is found that Ag_2O and AgO are the most observable phases. It has also been reported that they decompose at less than 250°C [22,23]. Their thin films show semiconducting behaviour [24]. Photo-activation of silver oxide leads to nano silver clusters; these nano clusters emit fluorescence and also exhibit plasmonic behaviour responsible for surface enhanced Raman scattering (SERS). These properties have a wide application range from ultra high density optical memories to single molecule detection [25,26]. Ag_2O being a versatile material, it has found application in oxidation catalyst, sensors, fuel cells, optical data storage systems, etcetera [27]. The present study investigates biological synthesis of silver oxide nanoparticles using *Lactobacillus mindensis* isolated from fixer solution and presents an insight on the possible mechanism involved in the synthesis. Further, it corroborates previous studies involving use of *Lactobacillus* in synthesis of silver nanoparticles.

2. Material and Methods

2.1 Chemicals

Silver nitrate ($AgNO_3$), peptone, yeast extract, beef extract, and agar were purchased from HiMedia laboratories and used as received without further treatment. All the solutions were freshly made, whereas all the microbiological media were steam sterilized by autoclaving at 15 psi at 121°C for 15 min. Nutrient broth (peptone 5 g/l, yeast extract 1.5 g/l and beef extract 1.5 g/l) of pH 7.2 was prepared without sodium chloride (unless otherwise mentioned).

2.2 Isolation of silver resistant bacteria

Isolation of silver resistant bacteria Fixer solution was collected from X-ray photographic laboratories in five sterile 10 ml syringes at various depths of the container. The sample was processed with two consecutive enrichments in nutrient broth, both containing 0.5 mM AgNO3 at 25°C for 48 hrs under static conditions. Aliquots were removed, serially diluted and spread on nutrient agar plates containing 0.5 mM AgNO3. Incubation was carried out at 25°C for 24 hrs in dark conditions. 2.3 Characterization of the isolate

The morphological and physiological characterization of the silver resistant isolate was performed according to the guidelines described in Bergey's Manual of Systematic Bacteriology. Further, PCR amplification and sequencing of 16S rRNA genes was carried out to substantiate the result. PCR amplification of 16S rDNA from cell lysates of the strains was performed using 16S rDNA specific universal oligonucleotide primers 16F27N (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 16R1488 (5′-CGG TTA CCT TGT TAC GAC TTC ACC-3′) hybridizing respectively at positions 8–27 and 1488–1511 relative to *E. coli* 16s rDNA numbering. The PCR reactions for all strains were carried out in a PE 9700 thermal cycler (Perkin Elmer, USA). The amplification conditions were: an initial denaturation at 94°C for two minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute, and final extension at 72°C for 10 minutes. PCR was carried out in 25 µl reaction mixture consisting of 10 x Taq polymerase buffer (New England Biolabs), 2 mM dNTPs, 10 pM primers, 1 unit Taq polymerase (New England Biolabs), and 10 ng DNA. The PCR amplification products were checked on 1% wt/vol agarose gels. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, HildeGermany) following the manufacturer's protocol. Purified PCR products were sequenced on both strands on an AB 3730 DNA analyser using the Big Dye terminator kit (Applied Biosystems, Inc.Foster City, CA). Sequences were analysed at the National Center for Biotechnology Information (Bethesda MD) (http//: www.nbi.nlm.nih.gov/BLAST) for closed homology using a BLASTn algorithm.

2.4 Biosynthesis of silver oxide nanoparticles

For inoculum development, the isolate was inoculated in 200 ml of sterile nutrient broth (pH 7.2) for 24 hrs at 25°C on an Orbitek shaker (1200 rpm). On the following day, the cells were separated and the wet biomass obtained was added to 200 ml of half-strength sterile nutrient broth (pH 8.0) containing 0.5 mM $AgNO_3$. Wet biomass was added until the broth reached final optical density of 0.55 units at 540 nm. The reaction mixture was incubated at

25°C in dark conditions (to avoid photochemical reduction) and was routinely monitored for visual colour change as well as periodic sampling of aliquots (2 ml) of the reaction mixture being subjected to UV–vis spectroscopy analysis. In parallel, a blank and control were examined. Blank consisted of 200 ml of half-strength sterile nutrient broth (pH 8.0) containing 0.5 mM $AgNO_3$, whereas control consisted of 200 ml of half-strength sterile nutrient broth (pH 8.0) containing 0.5 mM $AgNO_3$, to which heat-killed cells were added until the same optical density was attained in both test and control.

2.5 UV-vis spectral analysis

All the UV-vis spectroscopy measurements were performed on a 'Genesis-8', spectrophotometer operated at ambient temperature in solution form. The samples were analysed from 200 to 500 nm.

2.6 Transmission electron microscopy

Estimation of the size, shape and state of assembly of the metal nanoparticles was done using transmission electron microscopy (TEM). The sample was prepared by placing a drop of the nanoparticle solution onto a carbon-coated copper TEM grid. The sample was then dried under an infrared lamp for a period of 45 min. TEM measurements were performed on a 'Philips CM200' instrument operated at an accelerating voltage of 120 kV. The electron diffraction pattern was also recorded for the selected area. The images were analysed using Image J software and average particle size of nanoparticles was calculated. (http://rsbweb.nih.gov/ij/download.html)

2.7 X-ray diffraction

The powder obtained after drying of nanoparticles containing solution at 60°C was subjected to X-ray diffraction study. X-ray diffraction analysis was carried out using Philips X'Pert. Powder X-ray diffraction technique was used to determine phase purity, crystal structure and to roughly estimate the size of the nanocrystallites. X-ray diffraction patterns were obtained with a diffraction mode instrument using monochromatic high intensity CuK_α radiation (Lambda λ=1.54060 A^0). The average crystallite size of the nanoparticles was calculated from the XRD pattern using the Deby-Seherrer formula.

3. Results and Discussion

Silver resistant bacterial species obtained from fixer solution of an X-ray photographic laboratory were screened for the ability to produce silver nanoparticles. The isolate which gave an early onset of silver nanoparticles was identified to be of *Lactobacillus* sp., based on the cultural and biochemical tests as described in Bergey's Manual of Systematic Bacteriology [28]. Further, 16S rRNA sequencing confirms it to be *Lactobacillus mindensis* [29].

The production of silver nanoparticles was achieved by challenging aqueous silver ions with biomass of *Lactobacillus mindensis*, after incubation of five days in dark conditions. This was indicated by the visual change in colour from yellow to brown [30-32]. Control experiments without the addition of biomass (blank) and killed cells (control) showed no formation of brown colour, indicating that the colour change is due to live biomass (Figure 1).

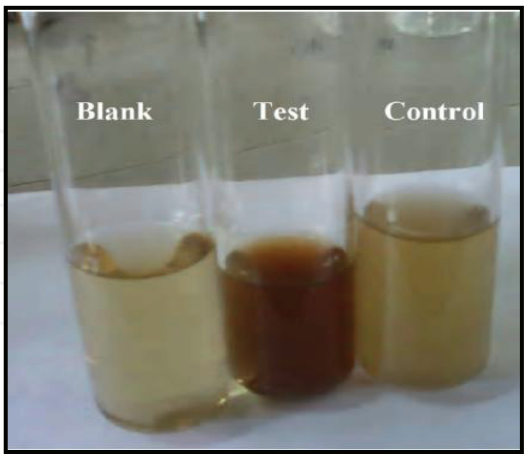

Figure 1. Biosynthesis of silver nanoparticles observation. Test: Live cells after exposure to $AgNO_3$ solution in nutrient broth (brown colour); Blank: $AgNO_3$ in nutrient broth without cells (no colour change); Control: Heat-killed cells after exposure to $AgNO_3$ solution in nutrient broth (no colour change).

Preliminary characterization was carried out using UV-vis spectrophotometer. UV-vis spectra (Figure 2) obtained after treating 0.5 mM $AgNO_3$ with a pellet of *Lactobacillus mindensis* showed a strong peak at 430 nm, which is characteristic for surface plasmon resonance of silver.

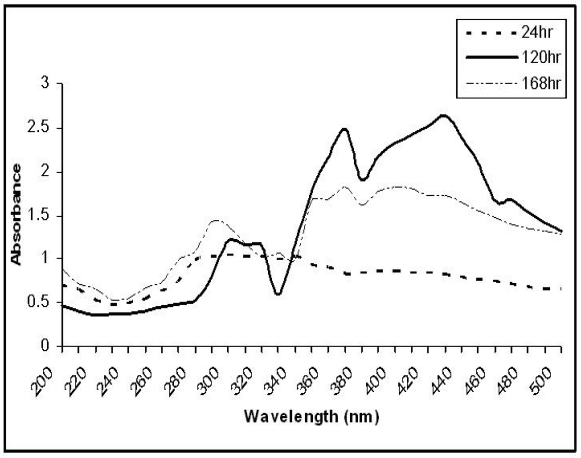

Figure 2. UV-vis spectra recorded as a function of time of reaction of biomass with 0.5 mM $AgNO_3$. The maximum absorbance obtained was at 430 nm, while an additional peak at 370 nm was due to out-of-plane quadrupole plasmon resonance.

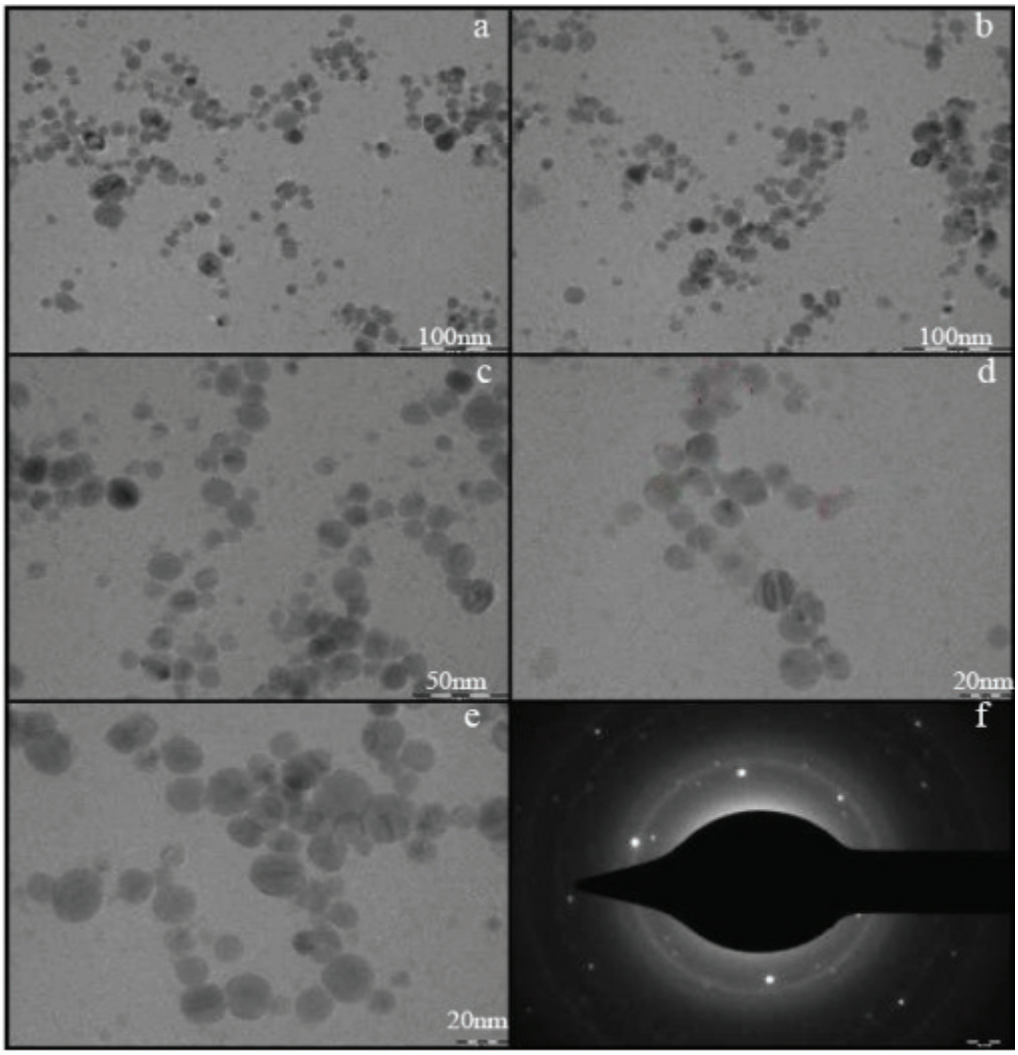

Figure 3. TEM images of silver nanoparticles synthesized by *Lactobacillus mindensis* [low resolution images (a-c) and high resolution images (d-e). Selected area electron diffraction pattern (f)]

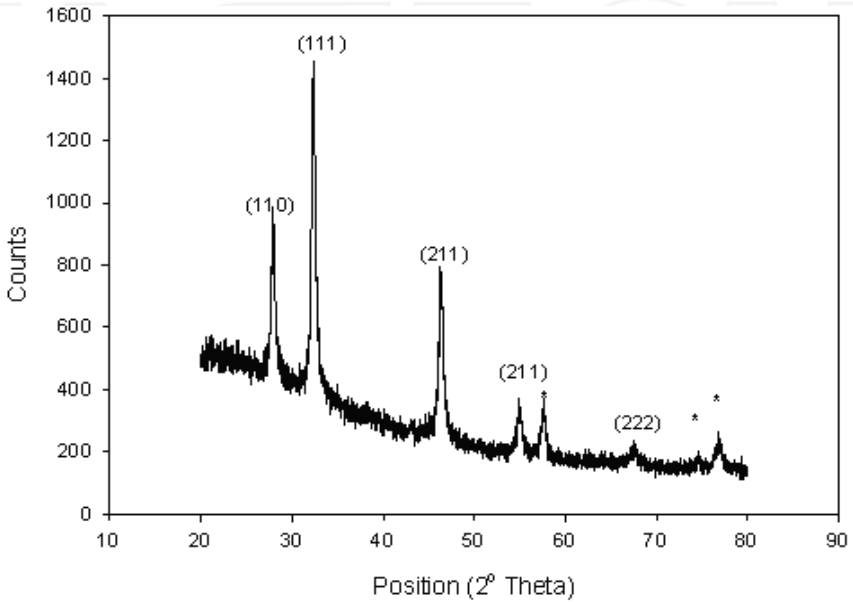

Figure 4. X- ray diffraction pattern of silver oxide nanoparticles. The samples were harvested on the fifth day. Cell filtrate was heated at 60°C to obtain powder and the X-ray diffraction pattern was observed

The results obtained are in accordance with previous studies of biologically synthesized silver nanoparticles using *Bacillus licheniformis* [32], *Aspergillus flavus* [33], *Bacillus subtilis* [34] and *Aeromonas* sp. SH10 [35], where the absorbance peaks were in the range of 400 to 440nm. The position and shape of the surface plasmon absorption of noble metal nanoclusters are strongly dependent on the particle size, dielectric medium and surface adsorbed species, and may shift due to various factors during the production process [36].

According to Mie's theory, small spherical nanocrystals should exhibit a single surface plasmon band, whereas anisotropic particles should exhibit two or three bands, depending on their shape. Absorption spectra of larger metal colloidal dispersions can exhibit broad or additional bands in the UV-vis range due to excitation of plasmon resonances or quadrupole and higher multiple plasmon excitation [36]. The UV-vis spectra from this work showed a distinct quadrupole plasmon resonance of silver nanoparticles at 370 nm. The peak at 370 nm may be due to out-of-plane quadrupole resonance for the silver nanoparticles, reflecting a blue shift.

As the time elapses (seventh day of incubation), there is decrease in the surface plasmon absorption of silver nanoparticles (Figure 2), indicating simultaneous build-up of oxidized species of silver in the solution, visible in the form of greyish-black precipitate. Larger aggregrates were avoided by centrifuging the culture filtrate followed by heating in a boiling water bath at 100°C for 10 mins. The solution was then cooled and preserved in dark conditions. This solution was found to be stable for a period of two months.

2 theta	FWHM (dθ)	Average particle (nm)	
		XRD	TEM
27.94	0.3011	42.8 ± 3.9	32.5 ± 2.8
32.27	0.1836		
46.34	0.2676		
54.92	0.3346		
67.48	0.4684		

Table 1. Particle sizes of silver oxide nanoparticles from X-ray diffraction and TEM measurements. Standard Error (SE) for particle size in TEM and XRD is 2.79 and 3.85 respectively.

The TEM images show some variability in shape and size as well as slight signs of aggregation (Figures 3a-e). The morphology of nanoparticles is variable, spherical form being dominant with respect to the few triangular particles also present. In the higher magnification image [Figures 3d and 3e], biogenic nanoparticles of 2-20 nm size were also observed. However, the average size of the nanoparticles was found to be 32.5 nm. This small size may be due to different cell growth and metal incubation conditions. The selected area electron diffraction pattern [Figure 3f] confirms the plane (111) of silver oxide nanoparticles and thus the nanoparticles formed are crystalline in nature. These results compare well with the previous observations reported in the studies of silver nanoparticles on *Fusarium oxysporum* [37] and *Lactobacillus fermentum* [41].

The X-ray diffraction (Figure 4) shows two intense peaks at 27.94 and 32.27, which corresponds to (110) and (111) of Ag_2O. Apart from this, diffraction peaks at 46.34, 54.92 and 67.48 can be indexed to (211), (220) and (222) planes of face-centre cubic silver, respectively. These peaks corroborate with the standard Ag_2O (JCPDS 76-1393) [38-39]. Three unknown peaks, 57.58, 74.62 and 76.86, were also observed, which may correspond to bacterial pellet.

The discrepancy regarding the difference in the diameter between the value determined from the XRD patterns and the one from TEM observations is unavoidable, as it is associated with the limitations coming from the use of Deby-Seherrer formula, which is applicable to particles of near-spherical shape.

Previous observations show that *Lactobacillus* is known to produce nitrate reductase only above pH 6 [40], which may be responsible for bioreduction of Ag^+ to Ag^0 and the subsequent formation of silver nanoparticles. The exact mechanism resulting in the formation of silver oxide nanoparticles by *Lactobacillus* sp. has not been reported. There is a possibility that the aldehydic group present in the extra polysaccharides secreted by the *Lactobacillus* sp might be involved in the reduction of silver ions to zerovalent silver [Ag(0)] [41,42]. This zerovalent form of silver was converted into silver oxide by an unknown oxidizing agent secreted by *Lactobacillus mindensis*. Further investigation is required in this direction to prove the exact mechanism involved in the synthesis of silver oxide nanoparticles.

4. Conclusion

Silver oxide nanoparticles in the range of 2-20 nm were synthesized using *Lactobacillus mindensis*, which was isolated using fixer solution from the X-ray photographic laboratory. The process used nontoxic chemicals, environmentally benign solvents and renewable materials, thus following the principles of green chemistry. In future, it would be important to design an approach involving rapid synthesis of stable, monodisperse silver oxide nanoparticles mediated through biological species, so as to compete with existing physical and chemical methods. Photo-activation study in relation to surface enhanced Raman scattering (SERS) can also be investigated in future.

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