



SYNTHESIS, CHARACTERIZATION AND ANTI MICROBIAL STUDIES OF AgNP'S USING PROBIOTICS

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ABSTRACT

Recent researches speak about the utilization of biological systems especially enterobacteria to synthesize nanoparticles is a novel approach. The present study demonstrates an eco-friendly and low cost protocol for synthesis of silver nano particles using the cell free filtrate of *Lactobacillus bulgaricus* when supplied with aqueous silver ions. Characterization of silver nanoparticles was done by UV-Visible absorption spectroscopy shows maximum absorption at 420 nm, Transmission electron microscope (TEM) revealed the formation of spherical nanoparticles with size ranging between 40 – 50nm, X-Ray diffraction (XRD) pattern shows spectrum, which reveals the FCC structure of silver nanoparticles. Energy dispersive spectroscopy (EDS) analysis was done using SEM instrument equipped with thermo EDS attachment shows the optical absorption peak at 3keV. Microbial nanoparticles showed very strong inhibitory action against *Staphylococcus aureus*, *Staphylococcus albus*, & *Pseudomonas aeruginosa*.

Key words: *Lactobacillus bulgaricus*, Probiotic, Enterobacteria, TEM, SEM, EDS.

INTRODUCTION

A significant national challenge for Indian scientists is achieving fundamental ingredients in establishing a healthy, happy and peaceful life not only to Indians but also to the people of the world. In order to achieve prosperity and all-inclusive growth of people it is an imperative necessity to provide safe and ecofriendly drugs to avoid all pervading perils caused by microbial.

In the scenario a fresh rose is "Nanotechnology" within a short time after its birth it has embraced all other disciplines of science and more so life sciences. Applications of nanotechnology in the biomedical area are varied and distinctive. Nanoparticles exhibit highborn structural integrity as well as diversified properties. This uniqueness is derived from their specific characters like size, distribution, structure etc¹... The fast expanding field of nanotechnology provides a great impetus to research in varied fields like biological sciences, agricultural and manufacture of consumer goods².

Extensive and multiangular studies have been forging ahead towards identification of microorganisms as possible Nano factories^{3, 4}. The defensive mechanism for silver detoxification has been suggested as a biological pathway for synthesis of AgNP'S. Ahmed.et.al had shown that NADH-dependent enzymes are responsible for the synthesis of AgNP'S^{5, 6}. The reduction mechanism seems to be initiated by electron transfer from NADH by NADH – dependent reductase as electron carrier⁷.

Biomimetics is the study of application of biological principles for material formation⁸. The use of microbial is a novel method for synthesis of nanoparticles. Bacteria are known to produce inorganic materials either intracellular or extracellular⁹.

The development of resistant strains of bacteria is a big challenge for a scientist that requires evolving new and effective bactericides. Bacteria are generally classified as gram – negative or gram – positive^{10, 11}. Their structural difference lies in the organization of a key component of the membrane peptidoglycan. Gram – negative bacteria exhibit only a thin peptidoglycan layer (2-3nm) between the

cytoplasmic membrane and the outer membrane^{12, 13}. In contrast, gram – negative bacteria lack the outer membrane but have a peptidoglycan, layer of about 30nm thickness. Silver is known to exhibit a strong toxicity to a wide range of bactericidal applications^{14, 15}.

For the synthesis of AgNP'S, biological methods have an advantage over physical and chemical methods. In this paper, synthesis of AgNP'S using bacteria *Lactobacillus bulgaricus*, which is ecofriendly, and cost effective is discussed. It is so selected because a gastrointestinal bacterium, which is useful and is capable of synthesizing AgNP'S extracellularly. *L.bulgaricus* has been associated with number of beneficial reaction in the alimentary canal. The ability of this bacterial strain to digest lactose is particularly helpful for those who suffer from lactose intolerance. *L.bulgaricus* can help to restore normal intestinal flora and may be helpful in preventing food – borne diseases.

Here we report the synthesis of stabilized AgNP'S using a cell free aqueous filtrate from *Lactobacillus bulgaricus*, its characterization by UV – Visible absorption spectra, XRD, SEM, EDS, TEM. Further studies were also attempted an antibacterial activity of biosynthesized AgNP'S against gram – positive & gram – negative bacteria like *S.aureus*, *P. mirabilis* and *P.aeruginosa* and found to have very strong inhibitory action.

EXPERIMENTAL PROCEDURE AND MATERIALS

Bacteria: The bacteria used in this study, *Lactobacillus bulgaricus* that is ecofriendly probiotic isolated from milk and was characterized in the laboratory of food technology, Gitam Institute of Technology, Gitam University, Visakhapatnam, India

Preparation of bacterial extract: To prepare cell free bacterial extract, culture medium was prepared, sterilized and inoculated with fresh culture of the bacteria. The cultured flask was incubated at 37°C for 24hours. After incubation time the culture was filtered and filtrate was used for further experiments.

Synthesis of silver nanoparticles: Silver nitrate was used as precursor in the synthesis of silver nanoparticles. 200ml of bacterial filtrate was added to 300ml of 1mM AgNO₃ aqueous solution for reduction of Ag ions and kept at room temperature for 24 hours. The primary detection of synthesized silver nanoparticles was carried out in the reaction mixture by observing the color change of the medium from pale yellow to reddish brown & observed optical density. After 24 hours incubation the reaction mixture was filtered & the suspension was hot air dried in the oven to make powder for the optical measurements. The filtrate concentrated by repeated centrifugation at 10,000rpm for 10 minutes. The supernatant was replaced by distilled water each time and stored for antibacterial assays.

Characterization of silver nanoparticles

UV – Visible Spectroscopy Analysis: The color change in reaction mixture was recorded through visible observation. The bioreduction of silver ions in aqueous solution was monitored by periodic diluted sampling of aliquots and subsequently measuring UV – Vis spectra of the solution on UV – Vis spectrophotometer UV – 2450 (Shimadzu).

XRD measurement: The silver nanoparticle powder thus obtained was analyzed by XRD and SEM to know the structure and composition. The dried mixture of silver nanoparticles was collected for the determination of the formation of Ag nanoparticles by an Xpert pro x-ray diffractometer (PANalytical BV) operation at a voltage 40kv and a current of 30mA with cu k α radiation in a Θ - 2 Θ configuration. The crystalline domain size was calculated from the width of the XRD peaks, assuming that they are free from non – uniform strains, using the Scherer formula.

Table 1: Color Change

Time	Bacterial Extract + AgNO ₃
0 minutes	-
10 minutes	-
30 minutes	+
1 hour	+
2 hours	++
4 hours	++
8 hours	++
16 hours	+++
24 hours	+++

- No Color Change, + Color Change, ++ Tinge Brown, +++ Brown

$$D = 0.94 \lambda / \beta \cos \Theta$$

EDS Analysis: For Energy Dispersive Spectroscopy (EDS) samples were prepared on a carbon film by drop coating of silver nanoparticles. Elemental analysis on single particle was carried out using SEM instrument equipped with a thermo EDS attachment.

SEM analysis: The thin film of the sample was prepared on a small aluminum plate by just dropping a very small amount of the sample on the plate, extra solution were removed using a blotting paper and then the film on the plate was allowed to dry overnight. The SEM analysis was performed on a JEOL; model JSM – 6610LV instrument operated at an accelerating voltage of 20 keV.

TEM analysis: The transmission electron microscopy (TEM) analysis of extracellular synthesized silver nanoparticles were prepared by drop – coating silver nanoparticles solution on carbon – coated copper TEM grids (40 μ m * 40 μ m mesh size). Samples were dried and kept under vacuum in desiccators before loading them on to a specimen holder. TEM measurements were performed on a JEOL model 1200EX electron microscope operated at an accelerating voltage at 120kv.

Antibacterial efficacy of silver nanoparticles: The antibacterial assays were done on gram + ve & gram – ve bacteria like *S.aureus*, *S.albus*, *P.mirabilis* & *P.aeruginosa* by Mueller Hinton (MH) agar plate – method. Fresh overnight cultures are used for inoculating 5 μ L of AgNP'S and the zone of inhibition was measured after 24 – 28 hours.

Table 2: Zone of inhibition

Micro Organisms	Antibiotics Zone (nm)	AgNP'S Zone (nm)
<i>S.aureus</i>	11nm	17nm
<i>S.albus</i>	11nm	18nm
<i>P.mirabilis</i>	-	-
<i>P.aeruginosa</i>	13nm	18nm

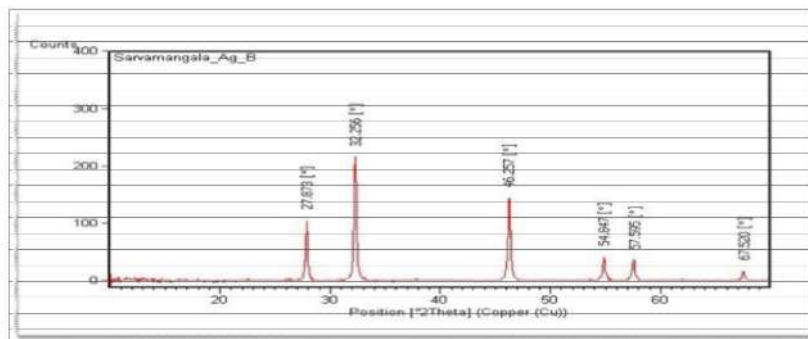


Figure 1: XRD pattern

XRD pattern shows intense peaks in the spectrum corresponding to different lattice planes

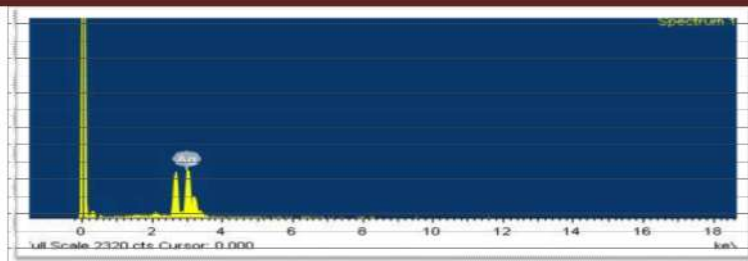


Figure 2: EDS Analysis
EDS analysis shows peak at 3keV that represents the presence of silver

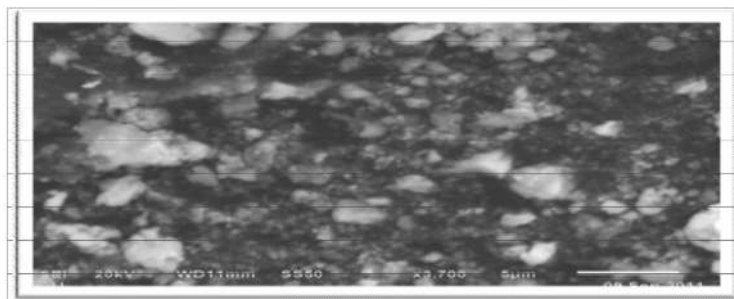
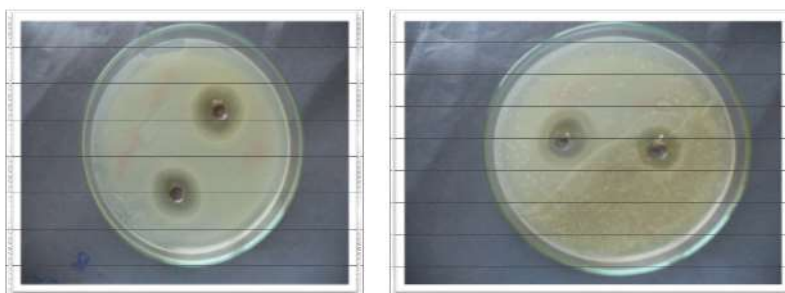


Figure 3: SEM Analysis
SEM analysis shows the individual and aggregated silver nanoparticles



Figure 4: TEM Analysis
TEM analysis shows spherical shaped individual nanoparticles ranging between 40nm to 50nm



Staphylococcus aureus

Pseudomonas aeruginosa

Figure 5: Antibacterial assay of Ag nanoparticles by using *Lacto Bacillus bulgaricus*
Antibacterial activities of Ag nanoparticles by using *Bacillus bulgaricus* extract with 5μl and 10μl of sample inoculating on MH agar plates showed Zone of Inhibition.

RESULTS AND DISCUSSION

The extra cellular synthesis of silver nanoparticles occurred during the exposure of *Lactobacillus bulgaricus* bacterial extract to 1mM aqueous silver nitrate solution. The complete reduction of silver ions was observed after 24 hours of reaction at 37°C under shaking condition. The color change in reaction mixture was observed during the incubation period, the appearance of color change from pale yellow to reddish brown is a clear indication of the formation of silver nanoparticles in the reaction mixture (Table 1). The color exhibited by metallic nanoparticles is due to the coherent

excitation of the entire free electrons within the conduction band, leading to Surface Plasmon Resonance (SPR).

UV – Visible spectroscopy analysis showed that the SPR absorption band of silver nanoparticles synthesized using *Lactobacillus bulgaricus* bacterial extract centered at 420nm. The frequency and width of the Surface Plasmon Absorption depends on the size and shape of the metal nanoparticles.

XRD pattern showed three intense peaks in the spectrum of 2θ value ranging from 40nm to 50nm average sizes of the particles. A Bragg reflection corresponding to the (111), (200), (220) sets of lattice planes are observed which may be

indexed based on the face – centered cubic (fcc) structure of silver (Figure 1).

EDS analysis shows additional evidence for the reduction of silver nanoparticles to elemental silver. The optical absorption peak is seen approximately at 3 keV, which is typical for the absorption of metallic silver nano crystals due to Surface Plasmon Resonance, which confirms the presence of nano crystalline elemental silver (Figure 2).

SEM images have shown individual silver particles as well as a number of aggregates. The morphology of the AgNP'S was predominantly spherical and aggregated in to larger irregular with no well – defined morphology observed in the micrograph (Figure 3).

The representation TEM picture shows individual silver nanoparticles as well as a number of aggregates, indicating stabilization of the nanoparticles by a capping agent. The separation between the silver nanoparticles seen in the TEM image could be due to capping by proteins. The picture shows clear, round silver nanoparticles ranging 40nm to 50nm (Figure 4).

Antibacterial assays: Silver nanoparticles exhibited antibacterial properties against bacterial pathogens with close attachment of the nanoparticles with the microbial cells. The antibacterial activity of the synthesized silver nanoparticles has been investigated against *S.aureus*, *S.albus*, *P.mirabilis* and *P.aeruginosa*.

Microbial nanoparticles showed very strong inhibitory action against *Staphylococcus aureus* with 17nm zone of inhibition, *Staphylococcus albus* with 18nm zone of inhibition and *Pseudomonas aeruginosa* with 18nm zone of inhibition (Table – 2). There is no zone of inhibition was observed with *Proteus mirabilis* (Figure 5).

CONCLUSION

The present work indicates that the enterobacteria are useful in the extracellular synthesis of silver nanoparticles. *Lactobacillus bulgaricus* is said to be a “probiotic” and called “friendly bacteria”. This particular bacterium has ability to affect the host animal beneficially by improving its intestinal microbial balance.

Extra cellular synthesis of highly stabilized silver nanoparticles by using *Lactobacillus bulgaricus* and the antibacterial effect of biosynthesized AgNP'S against gram +ve & gram –ve bacteria are reported. UV – Visible absorption spectroscopy, which shows an intense peak at 420nm, made the characterization of AgNP'S. XRD pattern confirms the FCC structure of AgNP'S. EDS analysis gives the optical absorption peak approximately at 3 keV. The SEM & TEM micrograph shown spherical shaped structures with size ranging between 40 nm to 50 nm. The process that is discussed in the present work for production of nanoparticles is environmental friendly because it is free from toxic chemicals; it is also low cost protocol. Use of harmless bacteria is synthesis of other cost effective metal nanoparticles is an exciting, neoteric & tremendous future possibility.

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