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Preparation and characterization of polymer-stabilized silver nanoparticles for antibacterial applications

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ABSTRACT

Silver nanoparticles (Ag-NPs) stabilized by a polysaccharide, chitosan, (chitosan stabilized Ag-NPs), (C-Ag-NPs) have been synthesized by the reduction of silver ions with $NaBH_4$ in aqueous solutions. These Ag NPs were very stable at room temperature. The chitosan -stabilized Ag NPs were characterized by UV–vis spectroscopy and transmission electron microscopy. The antibacterial efficiency of the nanoparticles was investigated by introducing the particles into nutrient agar media containing E. coli, B. mycoides and B. subtilis. It was found that the Ag-NPs exhibited antibacterial effect even at very low concentrations. It was evident that silver nanoparticles displayed an excellent inhibitory effect on both gram positive as well as gram negative bacteria. Hence it might prove to be an effective antimicrobial agent to combat not only Bacterial Blight (BB), but also other bacterial diseases of rice.

Key words: *silver, chitosan, nanoparticles, antibacterial activity, Bacillus subtils, E.coli*

Recent advances in materials science and chemistry have produced mastery in nanoparticle technology, with wide ramifications in the field of agriculture. One area in particular is that of the development and use of a new class of antimicrobials based on metal nanoparticles (MNPs). Among inorganic antibacterial agents, silver has been used most extensively since ancient times to fight infections and control spoilage. The antibacterial and antiviral actions of silver, silver ion, and silver compounds have been thoroughly investigated (Oloffs *et al.,* 1994). However, in minute concentrations, silver is nontoxic to human cells. Thus, silver ions have been used as an antibacterial component in synthetic zeolites (Matsumura *et. al.,* 2003).

The use of silver nanoparticles as antibacterial agent is relatively new. Because of their high reactivity due to the large surface to volume ratio, nanoparticles play a crucial role in inhibiting bacterial growth in aqueous and solid media. The simplest and the most commonly used bulk-solution synthetic method for metal nanoparticles is the chemical reduction of metal salts (Chaudhari *et. al.,* 2007; Pal *et. al.,* 2007). In fact, production of nanosized metal silver particles with different morphologies and sizes (Chen *et. al.,* 2007) using chemical reduction of silver salts has been reported (Kumar *et. al.,* 2003).

Nanometer-sized metal particles or Metal Nanoparticles (MNP) are objects of great interest in modern chemical research due to their unique electrical, magnetic, optical and other properties. MNP can be so unstable that if their surfaces touch, they will fuse together, losing their special shape and properties. The development of Polymer-Stabilized MNP (PSMNP) is one of the most promising solutions to the MNP stability problem. Therefore, in this piece of research, we wish to report the preparation of C-Ag-NPs by the reduction of silver ions with $NaBH_4$ in aqueous solutions stabilized by a polysaccharide namely chitosan.

MATERIALS AND METHODS

The purity of silver nitrate and sodium borohydride used in the study were 99.8% and 97.1% respectively. Triple distilled water was used throughout the experiment. Glasswares were rinsed in ethanolic KOH and dried in a hot air oven before use. The nanoparticles were characterized using UV–vis spectroscopy and transmission electron microscopy. The antibacterial efficiency of the nanoparticles was investigated by

introducing the particles into nutrient agar media containing E. *coli*, *Bacillus mycoides* and B. *subtilis*. B. *subtilis* a phylloplane microorganism on rice is a potential biocontrol agent agaisnt rice borwn spot (Harish *et al.,* 2007).

Briefly, 30 ml of 0.004 M $NaBH_4$ was taken in a conical flask and was kept at 0° C for 30 min. 10 ml of 0.002M $AgNO_{3-}$ was added to it drop wise with constant stirring. By mixing both solutions, Ag ions were reduced and clustered together to form mono-dispersed nanoparticles as a transparent sol in aqueous medium. The Ag solution was yellow because of the absorption at ~390 nm. Appropriate volume of 1% chitosan solution (in 1% acetic acid) was added to the nanoparticle suspension in order to stabilize the nanoparticles and make them available in a mono-dispersed condition. Same procedure was followed for stabilization of the silver nanoparticles using starch. The resulting solutions were kept at 4° C until further characterization.

The UV–vis spectrum was recorded using UV-vis spectrophotometer, Systronics India Ltd. The absorbance was recorded and plotted against the corresponding wavelength. The surface morphology of the prepared sample was observed by a Transmission Electron Microscope (JEOL 200 CX TEM) at SPC Biotech P. Ltd., Hyderabad. The TEM microphotographs were taken at an acceleration voltage of 200 kV (magnification x 100, 000).

The antimicrobial activity of NPs stabilized with starch and chitosan were assayed against Gram (+) ve and Gram (–) ve bacteria according to Pelczar *et. al*.,(1957). *Bacillus subtilis and B. mycoides* were used as Gram (+)ve models and *E. coli* was used as Gram (–)ve model which are being maintained in Microbiology Laboratory, Crop Production Division, Central Rice Research Institute (CRRI), Cuttack, Orissa, India. The bacteria were grown on nutrient agar (NA) (peptone 5 gm.lit^{-1}, beef extract 3 gm.lit^{-1}, NaCl 3 gm.lit^{-1}, agar 18 gm.lit^{-1}, pH7) plates for 12h at 30 ± 0.1^{0}C in a BOD incubator. A loopful of each bacteria was suspended in sterile distilled water separately and vortexed. The bacterial suspensions were adjusted to about 10^8 cell/ ml with sterile distilled water; one ml was mixed with 100 ml NA medium, plated onto five plates, allowed to solidify and the put into a refrigerator for 2h for hardening. Agar cups were made with a 5 mm diameter sterile cork borer. The NPs were filled in the cups along with sterile distilled water as control. The plates were incubated at 30±0.1^{0}C for 12h and the halo zones formed around the cups were recorded.

RESULTS AND DISCUSSION

The absorbance spectrum of the NP solution shows an absorbance maxima (l_{max}) = 390 nm (Fig. 1), which is characteristic for 12 nm spherical Ag-NPs, the maximum wavelength of which is around near 390 nm.

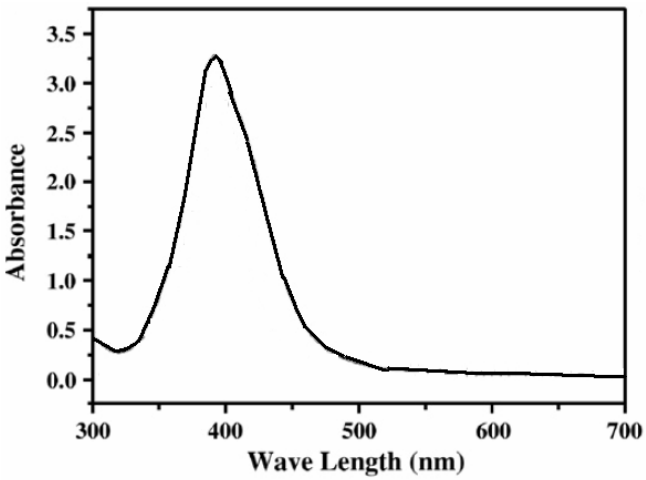

Fig. 1. Absorbance spectra of the prepared solution plotted against the corresponding wavelength. The peak of the curve (shown by an arrow), showing the absorbance maxima (l_{max}=390nm).

Transmission electron microscopic studies:
The TEM image of the silver NPs (Fig. 2) shows that the colloidal particles are highly mono-dispersed and size range of 12-14 nm.

Since Klabunde *et. al.,* (2002) demonstrated that the reactive metal oxide NPs show excellent bactericidal effects it is of great interest to investigate the usefulness of other inorganic NPs as antibacterial materials. Besides, knowledge on biocidal effects of noble metal particles is also negligible. It has been known for a long time that silver ions and silver compounds are highly toxic to most bacteria [Kreibig *et. al.,* 1995; van de Hulst, 1981; Mulvaney *et. al.,* 1996], but rarely silver-resistant [Moskovits *et. al.,* 1978; Spadaro *et. al.,* 1974]. Recently it was shown that highly concentrated and non-hazardous nanosize silver particles can easily be prepared in a cost-effective

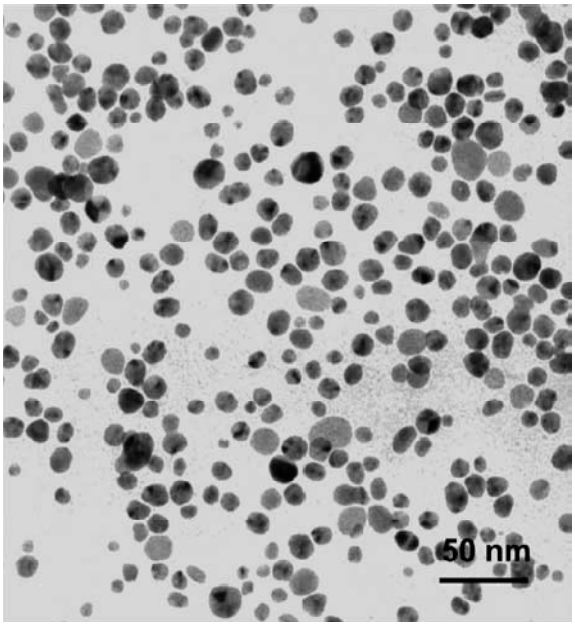

Fig. 2. Transmission electron micrograph of the silver nanoparticles

method [Cho *et. al.,* 2005] which was tested as a new type of bactericidal nanomaterial. In this study, application of the silver NPs as an antimicrobial agent was investigated by growing *E. coli, B. subtilis* and *B. mycoides* on agar plates supplemented with silver NPs in cups (Fig. 3, Table 1). Although, inhibition depends on the concentration and diffusion behaviour of the silver NPs, since the NPs resulted in significant inhibition on both groups of the microbes (Fig. 3, Table 1), it is evident that these particles could be effectively used to suppress bacteria for practical applications such as the formulation of various biocidal materials.

Bacterial sensitivity to nanoparticles was found to vary depending on the stabilizing agent used. Disk diffusion studies for chitosan stabilized AgNPs revealed greater effectiveness of the silver nanoparticles compared to the starch stabilized AgNPs. E. coli depicted the highest sensitivity to nanoparticles compared to the other strains and was more adversely affected by the chitosan stabilized AgNPs nanoparticles.

The stabilizing agent, i.e., chitosan used in this study also plays an important role in bacterial mortality [Tsai *et. al.,* 1999]. Therefore, the concentration of the nanoparticle was kept constant in all experiments. We found out that silver nanoparticles also inhibit the growth of Gram (+) ve bacteria and therefore, the silver NPs could be used against different microbes. However, the inhibitory effect of the AgNPs may also be attributed to chitosan as it is a well known antibacterial polymer. The Ag NP solution stabilized with 2% chitosan was found to inhibit all the strains used for the study, nevertheless, the unstabilized NPs, as well as those stabilized with starch showed a similar effect (Fig. 3, Table 1). The inhibition process is governed by the interaction of the NPs with intracellular substances of the disintegrated cells, causing their coagulation and removal from the liquid system.

The mechanism of inhibitory action of silver ions on microorganisms is partially known. It is believed that DNA loses its replication ability and cellular proteins become inactivated on Ag^+ treatment. In addition, it was also shown that Ag^+ binds to functional groups of proteins, resulting in protein denaturation [Sondi *et. al.,* 2003]. The obvious question is how nanosize silver particles act as biocidal material against *E. coli*. There are reports in the literature that show that electrostatic attraction between negatively charged bacterial cells and positively charged nanoparticles is crucial for the activity of nanoparticles as bactericidal materials [Feng *et. al.,* 2003]. While the mechanism of the interaction between these particles and the constituents of the outer membrane of *E. coli* is unfortunately still unresolved, it would appear that, despite their positive surface charge, they somehow interact with "building elements" of the bacterial membrane, causing structural changes and degradation and finally, cell death. A similar effect was described (Klabunde *et. al.,* 2002) when *E. coli*

Table 1. Antimicrobial potential of silver nanoparticles stabilized with biodegradable polymers

Sample	E. coli	B. subtilis	***B. mycoides***
Control (water)	-	-	-
Unstabilized silver NPs	+	+	-
Silver NPs stabilized with2% chitosan	+	+	+
Silver NPs stabilized with2% starch	+	-	+

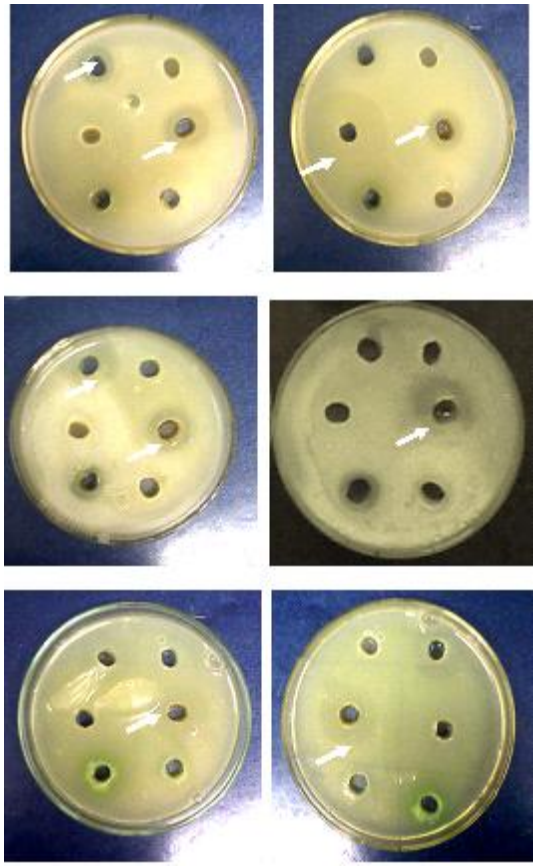

Fig. 3. Plates showing zones of inhibition (arrow marked) for bacteria treated with silver nanoparticles. Top: *Bacillus subtilis;* Middle: *Bacillus mycoides;* Bottom: *Escherichia coli*

bacteria were treated with highly reactive metal oxide nanoparticles.

Findings of the present investigation indicate that silver nanoparticles have excellent antibacterial activity against *E. coli and B. subtilis* leading to possible advances in the formulation of new types of bactericides. However, future studies on the biocidal influence of Ag-NPs on other Gram positive and Gram-negative bacteria of economic importance are necessary in order to fully evaluate its possible use as a new bactericidal material for pathogens of agricultural concern.

The deployment of bacterial antagonists to *Xanthomonas oryzae* might be an effective strategy, bringing about the disease suppression. It is evident that silver nanoparticles display an excellent inhibitory effect on both gram positive as well as gram negative bacteria. Hence it might prove to be an effective antimicrobial agent to combat not only Bacterial Blight, but also other bacterial diseases of rice.

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