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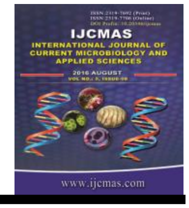

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Characterization and Antimicrobial Efficiency of Silver Nanoparticles Based Reduction Method

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This work demonstrates the use of a new Ag-NPs synthesized from reduction method of silver nitrate, for efficient ability and activity against various pathogenic bacteria. Here, this research is done in with the purpose of synthesizing silver nanoparticles while studying its antimicrobial properties on Gram positive and Gram negative bacteria. Sodium borohydride has been used for the reduction and capping of silver nanoparticles. The reduction process of Ag^+ to Ag^0 was observed by the change of color from clear yellow to cloudy gray. In addition, the synthesized of Ag-NPs followed by the characterization of the silver nanoparticles using UV-Vis, XRD pattern and CFU have been calculated. Antimicrobial activity of Ag-NPs was done by well diffusion method against pathogenic organism like *Salmonella typhi*. Finally, the optimum temperature has also determined which was 40°C. This work confirms that Ag-NPs have the antimicrobial efficacy.

Introduction

Silver nanoparticles are one of the promising products in the nanotechnology industry (Roco, 2003). The development of consistent processes for the synthesis of silver nanomaterials is an important aspect of current nanotechnology research. One of such promising process is green synthesis (Singh *et al*., 2008). Silver nanoparticles can be synthesized by several physical, chemical and biological methods (Rathi, 2009).

However for the past few years, various rapid chemical methods have been replaced by green synthesis because of avoiding toxicity of the process and increased quality. Ag-NPs highly antimicrobial to several species of bacteria, including the kitchen microbe, E. coli. According to the mechanism reported (Okada *et al*., 2001), Silver nanoparticles interact with the outer membrane of bacteria, and arrest the respiration and some other metabolic pathway that leads to the death of the bacteria. New technology advances in reducing Silver compound chemically to nanoscale sized particles have enabled the integration of this valuable antimicrobial into a larger number of materials—including plastics, coatings, and foams as well as natural and synthetic fibers. Nano-sized silver have already provides a more durable antimicrobial protection, often for the life of the product (Rathi, 2009; Okada *et al*., 2001; Qian *et al*., 2008). Current research in inorganic nanomaterials having good antimicrobial properties has opened a new era in pharmaceutical and industries. Silver is the metal of choice as they hold the promise to kill microbes effectively. Silver nanoparticles have been recently known to be a promising antimicrobial agent that acts on a broad range of target sites both extracellularly as well as intracellarly (Yakes *et al*., 2010). Silver nanoparticles shows very strong bactericidal activity against gram positive as well as gram negative bacteria including multiresistant strains, and also it was found to be in few studies (Esaki, 1999). Hence there is a huge scientific progress in the study of biological application of ZnO and Ag and metal NPs (Ren *et al*., 2009). Ag-NPs can be synthesized through many different routes. The routes can be divided into three broad categories, which are physical vapor disposition, ion implantation and wet chemistry. The Ag^{1+} in silver nitrate undergoes reduction to become silver molecule. The formula is shown in Equation 1.

$$Ag^{1+} \rightarrow Ag^{0} \text{ -------- (1)}$$

Materials and Methods

Silver nanoparticles was synthesized and developed for the present work consistently produced stable yellow colloidal silver, provided the conditions are properly controlled. The silver nitrate (>99% AgNO3) and sodium borohydride (99% $NaBH_4$) were available from chemistry department/ Koya University. Double distilled water was used. A large excess of sodium borohydride is needed both to reduce the ionic silver and to stabilize the silver nanoparticles that form. A 10 ml volume of 1.0 mM silver nitrate was added dropwise (about 1drop/second) to 30 ml of 0.5 mM sodium borohydride solution that had been chilled in an ice bath (Eq. 2). The reaction mixture was stirred vigorously on a magnetic stir plate. The solution turned light yellow (Fig. 1 and 2) after the addition of 2 ml of silver nitrate and a cloudy gray when all of the silver nitrate had been added. The entire addition took about three minutes, after which the stirring was stopped and the stir bar removed.

$$AgNO_3 + NaBH_4 \rightarrow Ag + \frac{1}{2} H_2 + \frac{1}{2} B_2H_6 + NaNO_3 \text{ ----------(2)}$$

The Ag nanoparticles that form are stabilized by a protective layer of borohydride ions.

Antibiotic disc preparation

The stock was prepared from 100 mg of Ag-NPs dissolved in 10 ml distilled water and filter paper discs. Then, filter paper discs immersed with stock of Ag-NPs, therefore kept the impregnated discs in incubator at 37°C for 2 h further drying (Nam *et al*., 1886).

Antibacterial assay

Antibacterial activities of the synthesized Ag-NPs were determined using the disc diffusion assay method, is a means of measuring the effect of an antimicrobial agent against bacteria grown in culture. The bacteria in question are swabbed uniformly across a culture plate (Cui *et al*., 2001). A filter-paper disk, impregnated with the compound to be tested, is then placed on the surface of the agar. The compound diffuses from the filter paper into the agar.

The concentration of the compound will be highest next to the disk, and will decrease as distance from the disk increases. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is the zone of inhibition (Chung *et al*., 2014). Thus, the size of the zone of inhibition is a measure of the compound's effectiveness: the larger the clear area around the filter disk, the more effective the compound. Approximately (20 ml) of molten and cooled media (Nutrient agar) was poured in sterilized Petri dishes. The plates were left overnight at room temperature to check for any contamination to appear.

The bacterial test organisms were grown in nutrient broth for 24 h (Salih *et al*., 2016; Chung *et al*., 2014; Franci *et al*., 2015). A (100 ml) nutrient broth culture of each bacterial organism was used to prepare bacterial lawns. Antibiotic disks of 5 mm in diameter were impregnated with Ag-NPs solution (100µg/ml).

The plates were incubated at 37°C and were evaluated for evidence of zones of inhibition, which appeared as a clear area around the wells. The diameter of such zones of inhibition was measured using a meter ruler and the mean value for each organism was recorded and expressed in millimeter (Mirzajani *et al*., 2011).

Results and Discussion

Characterization of UV-Visible Spectrophotometers

Generally metal nanoparticles such as in Ag, the conduction band and valence band lie very close to each other in which electrons move freely. These free electrons give rise to a surface Plasmon resonance (SPR) absorption band (Mohanty *et al*., 2012; Markowska *et al*., 2013) occurring due to the collective oscillation of electrons of Ag-NPs in resonance with the light wave. Classically, the electric field of an incoming wave induces a polarization of the electrons with respect to much heavier ionic core of silver nanoparticles. As a result a net charge difference occurs this in turn acts as a restoring force. This creates a dipolar oscillation of all the electrons with the same phase (Salih *et al*., 2016).

When the frequency of the electromagnetic field becomes resonant with the coherent electron motion, a strong absorption takes place, which is the origin of the observed color. Here the color of the prepared Ag-NPs is dark reddish brown. This absorption strongly depends on the particle size, dielectric medium and chemical surroundings. Small spherical nanoparticles (< 20nm) exhibit a single surface plasmon band[8]. The UV/Vis absorption spectra of the Ag-NPs are shown in the Fig. 4. The absorption peak is obtained in the visible range at 417 nm. With the above mentioned concentration.

Examine of Sodium Borohydride Dosage

The absorbance maxima of silver nanoparticles were synthesized from 0.5mM sodium borohydride at 417nm by reduction method.

XRD Analysis

The typical powder XRD pattern of the prepared nanoparticles is shown in Fig. 6. The data shows diffraction peaks at 2θ = 38.2°, 44.4°, 64.6°, 77.5°, and 81.7°, which can be indexed to (111), (200), (220), (311), and (222) planes of pure silver. Thus, confirmed that the main composition of the nanoparticles was silver. It is also found that there are some little peaks which match with the standard XRD data. The silver carbonate formation may be attributed to carbon dioxide in atmosphere. Therefore the product should be conserved in an airproof condition.

Ag-NPs efficacy Against Pathogenic Bacteria

The antibacterial activity of silver nanoparticles was evaluated against S. Typhi bacteria of using well diffusion technique.

The minimal bactericidal concentration values of Ag-NPs against S. Typhi were investigated in the range of 30 µl/5ml or 1.08 µg/1ml.These results suggest that Ag-NPs can be used as effective growth inhibitors in various microorganisms, making them applicable to diverse medical devices and antimicrobial control systems.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was read after 24 h of incubation at 37°C. The MIC was determined as the lowest concentration that inhibited the visible growth of the used bacterium.

The absorbance values for both blank and the test tubes were measured at 600nm[17].The MIC values of Ag-NPs against the bacterial strains were observed in the range of 20-30 µl, indicating very well bacteriostatic activity of the antibacterial agents. Against S. Typhi, the MIC value was found to be 20 µl/5ml (v/v), or 0.72 µg/1ml (w/v).

The obtained data were confirmed by CFU (which is measure of viable bacteria) after loading Ag-NPs with adequate dilution and plating on proper growth media followed by incubating at 35°C. In addition, colony forming units calculated by the number of bacteria per milliliter or gram of sample via dividing the number of colonies by the dilution factor.

The CFU/ml can be calculated using the equation below:

CFU/ml=(No. of colonies x dilution factor)/Volume of culture plate (3)

Table.1 Data measured of Ag-NPs and zone diameter

Conc. of Ag-NPs (mg/L)	Inhibition zone dimeter (mm)		
X	**Median**	**Upper Limit**	**Lower Limit**
0.000	0.000	0.000	0.000
20.000	15.940	15.940	15.940
40.000	23.050	23.050	23.050
60.000	31.720	31.720	31.720
80.000	37.010	37.010	37.010
100.000	42.590	42.590	42.590

Fig.1 Solution of Ag-NPs (color change from clear yellow to cloudy gray)

Fig.2 Prepared Ag-NPs (gray color)

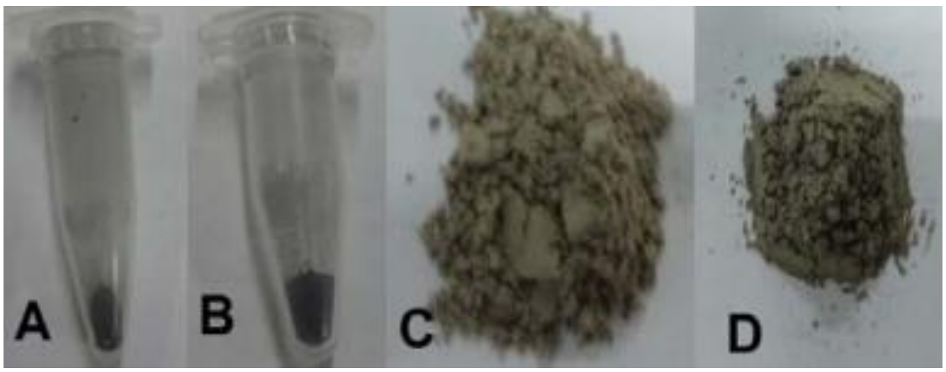


Fig.3 Repulsive forces separate Ag-NPs with adsorbed borohydride

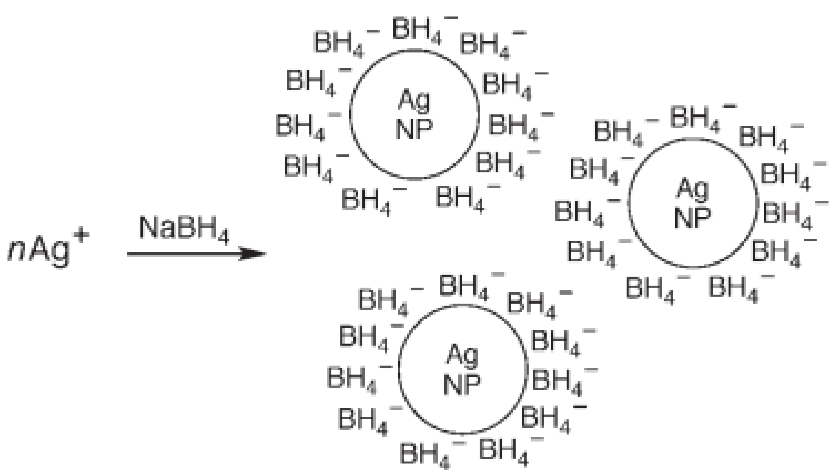


Fig.4 Spectrum of Ag-NPs (λmax = 417 nm)

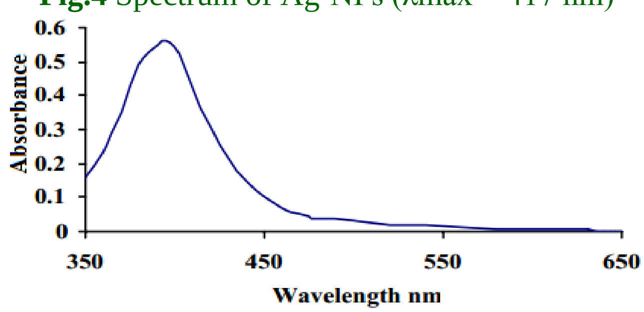


Fig.5 At 417 nm reduction reaction with 0.5 mM Sodium Borohydride

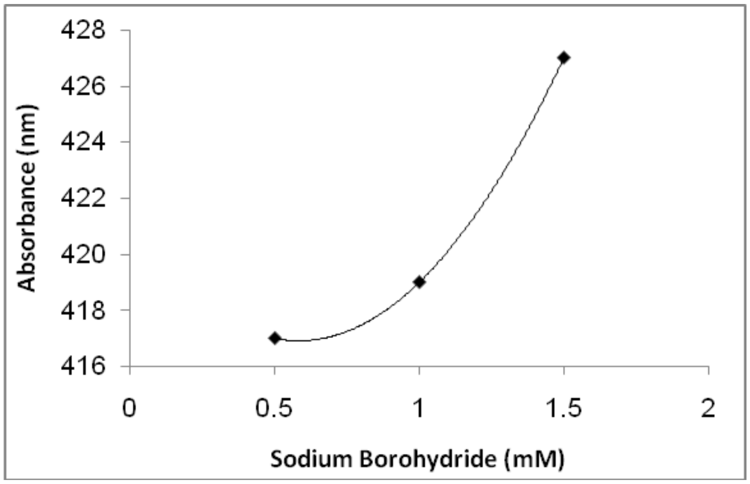


Fig.6 X-ray diffraction of Ag-NPs

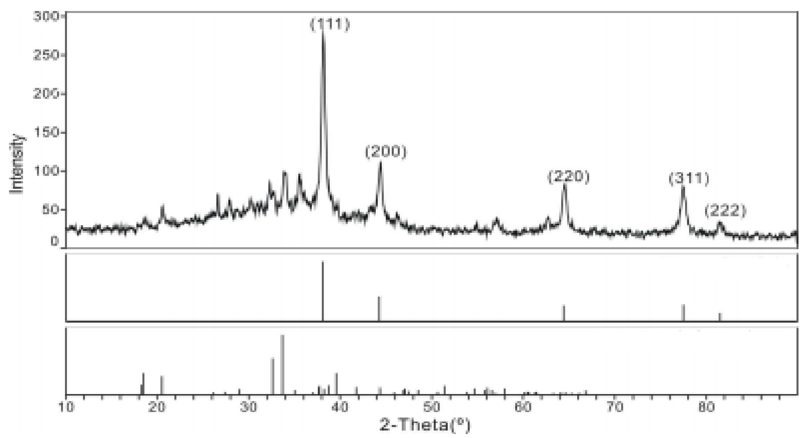


Fig.7 Image of *S. typhi* control and loaded Ag-NPs in different dosage

Fig.8 Effect of various concentration of synthesized Ag-NPs

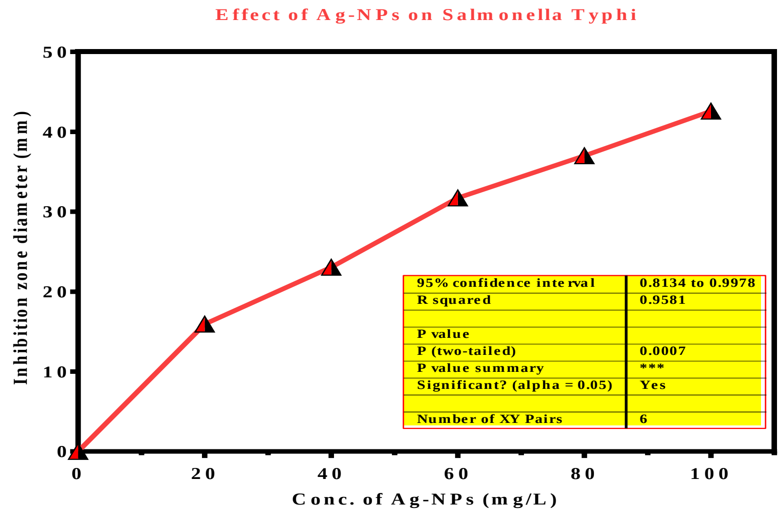


Fig.9 Effect of Ag-NPs on intensely growing *S. typhi*

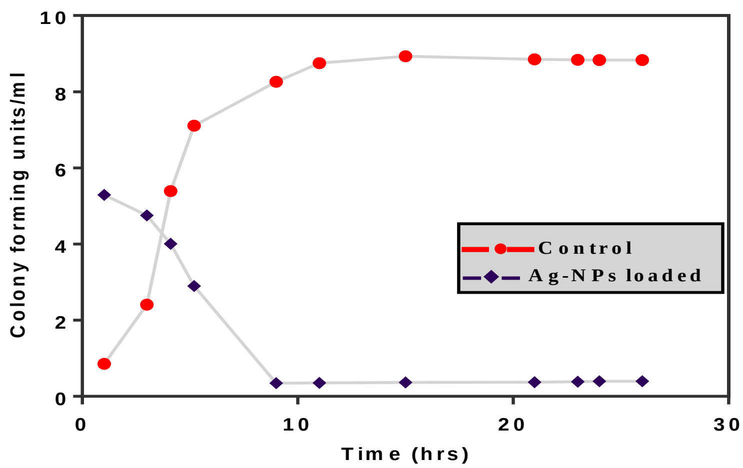
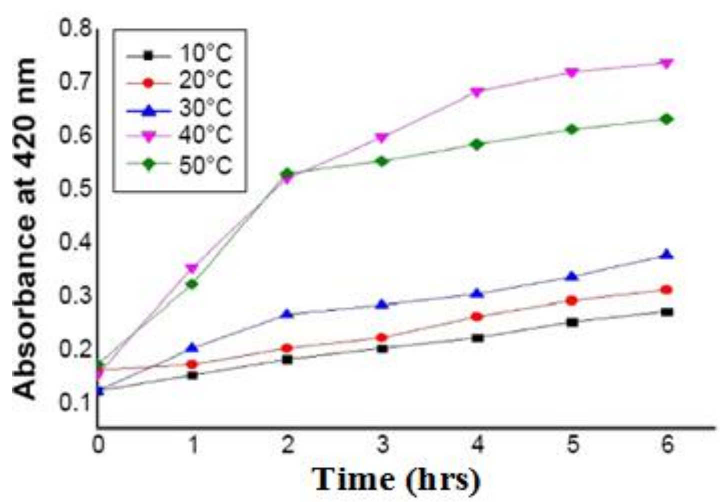


Fig.10 Study of Temp. on synthesized Ag-NPs



Study of Temperature on Synthesized Ag-NPs

The temperature have significant effect on the rate of chemical synthesis, the effect of temperature was investigated by using various temperatures for the chemical reaction, from 10 °C to 50°C/ 6 hrs, and then evaluated by measuring the absorbance at 417 nm using a UV–Vis spectrophotometer. At 10-30 °C it was found that the rate of reaction was quite slow. The rate of chemical reaction did not increase significantly with increase in temperature, but as the temperature was raised to 40°C, notice that a significant increase in the rate of reaction was observed. The reaction rate of synthesized Ag-NPs was found to be quite similar at 40°C and 50°C. The reduction of Ag ions reached its maximum after 5 hrs, which could correspond to complete reduction of silver ions. The maximum reaction rate and reduction was obtained at 40°C. Consequently, it was considered as optimum temperature for reaction.

In conclusion, silver has always been superior as antimicrobial and has been used for this purpose for ages. This work demonstrates of synthesized silver nanoparticles was done by chemical reduction of silver nitrate used Sodium Borohydride. The ideal size of silver nanoparticles was achieved by 1 mM silver nitrate concentration. It also clearly demonstrated that the Ag-NPs showed increased antibacterial efficiency with increased dosage in comparison with the low concentration. The results also demonstrated that higher antibacterial activity was observed against S. Typhi in qualitative and quantitative tests. The synthesized nanoparticles offer stabilized nanoparticles suitable for both biomedical and industrial application. The studies on the combined use of Ag-NPs with other antimicrobial agents can help reduce the problem of toxicity and to avoid the potential for development of resistance and, above all, strongly enhance the microbicidal effect. The broad spectrum of bioactivity of Ag-NPs makes them promising agents not only to fight infections, but in many other biomedical areas It can be expected that interest in Ag-NPs will continue to grow, and that this will lead to the development of new possibilities for its applications.

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