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Analysis of Antimicrobial Activity of Silver Nanoparticles Synthesized by *Klebsiella pneumoniae* and *Staphylococcus aureus* against Food Borne Pathogens

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Silver nanoparticles synthesized from supernatants of different organisms exhibit differential microbicidal activity against different pathogens. In this study the comparison of the antimicrobial activity of silver nanoparticles produced by supernatants of *Klebsiella pneumoniae* and *Staphylococcus aureus* against gram negative food pathogens *Salmonella typhi*, *Pseudomonas putida* and *Escherichia coli*. The silver nanoparticles produced by *Klebsiella pneumoniae* and *Staphylococcus aureus* were characterized according to size and shape using UV-vis spectroscopy and AFM(Atomic Force Microscopy). Silver nanoparticles produced by supernatants of *Klebsiella pneumoniae* showed complete antibacterial activity against all the gram negative pathogens tested while *Staphylococcus aureus* showed lesser activity or no activity. This is striking in view of the fact that supernatants of *Staphylococcus aureus* showed significant inhibition of gram positive MRSA(Methicillin- resistant *Staphylococcus aureus*) and MRSE(Methicillin-resistant Staphylococcus epidermidis) multidrug resistant strains of *Staphylococcus aureus* (reported by other investigators). This difference in the antimicrobial inhibition could be due to variations in size or shape of the nanoparticles synthesized by the two microorganisms.

Key words: Silver nanoparticles, Antimicrobial, *Klebsiella pneumoniae*, *Staphylococcus aureus*, Food borne pathogens, Spherical.

Silver nanoparticles have emerged as effective anti-microbicidal agents in the management of a wide range of bacteria, fungi and virus. Most significantly it has been demonstrated that its use eliminates the emergence of resistant strains of microorganisms. Thus it is most valuable in the management of wound infections in burns, diabetic ulcers etc[1-2]. The efficacy of silver nanoparticles in the control of infection has also been demonstrated against microorganisms resistant to commonly used antibiotics[3-4].

Thus it is pertinent to investigate different modalities of synthesis and parameters involved in its application to increase the susceptibility of wide range of pathogens and at the same time ensuring that it is nontoxic to normal cells and tissues. The majority of the current chemical production processes are regarded as having a relatively high environmental cost. There is increasing pressure to develop clean, nontoxic, and environmentally benign synthetic technologies.

Factors that favor biological method of synthesis have slower kinetics that better controls crystal growth, miminum use of hazardous

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chemical and lower cost of production. Bacteria, fungi, algae and plants have been used for both the intracellular and extracellular synthesis of silver nanoparticles.

In this study for the first time we have explored the idea of whether differential antimicrobial activity exists between the silver nanoparticles extracellularly synthesized by two microorganisms *Klebsiella pneumoniae* and *Staphylococcus aureus*. The silver nanoparticles synthesized were characterized by UV-spectroscopy and AFM. The antimicrobial activity was investigated against three food pathogen *Salmonella typhi*, *Escherichia coli* and *Pseudomonas putida*. Since all these were gram negative we also tested them with gram positive bacteria *Staphylococcus aureus*.

MATERIALS AND METHODS

Microbial cultures

The cultures like *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas putidus* were brought from MTCC (Microbial type Culture Collection), Chandigarh.

Media and chemicals

All the media constituents and analytical reagents required for the work were purchased from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India) and Sigma Chemicals (St. Louis, USA)

Synthesis of Silver Nanoparticle

Klebsiella pneumoniae, Staphylococcus aureus, Proteus mirabilis were inoculated in 2.5% Luria Bertani Broth and incubated at 37°C for 48hours. The cultured broth was centrifuged at 5,000 rpm for 10 min. 50ml of supernatant each culture was taken and 10^{-3} M of AgNO3 was added. This was incubated at 37° C in the rotating shaker at an rpm of 150 for 5 days. The absorbance values were measured Spectrophotometrically, every 24 hours interval. The colour of the supernatant changed from yellow to brown after 48hrs in case of *Klebsiella pneumoniae* and *Staphylococcus aureus* indicating the synthesis of silver nanoparticles

Characterization of Ag bionanoparticles

The synthesized Ag bionanoparticles were first characterized by Elico UV–visible spectrophotometer in the range of 250–650 nm. (Elico Ltd., Bangalore), using a quartz cuvette with the control as the reference. The surface plasmon resonance peaks were noted around 420–430 nm region.

Determination of size and shape of the silver nanoparticles that synthesized was done using the morphological characterization of the synthesized bio-nanoparticles and studied using Atomic Force Microscopy (Ajilent Technologies) in the contact mode. The samples were dissolved in acetone and spin coating the sample using apex instruments spin coater at 9000rpm. This was followed by drying the samples for 30mins.

Antibacterial Activity of Silver Nanoparticle

Sterilized Luria Agar plates were uniformly swabbed with test cultures *Salmonella typhi*, *Pseudomonas putida* and *E.coli*. 10µl of synthesized silver nanoparticles was added to each plate along with Luria Agar. Control plates had only the identical cultures of these organisms but without silver nanoparticles. It was kept for incubation at 37° C for 24 hrs. Zone of inhibition was measured.

RESULTS

Synthesis of Silver Nanoparticles

The biomass of *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Proteus mirabilis* that was grown for 48 hrs and centrifuged and a constant amount of 50ml of supernatant from all three bacteria was taken and 10^{-3} M $AgNO_3$ was added. Only the supernatant of *Klebsiella pneumoniae* and *Staphylococcus aureus* along with $AgNO_3$ changes from yellow to brown in 48hrs [Fig. 1B and 1C].The supernatant of *Proteus mirabilis* along with $AgNO_3$ did not change from yellow to brown [Fig. 1A].

Characterization

UV-visible spectral analysis

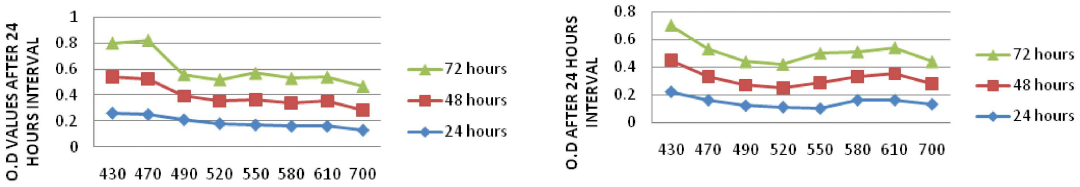
The UV–visible spectrum [Fig. 2] recorded in the aqueous solution of 1 mM $AgNO_3$ with the bacterial filtrate of *Klebsiella pneumonia* [A] and *Staphylococcus aureus* at pH 7[B]. Curves correspond to readings taken at 24 h, 48 h, and 72 h, respectively. The peak was noted around 439 nm.

AFM

The structure of the Silver nanoparticles synthesized by the organism *Klebsiella*

(A) *Proteus mirabilis* (A) *Klebsiella pneumoniae* (A) *Staphylococcus aureus*

Fig. 1. Synthesis of silver nanoparticles



(A) *Staphylococcus aureus* (B) *Klebsiella pneumonia*

Fig. 2. Absorbance of Silver Nanoparticles at pH 7

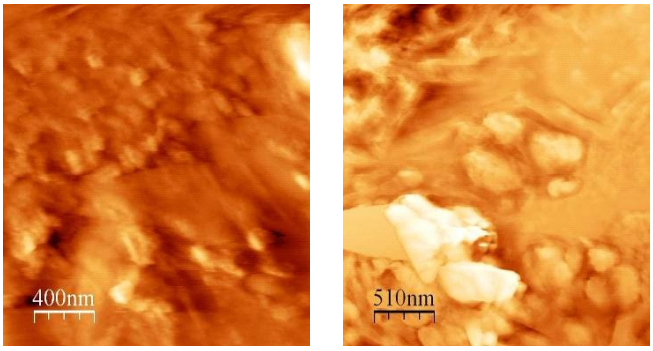


Fig. 3. Silver nanoparticles synthesized by *Klebsiella pneumoniae* at pH 7

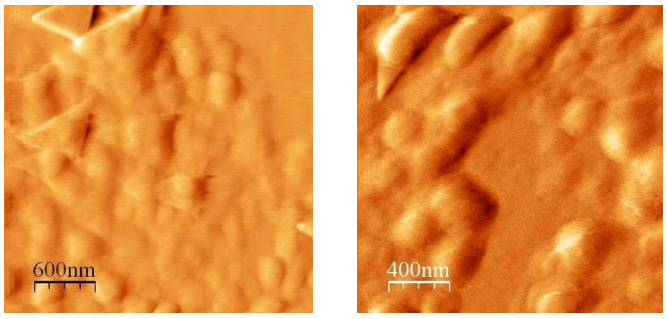


Fig. 4. Silver nanoparticles synthesized by *Staphylococcus aureus* at pH 7

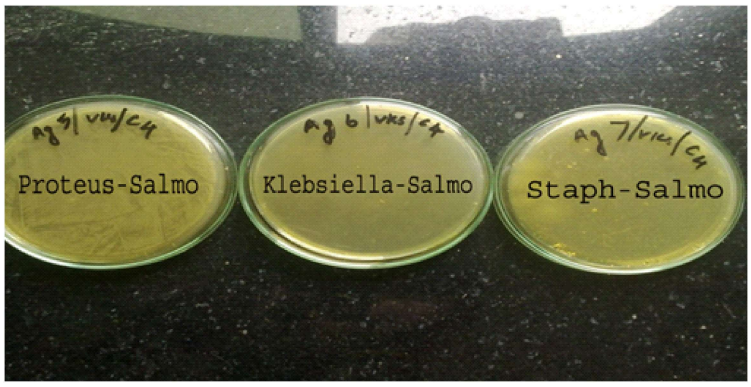


Fig. 5. Antibacterial activity of Silver Nanoparticle synthesized by *Klebsiella pneumonia, Proteus mirabilis & Staphylococcus aureus* on *Salmonella typhi*

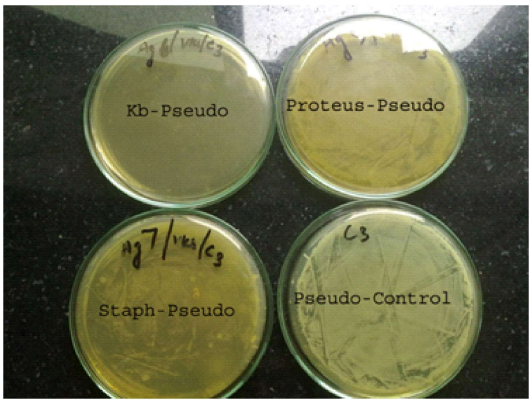


Fig. 6. Antibacterial activity of Silver Nanoparticle synthesized by *Klebsiella pneumoniae* (A), *Proteus mirabilis* (B) &*Staphylococcus aureus* (C) on *Pseudomonas putida*

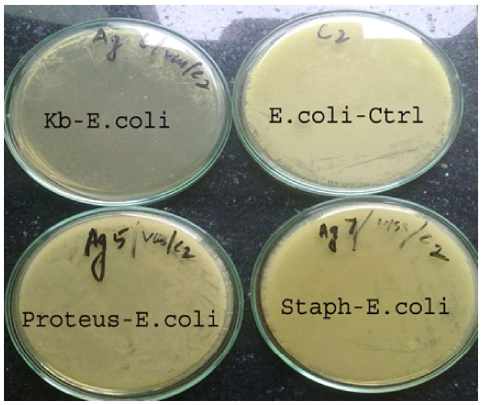


Fig. 7. Antibacterial activity of Silver Nanoparticle synthesized by *Klebsiella pneumoniae*(A), *Staphylococcus aureus* (D), *Proteus mirabilis* (C) on *E.coli*

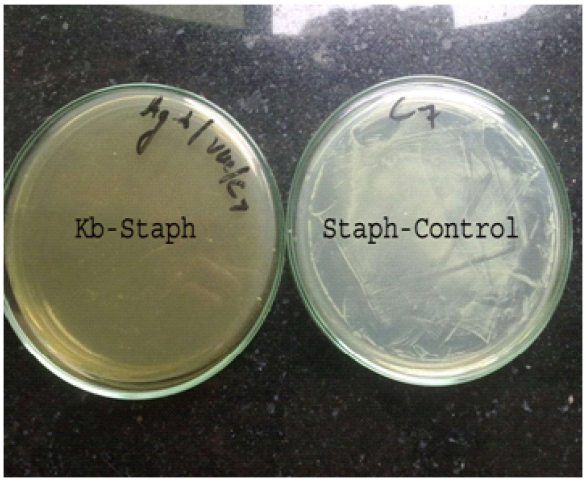


Fig. 8. Antibacterial activity of Silver Nanoparticle synthesized by *Klebsiella pneumoniae* on *Staphylococcus aureus*

pneumoniae [Fig.3] and *Staphylococcus aureus* at pH 7 [Fig4] were analyzed by using the AFM. The silver nanoparticles size synthesized by supernantants of Klebsiella pneumoniae is approximately 125nm and the shape is nanospheres irregularly shaped. In contrast the silver nanoparticle size synthesized by supernatants of Staphylococcus aureus is approximately 200-300nm and the shape is spherical and regular.

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Antibacterial activity of silver nanoparticles

The supernatant of *Klebsiella pneumoniae* and *Staphylococcus aureus* containing the silver nanoparticles were compared for antibacterial activity against three food pathogens- *Salmonella typhi*, Proteus mirabilis, *Pseudomonas putida* and *E.coli*.

As shown in [Fig.5], it is evident that silver nanoparticles from supernatants of *Klebsiella pneumoniae* [B] had complete inhibitory growth activity against *Salmonella typhi*, while supernatants of *Staphylococcus aureus* [C] showed moderate activity against it. The control was supernatant from *Proteus mirabilis* [A] that showed no antibacterial activity.

The supernatants of *Klebsiella pneumoniae* [A] also showed complete inhibitory activity against *Pseudomonas putida* while silver nanoparticles in supernatants of *Staphylococcus aureus*[C] showed very weak activity against it – even lesser than that showed against *Salmonella typhi*. The control was supernatant from *Proteus mirabilis* [B] that showed no antibacterial activity and the other plate [D] contains only *Pseudomonas putida* and shows full growth [Fig 6].

Complete inhibitory activity against E.coli was also seen using supernatants of *Klebsiella pneumonia*[A] while supernatants of *Staphylococcus aureus*[D] and control supernatant from *Proteus mirabilis*[C] showed absolutely no activity and was similar to growth as in the plate containing *E.coli*[B] [Fig.7].

Earlier reports have indicated that supernatants of *Staphylococcus aureus* were more active against gram positive organisms and drug resistant strains of *Staphylococcus aureus* -MRSE and MRSA than gram negative bacteria *Salmonella typhi*. In this study we also investigated the antibacterial activity of *Klebsiella pneumoniae* supernatant against gram positive *Staphylococcus aureus* and found that it was equally active against gram positive bacteria [Fig.8]

DISCUSSION

Silver nanoparticles have a wide application as antimicrobial agents in medical catheters, wound dressing, dental work, textiles, cosmetics[6-9].

There are several methods documented for the synthesis of silver nanoparticles-both chemical and use of biological agents such as bacteria, fungi, algae and plant extracts. Intracellular and extracellular synthesis of silver nanoparticles by different microorganisms has been documented[10]. Intracellular synthesis on a large scale would require elaborate energy resources for agitation and harvesting of the biomass and purification of the nanoparticles. Extracellular synthesis offers a far more viable and efficient source that can be further manipulated to get the desired size and shape of nanoparticles[11].

A few studies have reported extracellular synthesis of silver nanoparticles from microorganisms[12-13], but simultaneously with the search new biotemplate sources for microorganisms. The questions that need to be addressed is whether the supernatants from different microorganism (from same amount of biomass) have differential microbicidal activity against pathogens that infect particular sites such as food, water, wounds, burns, dental, acne so that the organisms that produce the silver nanoparticles with maximum antibacterial activity against all the pathogens in that site can be used as the biotemplate source .The second question that needs to be addressed is the possible reasons for the differential activity to obtain a good understanding of characteristics of silver nanoparticles required. In this study we addressed the first question by comparing the antimicrobicidal activity of supernantants of Klebsiella and Staphylococcus against pathogens that infect certain food items –*Salmonella typhi, Pseudomonas putida* and *E.coli*

An earlier study reported the synthesis of silver nanoparticles extracellularly from *Klebsiella pneumoniae* but it was examined for antimicrobial activity against *Staphylococcus*

aureus and *E.coli*[12]. The silver nanoparticles synthesized from supernatants of *Staphylococcus aureus* have been also been shown in an earlier study to be active against gram positive organisms MRSA and MRSE but only moderately active up against *Salmonella typhi*[13].

In this study supernatants of Klebsiella pneumoniae are completely inhibitory against all the three food pathogens Salmonella typhi, Pseudomonas putida and E.coli. While the supernatants of staphylococcus aureus are not at all or only moderately and weakly active against all three food pathogens. This is in contrast to the widely held observation that silver nanoparticles synthesized by microorganisms are more inhibitory to gram negative microorganisms than gram positive microorganisms because Gram negative bacteria have a lipopolysaccharide exterior followed by a thin layer of rigid peptidoglycan, whereas gram-positive bacteria have a thick peptidoglycan layer that are extensively cross linked that make it difficult for silver nanoparticles to penetrate.

The characterization of the silver nanoparticles synthesized by Klebsiella and staphylococcus aureus by UV spectroscopy and AFM revealed that the silver nanoparticles synthesized by *Klebsiella pneumoniae* are irregularly spherical in shape and range from 50nm-125nm in size while silver nanoparticles synthesized by staph are regularly spherical and range from 200nm-250nm. The UV-Vis spectra showed the presence of a single peak that centered at about 439 nm for Klebsiella or 431 nm for staph respectively.

According to Liz-Marzán et al, the optical absorption spectra of metal nanoparticles are dominated by Surface Plasmon Resonances (SPR), which are strongly dependent on the particle size, dielectric medium, and surface-adsorbed species[14]. A single SPR band is more indicative of spherical nanoparticles[15], whereas anisotropic particles could result in two or more SPR bands depending on the shape of the particles. Further it has been demonstrated that the number of SPR increases as the symmetry of the nanoparticle decreases[16].

Thus, spherical nanoparticles, disks, and triangular nanoplates of silver show one, two, and more peaks, respectively. Since we obtained only a single peak at 430nm with both the supernatants from *Klebsiella pneumoniae* and *Staphylococcus aureus* it signifies that the silver particles in both cases are predominantly spherical in shape. It must be noted however that different peaks of optical absorption spectra has been observed in preparations of silver nanoparticles purified for a particular shape – rods, triangle, spherical synthesized by chemical synthesis.

The AFM images indicate that the silver nanoparticle size ranged from 50-125 nm in supernatants obtained from *Klebsiella pneumoniae* and between 200-300 nm in supernatants from *Staphylococcus aureus*. Investigators have reported a single peak at wavelength 430nm under UV-VIS spectroscopy[12] and size of 50nm-100nm in supernatants of klebsiella. The synthesis of 160-180nm silver nanoparticles from *Staphylococcus aureus* has been reported[13].

It seems noteworthy that supernatants of *Klebsiella pneumoniae* were equally effective against gram negative and gram positive bacteria. However the work in our lab and other workers demonstrated that the supernatants of *Staphylococcus aureus* were more active against gram positive bacteria than gram negative[13].This converse antimicrobial effects of supernatants of Klebsiella and staphylococcus, indicate that silver nanoparticles of produced by the two microorganisms are distinct. It is possible that while silver nanoparticles of size 50 nm-200nm can kill gram positive microorganisms smaller sized nanoparticles less than 125nm are perhaps required for killing of gram negative microrganisms as produced by supernatants of Klebsiella. Also the shape of the silver nanoparticles may play a role and the irregularly spherical nanoparticles produced by supernatants of *Klebsiella pneumoniae* may contain more {111} facets than {100} facets that are required for killing of gram negative bacteria. On the other hand silver nanoparticles produced by *Staphylococcus aureus* are more regularly spherical and may have fewer {111} facets required for killing gram negative bacteria but nonetheless having the shape and size for killing gram positive bacteria. XRD analysis of the supernatants of klebsiella and staph may throw more light on the facets present.

It seems there are more than one shape or one size that contribute to antimicrobial activity of

gram negative microorganism –e.g. for truncated triangles a different size may be required (0) than for spherical nanoparticles for efficient killing. Thus various permutations and combinations are possible and the shape and size best suited for efficient killing of all organisms that infect a given site such as food, water, burn wound, ulcers, dental carries, with the least amount of cytotoxicity to the surrounding tissues and cells need to be defined and worked out. Smaller size of silver nanoparticles 10nm-60nm has been reported in literature to by toxic to mammalian cells and tissues. Most studies using microorganisms have reported smaller size of silver nanoparticles less than 100nm are inhibitory against gram negative and gram positive microorganisms but this could be toxic to human cells. In this study the silver nanoparticles were of the range 100nm-200nm and may be more tolerated by mammalian cells. Though our main study was on food pathogens (all gram negative) the interesting finding that indeed the supernatants of staph show only minimum activity against gram negative unlike supernatants of Klebsiella have prompted future studies in our lab to investigate mechanisms of antibacterial activity exclusive to gram positive microorganisms. Most mechanisms put forth such cell membrane rupture or inhibition of DNA replication or inhibition by binding to thiol groups of respiratory proteins it has been mostly investigated in gram negative microorganisms. It is also possible that changes in surface morphologies not distinguishable by AFM may be a contributing factor to killing of gram positive organisms.

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