ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES AGAINST *E. coli* AND *Staph. aureus*

A thesis submitted to the Quaid-i-Azam University in partial fulfillment of the requirements for the degree of Masters of Philosophy in Biology (Microbiology)

By

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CERTIFICATE

This thesis submitted by Ms. Fouzia Hussain is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, (Pakistan) as satisfying the thesis requirement for the degree of Master of Philosophy in Microbiology.

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Dated: 28th Feb. 2006

DECLARATION

The material contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Fouzia Hussain

Dedicated To My Late

Father,

My Beloved Mother

And

My Brother Mehboob

Hussain

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviations	
AG	Acid gas
Ag^+	Silver ions
Ag^0	Metallic silver
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CFU	Colony forming unit
conc.	Concentration
E. coli	*Escherichia coli*
hrs	Hours
IGC	Inert gas condensation
K. pneumoniae	*Klebsiella pneumoniae*
L	Liter
ml	Milliliter
OD	Optical density
P. aurgensia	Pseudomonas *aurgensia*
rpm	Revolution per minutes
S. aureus	*Staphylococcus aureus*
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UTI	Urinary tract infection
UV	Ultra violet
VP	Voges-proskauer
wt/vol	Weight/volume
µg	Micro gram

We did not create the heavens and the earth and everything between them as a game. We did not create them except with truth but most of them do not know it.

(Surat-Dukhan 38- 39)

ACKNOWLEDGEMENTS

They (angels) said: "Glory be to you, we have no knowledge except you have taught us. Verily, it is you, the knower, the All Wise".

(Surah Al-e-Imran, verse #32)

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Abstract

Introduction

INTRODUCTION

The basic science at the nanoscale is not new. Scientists have known that matter is made of atoms for over a century, and for decades scientists have known how to calculate many properties of the matter. However, only recently developments in instrumentation and computing power have made atomic level measurements possible. The ability to measure, manipulate, visualize and simulate matter at the atomic scale has the potential of redefining our interaction with the world around us. This is why nanotechnology is considered revolutionary, like the Industrial Revolution, rather than just another step in technological progress. Nanoscience and nanotechnology are an extension of the field of materials science, and materials science in conjunction with physics, mechanical engineering, bioengineering, and chemical engineering departments are leading the breakthroughs in nanotechnology. (Eelstein and Cammarata, 1996).

What is Nanotechnology?

"Nanotechnology is the understanding and control of matter at dimensions of roughly 1 to 100 nanometers, where unique phenomena enable novel properties and applications (www.sciencedirect.com).

Richard Feynman (1959), an American Physicist, first presented the idea of nanotechnology. In a lecture entitled "There is Plenty of Room at the Bottom", delivered at the California Institute of Technology (Caltech), he unveiled the possibilities available in the molecular world. Because ordinary matter is built of so many atoms, he showed that there is a remarkable amount of space within which to build. Feynman described the process by which the ability to manipulate individual atoms and molecules might be developed, using one set of precise tools to build and operate another proportionally smaller set, so on down to the needed scale. In the course of this, he noted, scaling issues would arise from the changing magnitude of various physical phenomena: gravity would become less important, surface tension and Van der Waals attraction would become more

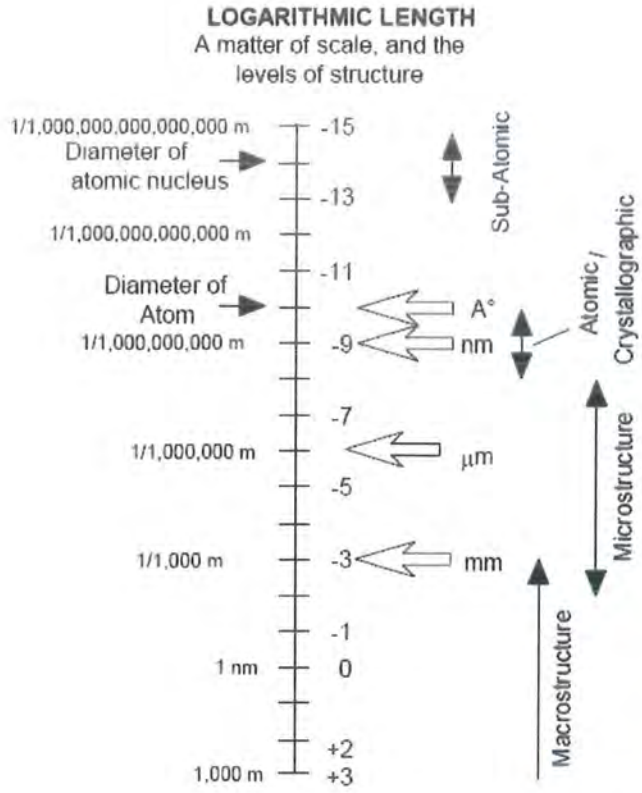


Figure: A. Logarithmic length scales and levels of structures (J. Dutta and H. Hoffmann, 2003)

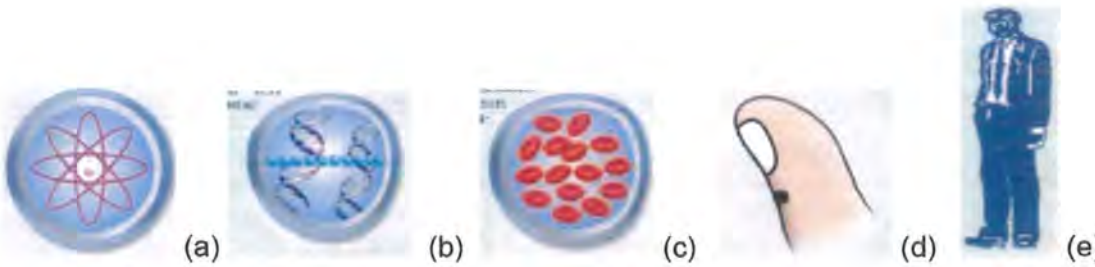


Figure: B. Length scales (a) an individual atom up to few angstrom (b) shoulder t shoulder 10 hydrogen atoms spans to one nanometer and DNA molecules about 2.5 nanometers wide (c) Red blood cells in diameter range- thousands of nanometers (d) a pinhead sized patch on thumb- millions of nanometers (e) two meter tall person two billion nanometers (J. Dutta and H. Hoffmann, 2003)

important, etc. This basic idea appears feasible, and exponential assembly enhances it with parallelism to produce a useful quantity of end products. Feynman's vision spawned the discipline of nanotechnology and now we are amassing the tools to make his dream a reality (Stoimenov *et al.*, 2002).

Nanotechnology involves the creation and/or manipulation of materials at the nanometer (nm) scale either by scaling up from single groups of atoms or by refining or reducing bulk materials. A nanometer is 1 x 10^{-9} m or one millionth of a millimeter. To give a sense of this scale, a human hair is of the order of 10,000–50,000 nm, a single red blood cell has a diameter of around 5000 nm, viruses typically have a maximum dimension of 10–100 nm and a DNA molecule has a diameter of 2–12 nm. The use of the term "nanotechnology" can be misleading since it is not a single technology or scientific discipline. Rather it is a multidisciplinary grouping of physical, chemical, biological, engineering, and electronic, processes, materials, applications and concepts in which the defining characteristic is one of size (Health and Safety Executive Britain 2004).

Nanotechnology is more than the study of small things; it is the research and development of materials, devices and systems that exhibit physical, chemical and biological properties that are different from those found at larger scales. Thus nanotechnology can be best understood as a broad collection of technologies from diverse fields such as physics, materials science, engineering, chemistry, biochemistry, medicine and optics, each of which may have different characteristics and applications. Natural or man-made particles or artifacts often have qualities and capabilities quite different from their macroscopic counterparts. Gold, for example, which is chemically inert at normal scales, can serve as a potent chemical catalyst at nanoscales. One fundamental characteristic of nanotechnology is that nanodevices are self-assembled. That is, they build themselves from the bottom up (Siegel *et al.*, 1993).

Nanotechnology provides the ability to engineer the properties of materials by controlling size, and this has driven research toward a multitude of potential uses for nanomaterials. Nanomaterials exhibit quantum confinement, which can result in different

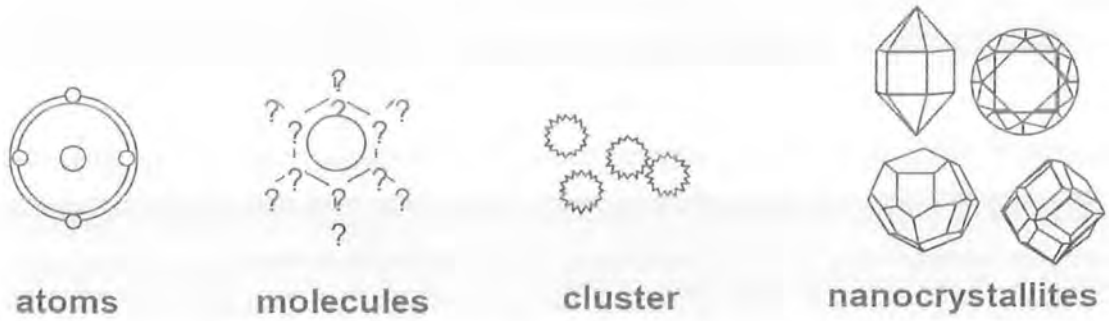


Figure: C. Schematic representation of various states of matter
(J. Dutta and H. Hoffmann, 2003)

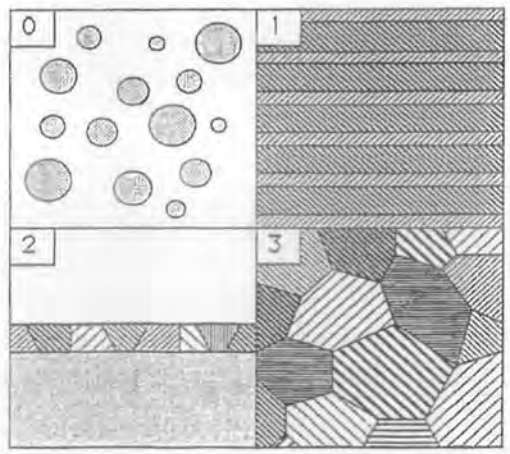


Figure: D. Definition of nanocrystalline materials on the basis of modulation dimensionality (0) clusters and powders (1) multilayers (2) ultra fine-grained layers (3) nanomaterials composed of equiaxed nanometer sized grains (Siegel, R. W. 1994)

electromagnetic and optical properties of a material between nanoparticles and the bulk material, the Gibbs-Thomson effect, which is the lowering of the melting point of a material when it is nanometers in size, and such structures including carbon nanotubes (Goodsell, 2004).

Nanomaterials exhibits new and superior size-dependent properties such as electronic, optical, electrical magnetic, chemical and mechanical, compared with larger particles of the same material. These materials have large surface to volume ratio. They have large number of grain boundaries per unit volume; a large number of atoms are available at the surfaces. Stepped surfaces, active surface sites, coordination number i.e. nearest neighboring atoms, decreased density, change the physical and chemical behavior of materials. Having size between molecular and bulk solid-state structures, nanomaterials have hybrid properties that are being investigated for understanding. Some examples of these novel properties are lower melting temperature, solid- solid phase transition pressure, lower effective Debye temperature, decreased ferroelectrics phase transition temperature, higher self diffusion coefficient, changed thermo physical properties and catalytic activity (Kruis, 1998).

Nanoparticles are either single particles of size 1 to 100 nm or the individual primary particles, grains or crystals of aggregates or agglomerates. These have distinct different properties than bulk materials because the number of atoms on their surface becomes comparable to that of inside the particles. In nanoparticles, melting point decrease, light absorption increases, electromagnetic, catalytic properties changes, compared with those of the bulk material (Kvitek and Prucek, 2005).

Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. A bulk material should have constant physical properties regardless of its size, but at the nano-scale this is often not the case. Nanoparticles are sometimes referred to as clusters, which are smaller nanoparticles with less than 10^4 molecules or atoms corresponding to a diameter of few nanometers. Metal, dielectric, and semiconductor nanoparticles have been formed, as well as hybrid structure

Full shell clusters	Total number of atoms	Surface atoms (%)
One shell 	13	92
Two shells 	55	76
Three shells 	147	63
Four shells 	309	52
Five shells 	561	45
Seven shells 	1415	35

Figure: E. Surface atoms decrease as the number of shells increase (Schmid, G. 1990)

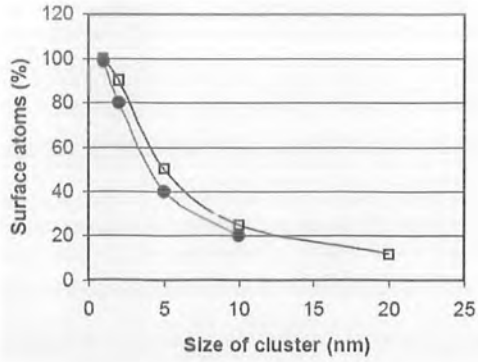


Figure: F. A graphical representation of surface area and cluster size (Jimbo, G and Preining, O. 1990)

(e.g., core-shell nanoparticles). Nanospheres, nanorods, and nanocups are just a few of the shapes that have been grown. Semiconductor quantum dots and nanocrystals are types of nanoparticles (Kimoto *et al.*, 1993).

Baker *et al.*, (2004) reported that the surface density required for a completely cytotoxic surface depends on the size on the nanoparticles. This is expected as smaller nanoparticles have a larger surface to volume ratio and, hence the same weight of nanoparticles can over a larger area. However, if the particles are agglomerated, then the effective is not the particle but the size of the agglomerate, which can be several times larger than the particle size.

Discoveries in the past decade have shown that once materials are prepared in the form of very small particles, they change significantly their physical and chemical properties, sometimes to the extent that completely new phenomena are established. However, little is yet known about how the biological activity of a certain material changes as the size of the constituting particles decreases to nanoscale dimensions. There are some reports in the literature that show encouraging results about the activity of different drugs and antimicrobial formulations in the form of nanoparticles (Stoimenov *et al.*, 2002).

According to www.cientifica.com, in the biological sciences, many applications for metal nanoparticles are being explored, including biosensors, labels for cells and biomolecules, and cancer therapeutics. Nanotechnology is expected to open new avenues to fight and prevent disease using atomic scale tailoring of materials. Among the most promising nanomaterials with antibacterial properties are metallic nanoparticles, which exhibit increased chemical activity due to their large surface to volume ratios and crystallographic surface structure. It has been demonstrated that, in the case of noble-metal nanocrystals, the electromagnetic, optical and catalytic properties are highly influenced by shape and size. This has driven the development of synthesis routes that allow a better control of morphology and size. Noble-metal nanomaterials have been synthesized using a variety of methods, including hard-template, bioreduction and solution phase syntheses.

Metals are generally polycrystalline, meaning that they consist of many randomly oriented crystalline regions or grains. Physical and chemical properties of bulk metals are dependent on the crystal structure and grain size. As the grain size is reduced, an increasing proportion of the atoms in the solid are available at the grain boundaries, where they behave differently from those not on the boundaries (www.cientifica.com).

Among noble-metal nanomaterials, silver nanoparticles have received considerable attention due to their attractive physicochemical properties. The surface plasmon resonance and large effective scattering cross section of individual silver nanoparticles make them ideal candidates for molecular labeling, where phenomena such as Surface Enhance Raman Scattering (SERS) can be exploited. In addition, the strong toxicity that silver exhibits in various chemical forms to a wide range of microorganisms is very well known, and silver nanoparticles have recently been shown to be a promising antimicrobial material (Jose *et al.*, 2005).

The antimicrobial activity of silver ions has been well established. Silver ions are significant antimicrobials by virtue of their antiseptic properties with only few bacteria being intrinsically resistant to this metal. Silver is well known being a significant resource for topical therapy because of its beneficial antimicrobial properties in medical devices such as catheters, cannulae etc. (Kumar and Munstedt, 2004).

Baker *et al.*, (2004), reported that silver ion is highly toxic to bacteria such as bacteria *E.coli* and *S. aureus* but has a low toxicity to animal cells. While other antibacterial medicines have failed due to the emergence of antibiotic resistance, these bacteria fail to develop immunity to silver.

According to Zhao and Stevens (1998) Silver is the most toxic element to microorganisms in the following sequence:

Ag > Hg > Cu > Cd > Cr > Pb > Co > Au > Zn > Fe > Mn > Mo > Sn

Silver is very good anti-bacterial agent because it is non-toxic and natural inorganic metal and besides, silver can kill many harmful microorganism against the human body. Such silver has a size of nano-level; the total surface area of silver becomes larger in identity volume. Then a chance, which is antibacterial action of nanosilver, is raised the antibacterial efficiency is consequently increased (Yeo et al., 2003). With the rise of antibiotic-resistant bacteria, silver is re-emerging as a modern medicine because all pathogenic organisms have failed to develop immunity to it. However, the mechanism for such bacterial sensitivity to silver is poorly understood (Zhao and Stevens, 1998).

Over the past few years, there has been a rapid increase in the number of silver dressing made available to physicians. Perhaps the most unique form of silver developed for wounds dressings is the nanocrystalline silver, which differs in both physical and chemical properties from micro- and macro-crystalline and from silver salts. This is, in part related to the increase in grain boundary atoms as a percentage of the total atoms in the material, which is due to the small crystal size. These grain boundaries may represent a third state of solid matter. Amorphous materials are made up of atoms or molecules that demonstrate short range order, interacting with their immediate neighbors. Crystals demonstrate long-range order due to the lattice structure of the atom or molecule. Grain boundary atoms or molecules, however, are not a part of a crystal lattice structure, but they are affected by the lattice structure of the crystals around them, which prevent them from free interaction with their neighbors. This suggest that grain boundary atom demonstrate neither short nor long range order, indicating that these atoms may behave differently from either amorphous or crystalline silver indeed confirm that the material structure is unique (Taylor *et al.*, 2005).

According to Taylor *et al.*, (2005), unique property of nanocrystalline silver is that, it dissolves to release Ag^{o} clusters and Ag^{+}, whereas other silver sources release only Ag^{+}. This difference in the dissolution properties of nanocrystalline silver dressing appears to alter the biological properties to the solution, including both antimicrobial and anti-inflammatory activity. Nanocrystalline silver dressing has been demonstrated in vitro as effective antifungal agent for antibiotic resistant bacteria. In vivo studies have shown that

nanocrystalline silver is very effective at preventing infections and healing wounds. Nanocrystalline silver's anti-inflammatory efficacy has also been demonstrated, both in vivo and in clinical studies. Demling and Burrel (as cited in Taylor *et al.*, 2005) have speculated that the enhanced biological properties of nanocrystalline silver are related to its nanostructure.

For the near-term, critics of nanotechnology point to the potential toxicity of new classes of nanosubstances that could adversely affect the stability of cell walls or disturb the immune system when inhaled or digested. Objective risk assessment can profit from the bulk of experience with long-known microscopic materials like carbon soot or asbestos fibres. There is a possibility that nanoparticles in drinking water could be dangerous to humans and/or other animals (www.wikipedia.com).

Aims and Objectives

Aims:

The main aim of present research is to characterize the effect of silver nanoparticles on growth of Gram-negative (*Escherichia coli* ATCC 15224 and locally Isolated *E. coli* UTI) and Gram-positive (*Staphylococcus aureus* ATCC 6538), strains.

Objectives:

Present study was planned for the following objectives.

- To conduct shake flask experiment for observing the inhibitory effect of silver nanoparticles of different concentrations on various bacterial strains.
- To check the growth pattern of bacteria by determination of optical density (OD) and colony forming units.
- To estimate the protein concentration for checking the inhibitory effect of nanoparticles
- To check the interaction of silver nanoparticles with the bacterial cells by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).
- To study the chromosomal/ plasmid mediated changes in the growth pattern of bacterial cells in the presence or absence of nanoparticles.

LITRATURE REVIEW

Over the last few years, much effort has been directed in to developing new materials that have specific antimicrobial activities that can be used to fight infections and to create sterile conditions. The antifouling properties of silver and copper are well known and their effectiveness at reducing the growth of various microorganisms has been reported. Nanotehcnology could become very important in this research field, providing the tools needed to synthesize copper-containing and silver-containing nanostructured films-layers embedding metal nanoparticles showing an enhanced bioactivity (Cioffi *et al.*, 2005).

Nanotechnology is applied in a variety of biomedical and bioengineering proceedings. Magnetic nanoparticles of different sizes with tailored surface chemistry are used in vitro in a routine setting for cell separation and in vivo as magneteic resonance imaging contrast agent, hyperthermia and drug delivery (Alexiou, *et al.*, 2004).

According to Kvitek and Prucek (2005), Nanoparticles are viewed by many as fundamental building blocks of nanotechnology. They are the starting point for many 'bottom-up' approaches for preparing nanostructured materials and devices. As such, their synthesis is an important component of rapidly growing research efforts in nanoscale science and engineering.

The preparation of uniform nanosized drug particles with specific requirements in terms of size, shape, and physical and chemical properties is of great interest in the formulation of new pharmaceutical products. Nanoparticles of a wide range of materials can be prepared by a variety of methods including dispersion methods (laser ablation), Condensation methods (chemical reduction), Condensation methods (photochemical reductions and radiolysis) and Condensation methods (the preparation of silverorganosols) (Kvitek and Prucek, 2005).

Resistance of bacteria to bactericides and antibiotics has increased in recent years due to development of resistant strains. Some antimicrobial agents are extremely irritant and toxic and there is much interest in finding ways to formulate new types of safe and cost-effective biocidal materials. Antimicrobial formulations in the form of nanoparticles could be used as effective bactericidal materials (Hamouda, *et al.*, 1999).

Silver has been widely used in the treatment of clinical diseases including newborn eye prophylaxis, topical burn wounds, orthopedic infection, it has been known to be disinfectant for about 1200 Years and has been widely used during this century in the treatment and so on (Zhao and Stevens, 1998). Silver ions, as an antibacterial component, have been used in formulation of dental resin composites and ion exchange fibers and in coating of medical devices (Sondi and Sondi, 2004).

Baker *et al.*, (2004) reported that product such as silver nitrate and silver sulfadiazine have been used in recent years to prevent bacteria growth in applications such as drinking water sterilization and burn care. Metallic silver has also been added to polymer fabrics and catheters for antimicrobial functionality. However, due to its high cost, it is not economically feasible to use large quantities of silver for obtaining antimicrobial surfaces.

According to Taylor *et al.*, (2005), silver serves as a potent antibacterial agent acting against an exceptionally broad spectrum of bacteria while exhibiting low toxicity to mammalian cells. Silver has long been used in the treatment of infection. Crede (1884, as cited in Taylor *et al.*, 2005) first used 1% silver nitrate to treat and prevent ocular infection. More recently, burn physician have used 0.5% silver nitrate and 1% silver sulfadiazine to treat major burn injuries. Similarly, Kumar and Munstedt (2004) reported that silver based antimicrobials capture much attention not only because of the non toxicity of the active Ag^+ to human cells but because of their novelty being a long lasting biocide with high temperature stability and low volatility.

Silver dressings are used regularly in the hospital setting to help control infections in major wounds and burns. Now, consumers can use silver for at-home first aid emergencies with the new Curad Silver Bandage line. New Curad Silver Bandages use silver in the wound pad as a natural antibacterial. Laboratory testing showed that silver in the dressing reduced the growth of bacteria like *S. aureaus, E. coli, E. hirae,* and *Pseudomonas aeruginosa*, a powerful germ that does not respond to many antibacterial, for 24 hours (Berger *et al.*, 1976).

The inert nature and antimicrobial efficiency of silver make it attractive option for the food processing and medical equipment industries. It is not toxic flammable or corrosive and will not cause bacteria to become resistant to antibiotics (www.addmaster.co.uk).

Catauro *et al.*, (2004) reported that there is much interst in silver containing glasses and cermics for use in bone replacement as well as wastewater treatment owing to the demonstrated antimicrobial effects. Jeong *et al.*, (2005), reported that sulfur is a good disinfectant and a toxic agent, but the Ag-sulfur composite is non-toxic. The addition of sulfur enhances the antibacterial properties and the stability of Ag^{+} ions.

Silver ions and silver based compounds are highly toxic to microorganisms showing strong biocidal effects on as many as 16 species of bacteria including *E. coli* (Spadaro *et al* ., 1974). In the last few decades, there has been increased interest antibacterial finishing on textile materials because of varieties environmental pollution. By the way, use of some organic antibacterial agents has been evaded up to now, since some unsafe halogen compounds of aromatic group have become a serious problem. From that point of view, silver at nano-level is very good antimicrobial agent (Yeo *et al.*, 2003).

According to Sondi and Sondi (2004), the preparation, characterization, surface modification, and functionalization of nanosized inorganic particles open the possibility of formulation of a new generation of bactericidal materials. They conducted a study in

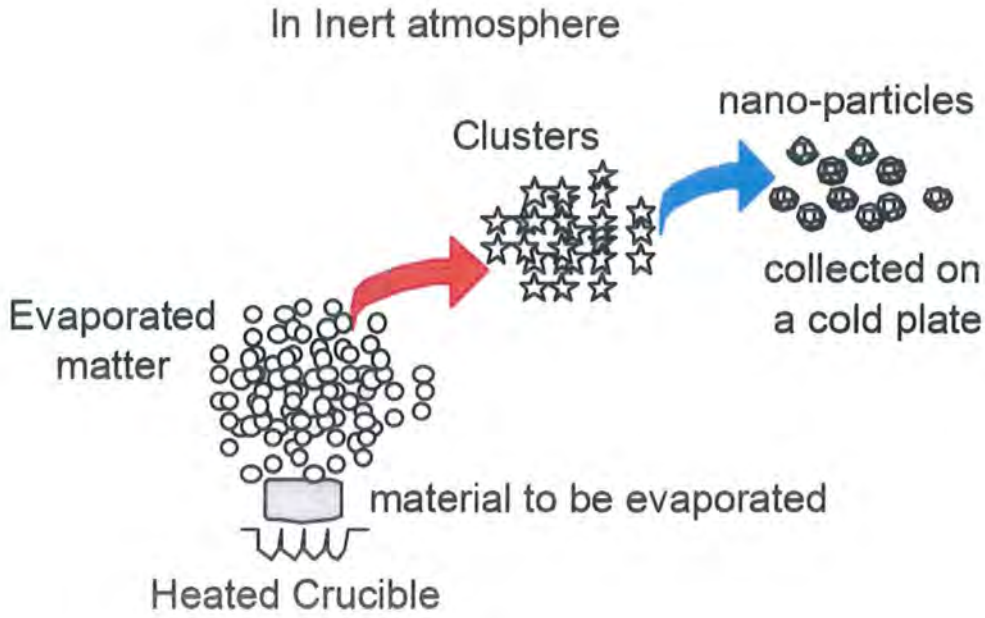


Figure: G. Schematics representation of Inert Gas Condensation synthesis method (J. Dutta and H. Hoffmann, 2003)

which the antimicrobial activity of silver nanoparticles against *E. coli* was investigated as a model for Gram-negative bacteria. These particles were shown to be an effective bactericide. Scanning and transmission electron microscopy (SEM and TEM) were used to study the biocidal action of this nanoscale material. Their results confirmed that the treated *E. coli* cells were damaged, showing formation of "pits" in the cell wall of the bacteria, while the silver nanoparticles were found to accumulate in the bacterial membrane. A membrane with such morphology exhibits a significant increase in permeability, resulting in death of the cell.

Jeong *et al.*, (2005), performed antibacterial tests using two types of bacteria; a Gram-positive microbe (*S. aureus*, ATCC 6538) and a Gram-negitive microbe (*E. coli*, ATCC 25922). The initial number of cultured bacteria was 1.6×10^5 cells/ml for the Gram-positive microbe and 1.2×10^5 cells/ml for the Gram-negative microbe. The cultured bacteria were inoculated with both the control polypropylene sample and the samples incorporating the various silver contents.The compounds containing very small amounts of Ag^+ nanoparticles exhibited almost perfect reduction against both kinds of Bacilli. For the silver nanoparticles (Silver I), they found that the compound having a 0.1% silver content displayed excellent antibacterial effect (bacterial reduction of 99.9%), but the micron-sized silver (Silver II) required a silver content >0.5 before exibiting good antibacterial activity. Therefore, the polymer compounds that contain the smaller silver particles have the stronger antibacterial activity, regardless of the Gram class. They concluded that for the production of compounds having antibacterial activity, the nano-sized silver is superior to micro-sized silver at the same weight.

Stoimenov *et al.*, (2002), reported that highly reactive metal oxide nanoparticles exhibit excellent biocidal action against Gram-positive and Gram-negative bacteria. Baker, *et al.*, (2005), synthesized nanometer sized silver particles by inert gas condensation and co-condensation techniques and the antibacterial efficiency of the nanoparticles was investigated by introducing the particles into a medium containing *E. coli*. The silver nanoparticles were found to exhibit antibacterial effect at lower concentrations. The antibacterial were related to the total surface area of the

nanoparticles. Smaller particles with a larger surface to volume ratio provided a more efficient means for antibacterial activity. The nanoparticles were found to be completely cytotoxic to *E. coli* for surface concentrations of as low as 8 $\mu g/cm^2$ of Ag.

Jeong *et al.*, (2005), prepared PE/PP nonwovens using various kinds of nano-sized silver colloids and compared their antibacterial efficacy against three kinds of bacteria: *S. aureus, Klebsiella pneumoniae*, and *E. coli* these silver colloids comprised of silver nanoparticles that are a non-toxic and non-tolerant disinfactant. PE/PP nonwovens are used as back sheats or cover stocks baby diapers, adult diapers, sanitary napkins, and wipes. These materials are readily contaminated by bacteria present in moisture and dirt and can cause disease. They concluded that antibacterial properties increase as the concentration of the nano-sized silver colloids increased. They observed many *S.aureus* were present on untreated nonwovens. Similarly *K. pneumoniae*, a Gram-negative, stronger bacterium, was infected by the silver nanoparticles.

Similarly another study conducted by Li *et al.*, (2005), assessed the antimicrobial activity of nanoparticles (consisting of a mixture of silver nitrate and titanium dioxide) and nanoparticle-coated facemasks to protect against infectious agents. They observed 100% reduction in viable *E. coli* and *S. aureus* in the coated mask materials after 48hrs of incubation. Skin irritation was not observed in any of the volunteers who wore the facemasks. They concluded that Nanoparticles show promise when applied as a coating to the surface of protective clothing in reducing the risk of transmission of infectious.

Lee, *et al.*, (2005) conducted a study in which antibacterial coatings based on hydrogen-bonded multilayers containing synthesized Ag^0 nanoparticles were created on planar surfaces and on magnetic colloidal particles. They reported the antibacterial properties of these coatings, as a function of the film thickness and the concentration of Ag^0 nanoparticles in the hydrogen-bonded multilayers. Results obtained for the values of the zone of inhibition depended linearly on the logarithm of the thickness of the silver-loaded films. They suggested that, in order to incrementally increase the zone of inhibition, an exponentially increasing amount of Ag^0 is required within the multilayers. In general,

there was no statistically significant correlation between the zone of inhibition and the number of Ag^0 loading and reduction cycles. The duration of sustained release of antibacterial Ag^+ ions from these coatings, however, could be prolonged by increasing the total supply of zero valent silver in the films via multiple loading and reduction cycles. These results indicate that the release of silver is controlled by an oxidation mechanism at the surface of the nanoparticles and that repeated loading and reduction of silver leads preferentially to growth of the existing silver nanoparticles in the film as opposed to nucleation of new Ag^0 nanoparticles. They show that magnetic microspheres coated with silver nanoparticles loaded hydrogen-bonded multilayer thin films can be used to deliver antibacterial agents to specific locations. Antibacterial magnetic microspheres with higher concentrations of Ag^0 nanoparticles exhibited lower minimum inhibitory concentration values.

Yeo *et al.*, (2003) prepared nanocomposite fibers for the attainment of permanent antibacterial activity to common synthetic textile. The antibacterial activity of nanosilver in fibers was evaluated after certain contact time and calculated by percent reduction of two kinds of bacteria; *S. aureus* and *K. pneumonieae.* Their SEM results showed the silver nanoparticles in the fibers have relatively good dispersion. In the results of antibacterial test, the fibers containing silver nanoparticles in core-part had not nearly significant antibacterial activity. However, they concluded that the fibers having silver in sheath-part showed excellent antibacterial effect.

According to Kokkoris *et al.*, (2002), composite $AgSiO_2$ thin coating containing nanoparticles prepared by sol-gel route show high antibacterial activity. Even at low metal concentration exhibit sufficient activity for *E. coli* bacteria elimination after 24 hrs treatments. It was concluded that the differences in the antibacterial activity were attributed to the difference in the distribution of silver in the coating, as well as to the formation of silver nanoparticles with a larger diameter and correspondingly lower surface area, due to the additional reductive thermal treatment.

Thermal stability of heat-treated nanocrystalline silver dressing was investigated using techniques and biological assays. Dressings were heat-treated for 24 hrs in a temperature range of 23 to110°C. It was clear from the research that the quantity of soluble silver decreased significantly with increased heat treatment temperatures. The results of these experiments indicate that in order to use a metallic silver dressings as an effective antimicrobial agent, soluble silver must be present in the nanocrystalline silver dressings. These results also indicate that nanocrystalline silver is thermally unstable, and this instability can have a significant impact on its chemical and biological properties (Taylor *et al.*, 2005).

Nanocrystalline silver dressings have been shown to releases both Ag^0 (possibly as clusters) and Ag^+ in to solution. Since silver in solution is generally thought to be antimicrobial, a complete lose of antibacterial activity indicates that the dressing has run out of soluble silver (Fan and Bard 2002). Nanocrystalline silver dressing heat-treated at temperatures from 90 to 110 °C, the decreased days of activity correlated with the decrease in silver dissolution. They concluded that the antibacterial activity of *P. aeruginosa* was less than *S. aureus*, most likely due to greater sensitivity of *P. aeruginosa* to silver (Yin *et al.*, 1999).

Silver ions, as an antibacterial component are used in the formulation of dental resin composites, ion exchange fibers and in the coating of medical devices. Recently, Tiller and co-workers (as cited in Sondi and Sondi, 2004) showed that hybrid of silver nanoparticles with amphiphilic hyper-branched macromolecules exhibit effective antimicrobial surface coating (Sondi and Sondi, 2004).

Lee *et al.*, (2004), reported that nanosized Ag^0 particles were entrapped successfully in mutihollow porous polymethyl methacraylate (PMMA) microspheres by water-in-oil-in-water emultion polymerization. In order to evaluate the biological function in the form of polymer composites, they performed antibacterial test of PMMA/Ag^0 microspheres in the aqueous dispersion. They evaluated the preservation performance by counting the number of bacteria in the sample with storage time at 32 °C. They found that in the

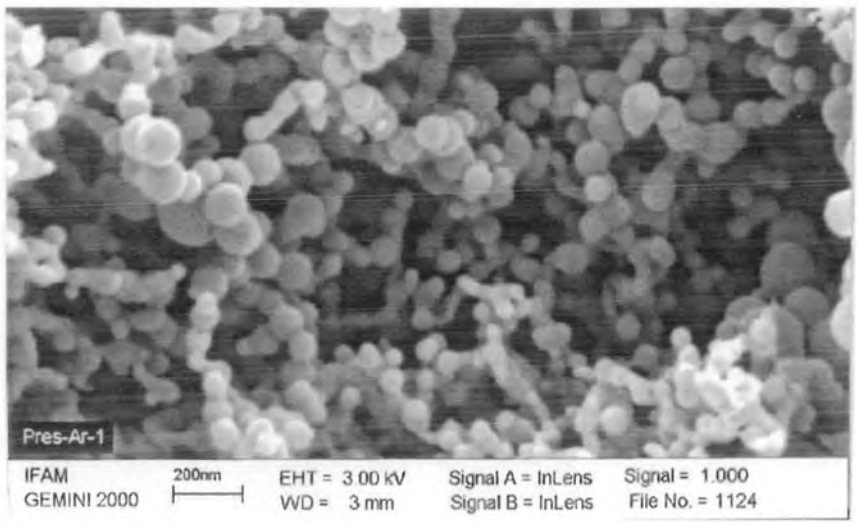


Figure: H. Scanning electron micrograph of silver nanoparticles (Mehmet Turker, 2004)

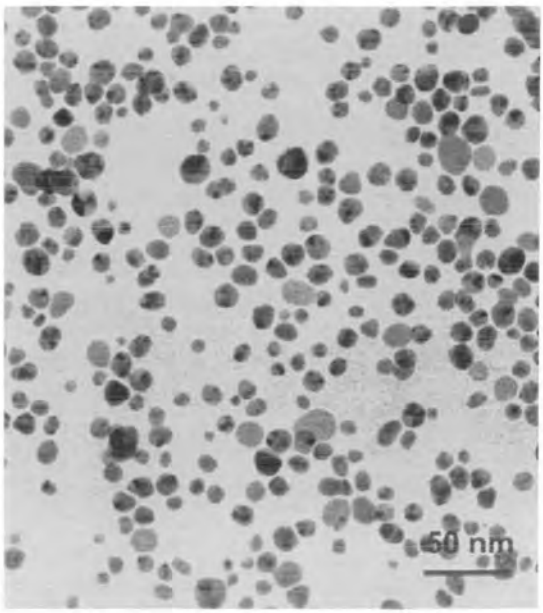


Figure: I. Transmission electron micrograph of silver nanoparticles (Ivan Sondi, 2004)

absence of Ag^0, the numbers of bacteria remain constant. However, on adding Ag^0 to the test formulations, the number of bacteria decreased dramatically. Moreover, as the concentration of Ag^0 increased, the number of bacteria decreased more sharply. Within 1 week, most of the initially inoculated bacteria disappeared.

Nanomedicine is the application of nanotecnology to the prevention and treatment of diseases in human body. This dicipline is in its infancy. It has the potential to change medical science dramatically in the 21st century. The most elementory nanomedical devices will be used for diagonosis. Chemical tests exist for this purpose; nanomedicine could be employed to monitor the internal chemistry of the body (Thalhammer and Heckl, 2004). The application of Biotecnology, such as medical implants of organic materials, cancer treatment by direct targeting of medicine that can navigate and detect the bad cells to destroy them, genetic therapy stemming from a better understanding of human genome, and new organically synthsized bone materials are other promising areas of research of and applications of nanotecnology (Olavarrieta, 2004).

Material & Methods

Materials and Methods

3.1 Microorganisms

Escherichia coli ATCC 15224, and *Staphylococcus aureus* ATCC 6538, were obtained from the American Type Culture Collection (ATCC) USA, and *Escherichia coli* strain locally isolated from urinary tract infectious patients used in the study were obtained from the Microbiology Research Laboratory, Department of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

3.2 Preparation of Silver Nanoparticles

Nanometer sized silver particles (Ag^0) (average particle size of 16 nm) were synthesized by inert gas condensation (IGC) technique. This technique is based on the evaporation of a metal into an inert atmosphere with the subsequent cooling for the nucleation and growth of nanoparticles. The obtained powder was fully dispersed in pre-sterilized deionized water through sonication for five minutes to make desired concentrations of the aqueous dispersions of silver nanoparticles. Ag^0 nanoparticle's samples were received from Mr. Muhammad Raffi, PhD Scholar, Pakistan Institute of Engineering and Applied Sciences (PIEAS), Islamabad.

3.3 Chemicals

The majority of the chemical compounds and media components were obtained from BDH laboratory chemicals division (Poole, Dorset, England), DIFCO Laboratories (Detroit, Michigan, USA), Fluka-garanite CH-9470 Buchs, Sigma Chemical Co., St.Louis, and E.Merck (Germany).

Indole Production Test

Solutions and reagents required to check the production of indole were prepared as follows:

Peptone Water (pH 6.9)

Contents	**g/l**
Peptone	7.0
Dextrose	5.0
Potassium phosphate	5.0

Kovac's Reagent (Indole)

Amyl Alcohol	75ml
P-dimethylamino benzaldehyde	2mg
Hydrochloric acid (conc.)	25ml

For Indole production autoclaved peptone water was aseptically inoculated with isolates of 24 hrs culture and incubated overnight at 28°C. This test was used to determine the ability of microorganisms to degrade the amino acid tryptophane and convert it to indole pyruvic acid and ammonia. The presence of indole was detected by adding Kovac's Reagent. Production of cherry-red reagent layer indicated the presence of indole while absence of the red layer confirmed its absence.

Citrate Utilization Test

Simmons Citrate agar slants were aseptically inoculated and incubated for 24 hrs at 28°C.This test was used to determine the ability of bacteria to ferment citrate as a sole source of carbon. Positive test was accompanied by blue coloration and negative results showed no growth and the slant remained green.

Solution: B

Contents	**g/100ml**
Potassium Hydroxide	40.0g
Distilled water	100.0ml

Upon completion of incubation period, isolates grown in test tubes were divided into two parts and transferred in to fresh sterile test tubes. To one part 1-2 drops of methyl red indicator were added. A bright red color was taken as positive test while no color change was considered as negative test.

To the second part, 1ml of potassium hydroxide (Solution B) and 3ml of alpha-naphthol were added. The positive reaction was a development of pink color within 2-5 minutes. During this time interval test tubes were constantly shaken to maintain reaction.

Oxidase Test

Filter paper was moistened with a few drops of 1% tetramethyl-p-phenylenediamine dihydrochloride. With a wooden applicator, growth from nutrient agar plate was smeared on the paper. A positive result was the development of pink color. No color change indicated a negative result.

Catalase Test

Single colony of each bacterial growth was taken by a sterilized wooden applicator and was put on a glass slide. Then a drop of H_2O_2 was poured onto the colony and the production of bubbles indicated positive result and the absence of bubbles was a negative catalase test.

Carbohydrate Fermentation (Gas Production)

Phenol red broth containing glucose was used for this test. Inverted Durham tubes were approximately inoculated with each of the isolate and incubated for 24 hrs at 28°C. The

pH indicator, phenol red, is red at neutral pH, and changes to yellow at a slightly acidic pH of even 6.8. Gas production is shown by the presence of air bubbles in Durham tubes.

3.5 Bactericidal Tests

Bactericidal effect of silver nanoparticles was examined on different bacterial strains. Six flasks of 100ml capacity in triplicate were used in the experiments. Each flask containing 45 ml of nutrient broth was autoclaved at 121°C at 15psi for 15 minutes. The 48 hrs old bacterial cells were compared with 0.5 McFarland standard solution (CFU 10^4/ml). 1ml of inoculum was added in all flasks. After inoculation, different concentrations of silver nanoparticles (20, 40, 60, 80 and 100µg/ml of Ag^0) and sterilized deionized water were added to maintain 50ml of volume of all the flasks for selected bacterial strains (*E. coli* ATCC, *E. coli* UTI, *S. aureus* ATCC). A flask containing 45ml of nutrient broth with 4ml- deionized water and Inoculum but without Ag^0 nanoparticles was used as control. OD of these samples was periodically recorded every two hours starting from zero hour up to 24 hours of incubation in shaker incubator at 37 °C, 150 rpm. OD was measured again at 48 hours of incubation. The OD and CFU values clearly indicated inhibition of these bacterial strains in the presence of silver nanoparticles.

3.6 Analytical Methods

Agilent model 9453 Japan UV-visible recording spectrophotometer UV was used in absorbance mode to determine the bacterial growth by measuring ODs of samples at λ600 nm and also by counting the colony forming units (**CFU**).

3.7 Viable Cell Counts

Viable cell counts were performed to determine the growth of bacteria. Serial dilutions of the cultures were made in pre-sterilized normal saline (0.9% wt/vol NaCl). One ml of culture was diluted in series, ten folds each time. A sterile pipette was used to dispense 0.1 ml of the dilutions in triplicate was used. The plates were incubated at 37 °C for 24

3.21 Scanning Electron Microscopy

The method of P. J. Goodhew and F. J. Humphreys (1988) was used for scanning electron microscopy. The method is described below.

Sample Preparation for SEM

- *E. coli* ATCC cultures were inoculated in nutrient broth medium with different concentrations of Ag^0 nanoparticles. The flasks were incubated at 37 °C and 150 rpm for 24 hrs.
- After 24 hrs, inoculum of *E. coli* ATCC, untreated and treated with various concentrations of Ag^0 nanoparticles was centrifuged at 10,000 rpm for 10 minutes, supernatant was discarded and pellet was recovered.
- The cell biomass was gradually treated with solution of deionized water and absolute ethanol to avoid direct shock of absolute ethanol to the cells that might physically damage the cells.
- Finally, bacteria cell treated with pure ethanol were fixed on the aluminum SEM stubs. These sample-coated stubs were allowed to get dry in desiccators. These stubs were then coated with a thin layer of gold. Surface morphology of the bacteria was analyzed on SEM LEO-441-I.

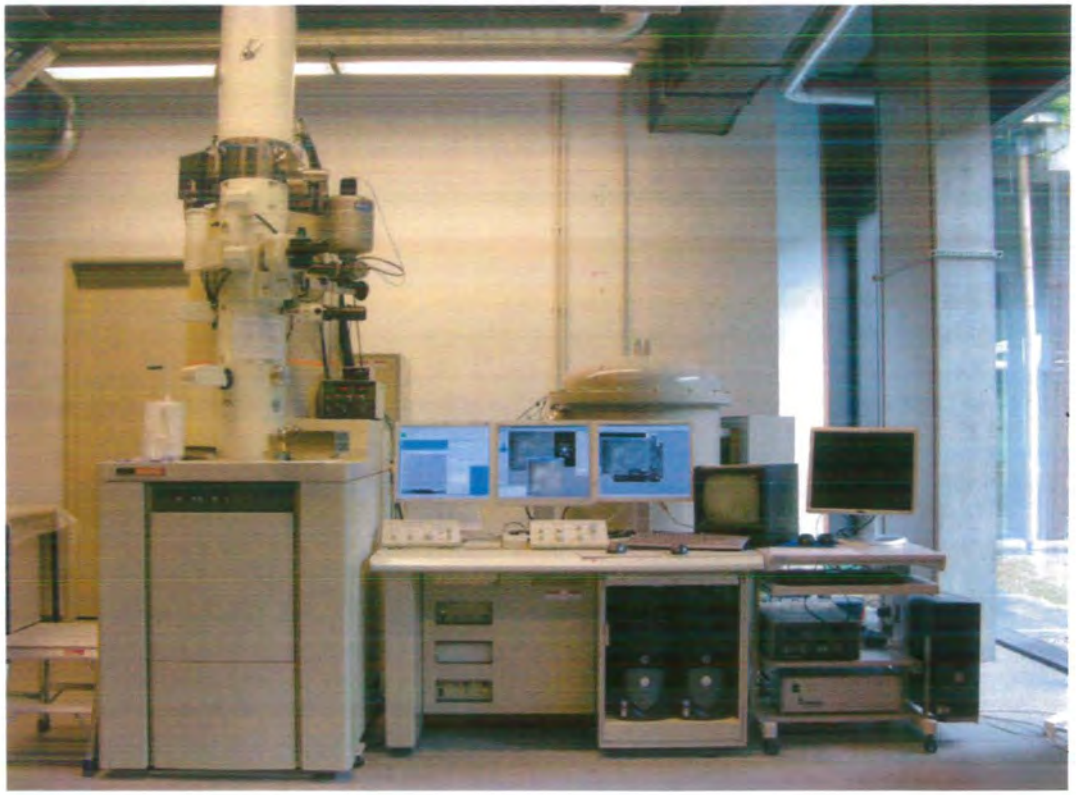


Figure: L. Transmission electron microscope (www.jeol.com)

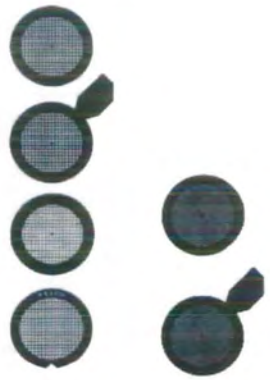


Figure: M. Copper grids used as sample holders for transmission electron microscopy (www.jeol.com)

3.13 Transmission Electron Microscopy

The method of Bechtel and Bulla (1976) was used for transmission electron microscopy. The method is described below.

Sample Preparation for TEM

- *E. coli* ATCC cultures were inoculated in nutrient broth medium with different concentrations of Ag^0 nanoparticles. The flasks were incubated at 37 °C and 150 rpm for 24 hrs.
- After 24 hrs, inoculum of *E. coli* ATCC, untreated and treated with various concentrations of Ag^0 nanoparticles was centrifuged at 10,000 rpm for 10 minutes, supernatant was discarded and pellet was recovered.
- To avoid the cell damage ethanol solution was used in which the concentration of ethanol was gradually increased.
- Treated samples of *E. coli* ATCC with absolute ethanol were then fixed on carbon-coated copper transmission electron microscopy grids.
- After fixing the samples on copper grids, a drop of double distilled water was used to disperse the samples uniformly on the carbon coated copper grid.
- For improving the image contrast uranyl acetate was used for staining the sample on grids.
- Samples were dried in the desiccators then these were fixed in the TEM (Joel- 100-CX-II) samples holders for analysis.

Results

RESULTS

The present research has demonstrated the efficiency of silver nanoparticles for inhibiting the growth of bacterial colonies. The bacterial strains were obtained from the Microbiology Research Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad, which were *E. coli* ATCC 15224, *S. aureus* ATCC 6538, and third strain *E. coli* that was isolated from urinary tract infectious patients. *E. coli* (UTI) has been identified as gram negatives rods by staining behavior Oxidase and catalase tests were positive. Pure cultures were maintained on nutrient agar plates and slants to perform experiments with different concentrations of silver nanoparticles.

4.1 Identification of *E. coli* Locally Isolated From Urinary Tract Infectious Patients

Isolated strains were identified on the basis of routine morphological and biochemical tests (Buchanan and Gibbons, 1974). Table 4.1a/ 4.1b: Morphology and biochemical test for identification of *E. coli.*

4.2 Effect of Silver Nanoparticles on The Growth of Different Bacterial Strains

Silver nanoparticles produced by IGC were added to nutrient broth medium containing bacterial strains to evaluate antibacterial efficacy of these nanoparticles. Nanoparticles were added at the beginning of bacterial cell growth at concentration ranging between 0 (control) and 100μg/ml of Ag^0. ODs of samples were measured after every 2 hrs up to 24 hrs and at 48 hrs.

In the sample containing 20μg/ml of Ag^0 nanoparticles, optical density decreased with respect to the control sample, indicating a reduced concentration of bacterial cell growth. For all higher concentrations of silver nanoparticles, there were gradual decreases in bacterial cell growth.

4.2.1 Effect of Silver Nanoparticles on Growth of *E. Coli* ATCC in the Nutrient Broth Medium

Antibacterial test were performed against Gram-negative bacterium *E. coli* ATCC on the nutrient broth medium containing different concentrations of Ag^0 nanoparticles. OD significantly changed at 6 hrs with respect to control (containing no Ag^0 nanoparticles). There was virtually no bacterial growth up to 8 hrs in the sample containing 40, 60, 80, and 100μg/ml of Ag^0. Bacterial growth was observed in the samples containing 20μg and 40μg/ml of Ag^0 up to 24 hrs but in these samples there were still delayed growth behavior of microbes with respect to control. In the samples that contained 60 to 100μg/ml of Ag^0, no bacterial growth was observed. Bacterial growth pattern observed up to 48 hrs showed different patterns as all samples showed growth except 100μg/ml of Ag^0 sample that had complete inhibition. In rest of the samples, OD showed the delayed growth pattern with the increase in Ag^0 nanoparticles concentration a compared to the control (Table 4.2.1a, Fig. 4.2.1b, Fig. 4.2.1c).

4.2.2 Effect of Silver Nanoparticles on the Growth of *E. coli* (UTI) in the Nutrient Broth Medium:

Same Antibacterial test were performed against Gram-negative bacterium *E. coli* (UTI) on the nutrient broth medium containing different concentrations of Ag^0 nanoparticles. OD significantly changed at 6 hrs with respect to control (contains no Ag^0 nanoparticles). There was virtually no bacterial growth up to 8 hrs in the sample containing, 60, 80, and 100μg/ml of Ag^0. Bacterial growth was observed in the sample containing 40 μg/ml of Ag^0 and showed slightly different behavior through some extent from *E. coli* ATCC. At 24 and 48 hrs of incubation, bacterial growth was monitored in all samples but ODs values of these samples showed delayed behavior of microbes with respect to control but there was no complete inhibition in any sample. (Table 4.2.2a, Fig. 4.2.2b, Fig. 4.2.2c).

The 98 X 10^7 CFU/ml cell count was observed by *S. aureus* ATCC in the absence of Ag^0 nanoparticles, which is the highest growth rate. At the concentrations of 20µg/ml of Ag^0, and 40µg/ml of Ag^0 the cell count was 50 x 10^7 CFU/ml and 45 x 10^7 CFU/ml respectively. The growth of bacteria gradually decreased by increasing the Ag^0 nanoparticles concentration but the *S. aureus* ATCC did not show 100% inhibition, as was the case in *E. coli* (UTI). At the concentrations of, 60, 80, and 100µg/ml of Ag^0, 41 x 10^7 CFU/ml, 35 x 10^7 CFU/ml, and 20 x 10^7 CFU/ml of cell counts was observed in 24 hrs of incubation respectively (Table 4.3a, 4.3b).

4.4 Estimation of Protein in Different Indicator Strains at 8 and 24hrs of Incubation with Various Concentrations of Ag^0 Nanoparticles

Proteins estimation in the samples was carried out by Lowery's method. In the supernatant of *E. coli* ATCC incubated for 8 hrs, it was observed that the sample without silver nanoparticles showed high concentrations of protein (56.8 µg/ml). A decreasing trend in protein estimation was observed as the concentration of silver nanoparticles increased in the samples. The lowest noted protein value was 48.7 mg/ml in the sample containing 100µg/ml of Ag^0 at 8 hrs. A similar pattern of protein concentration was observed in all samples at 24 hrs. The similar sequences of protein estimation were observed in *S. aureus* ATCC and *E. coli* (UTI) at 8 and 24 hrs of incubation, respectively (Table 4.4a, Fig. 4.4b).

4.5 Agar Well Diffusion Assay

Agar diffusion assay method shows the antimicrobial potential of Ag^0 nanoparticles against different indicator strains of bacteria. The zones of inhibition were measured and found to be 21mm for *E. coli* ATCC by incubating in the presence of 100µl of Ag^0 nanoparticles. The measurements of zones of inhibition of *E. coli* (UTI) was 17 mm by using 100µl of Ag^0 nanoparticles while *S. aureus* ATCC gave a measurement of 19 mm for the zone of inhibition by using the same concentration of Ag^0 nanoparticles (Table 4.5a, Fig. 4.5b).

4.6 Plasmid Curing

The CFU and OD values showed that after certain period of inhibition, the indicator strain (*E. coli* ATCC) starts its growth pattern. After plasmid curing, first highest concentration of ethidium bromide, in which *E. coli* ATCC showed turbidity, was selected as titer. Same experiment was revised by using titer as the inoculum with different concentration of Ag^0 nanoparticles, but no change in the growth pattern of bacteria was observed as shown by OD values. It is evident from the plasmid curing that this activity is not plasmid mediated.

4.7 Scanning Electron Microscopy

The SEM result showed surface interaction of *E. coli* ATCC with Ag^0 nanoparticles. It was observed that nanoparticles get attached with the cell wall of bacteria. The parts of damage bacteria also observed in the SEM micrograph (Fig. 4.7b).

4.8 Transmission Electron Microscopy

The TEM showed the penetration of Ag^0 nanopaticles inside the *E. coli* ATCC due to the penetration of Ag^0 nanoparticles inside the cell caused the loss of distinctiveness of the cell membrane. Flacks of ruptured and damaged cells were also observed in the micrograph. The black aggregates observed in TEM micrographs were the Ag^0 nanoparticles (Fig. 4.8b). .

Table 4.1a: Morphology of *E. coli*

Characteristics	*E. coli*
Shape	Rod
Gram staining	Negative
Agar slant culture characterization	White, moist, glistening, growth

Table 4.1b: Biochemical Test for Identification of *E. coli*

Test	Result
Citrate utilization	–
Litmus milk reaction	Acid, curd ±, gas ±, reduction ±
Indole production	+
Oxidase activity	–
Catalase activity	+
VP result	–
MR result	–
Fermentation (lactose, dextrose, sucrose, and H_2S production)	AG, AG, A±, negative respectively

Table 4.2.1a Effect of Ag^0 Nanoparticles on the Growth of *E. coli* ATCC in the Nutrient Broth Medium (n.d = not detected)

Various conc. of Ag^0 nanoparticles	Optical density at λ 600 nm					
	2hrs.	4 hrs.	6 hrs.	8 hrs.	24 hrs.	48 hrs.
W/O Ag^0	n.d	n.d	.468	.625	1.366	1.565
20 µg/ml of Ag^0	n.d	n.d	.195	.455	.631	1.445
40 µg/ml of Ag^0	n.d	n.d	n.d	n.d	.227	.418
60 µg/ml of Ag^0	n.d	n.d	n.d	n.d	n.d	.166
80 µg /ml of Ag^0	n.d	n.d	n.d	n.d	n.d	.146
100 µg/ml of Ag^0	n.d	n.d	n.d	n.d	n.d	n.d

Table 4.2.2a Effect of Ag^0 Nanoparticles on the Growth of *E. coli* (UTI) in the Nutrient Broth Medium

Various conc. of Ag^0 nanoparticles	Optical density at λ 600 nm					
	2hrs.	4 hrs.	6 hrs.	8 hrs.	24 hrs.	48 hrs.
W/O Ag^0	n.d	n.d	.463	.621	1.258	1.415
20 µg/ml of Ag^0	n.d	n.d	.227	.481	.931	1.341
40 µg/ml of Ag^0	n.d	n.d	.112	.327	.603	1.221
60 µg /ml of Ag^0	n.d	n.d	n.d	n.d	.322	.645
80 µg/ml of Ag^0	n.d	n.d	n.d	n.d	.125	.263
100 µg/ml of Ag^0	n.d	n.d	n.d	n.d	.110	.160

Table 4.2.3a Effect of Ag^0 Nanoparticles on the Growth of *S. aureus* ATCC in the Nutrient Broth Medium (n.d = not detected)

Various conc. of Ag^0 nanoparticles	Optical density at λ 600 nm					
	2hrs.	4 hrs.	6 hrs.	8 hrs.	24 hrs.	48 hrs.
W/O Ag^0	n.d	n.d	.453	.635	1.288	1.510
20 µg/ml of Ag^0	n.d	n.d	.265	.513	.875	1.421
40 µg/ml of Ag^0	n.d	n.d	.205	.437	.525	1.251
60 µg/ml of Ag^0	n.d	n.d	n.d	n.d	.483	.587
80 µg/ml of Ag^0	n.d	n.d	n.d	n.d	.391	.428
100 µg/ml of Ag^0	n.d	n.d	n.d	n.d	.160	.302

Table 4.3a: Cell Count (CFU/ml) of Indicator Strains at 24 hrs of Incubation (n.d = not detected)

Different conc. of Ag^0 of nanoparticles	*E. coli*, ATCC 15224	*E. coli*, UTI	*S. aureus*, ATCC 6538
W/O Ag^0 nanoparticles	95×10^7	94×10^7	98×10^7
20 µg/ml of Ag^0	51×10^7	56×10^7	50×10^7
40 µg/ml of Ag^0	30×10^7	52×10^7	45×10^7
60 µg/ml of Ag^0	n.d	30×10^7	41×10^7
80 µg/ml of Ag^0	n.d	20×10^7	35×10^7
100 µg/ml of Ag^0	n.d	18×10^7	20×10^7

Table 4.4a: Estimation of Protein in Different Indicator Strains at 8 and 24hrs. of Incubation With Various Concentrations of Ag^0 Nanoparticles

Different conc. of Ag^0 of nanoparticles (µg/ml of Ag^0)	Estimation of protein in different indicator strains with various concentrations of Ag^0 nanoparticles at λ 650 nm					
	E. coli ATCC		*E. coli* (UTI)		*S. aureus* ATCC	
	8 hrs.	24 hrs.	8 hrs.	24 hrs.	8 hrs.	24 hrs.
W/O Ag^0	56.8	44.3	57.6	47.5	72.2	41.6
20	55.6	40.5	53.7	42.4	67.7	36.4
40	53.7	39.8	52.1	39.2	63.1	35.0
60	51.2	48.8	51.3	38.1	53.7	32.3
80	52.4	48.5	50.7	36.9	52.4	29.9
100	48.7	47.0	49.7	35.4	49.7	26.8

Table 4.5a: Agar Well Diffusion Assay

Agar well diffusion assay	Zones of inhibition (mm)		
	E. coli ATCC	*E. coli* (UTI)	*S. aureus* ATCC
Ag^0 nanoparticles (100 µl)	21mm	17mm	19mm

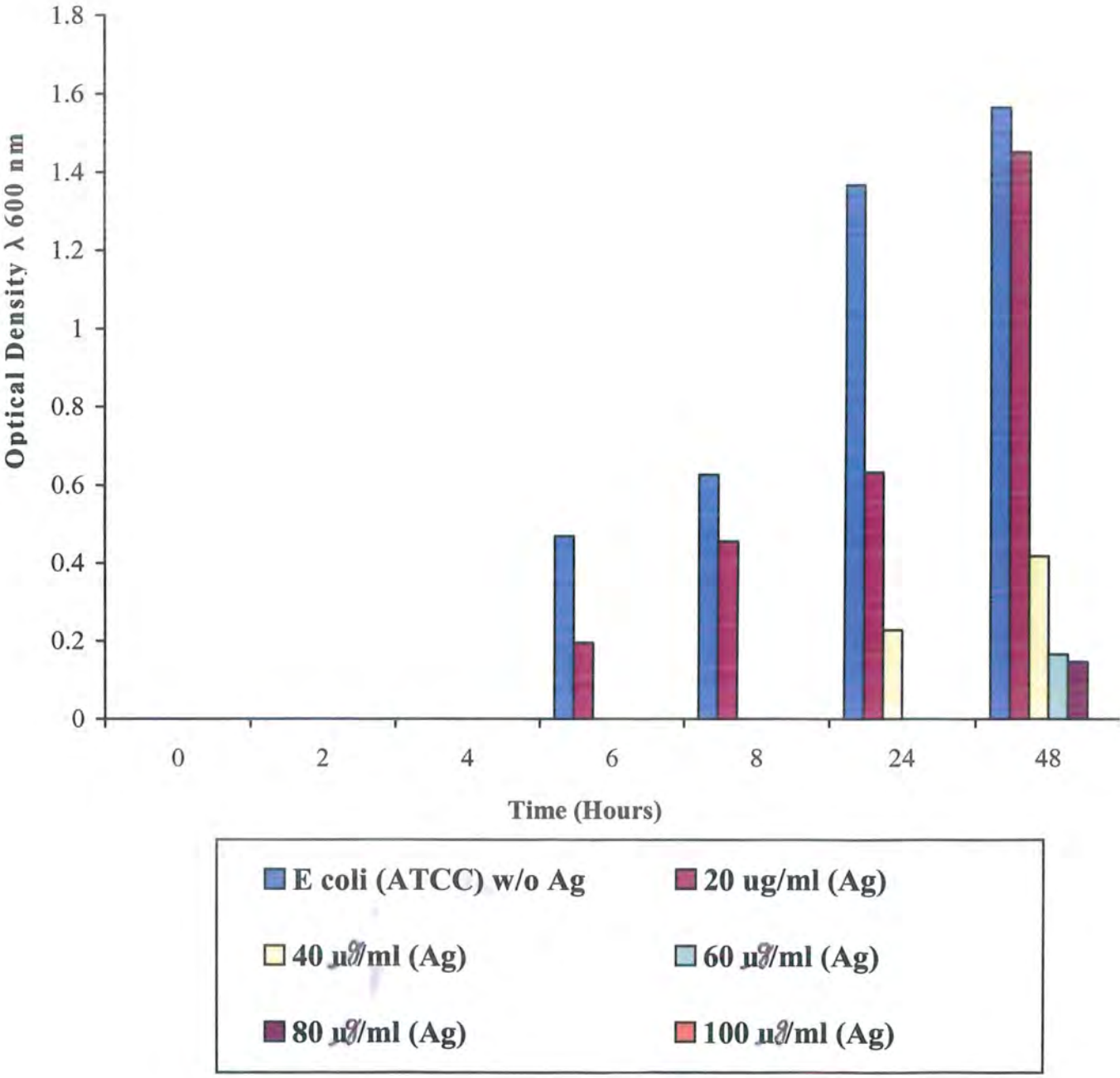


Figure: 4.2.1 b. Optical Density of *E. coli* ATCC solutions with varying concentrations of Ag nanoparticles synthesized by IGC

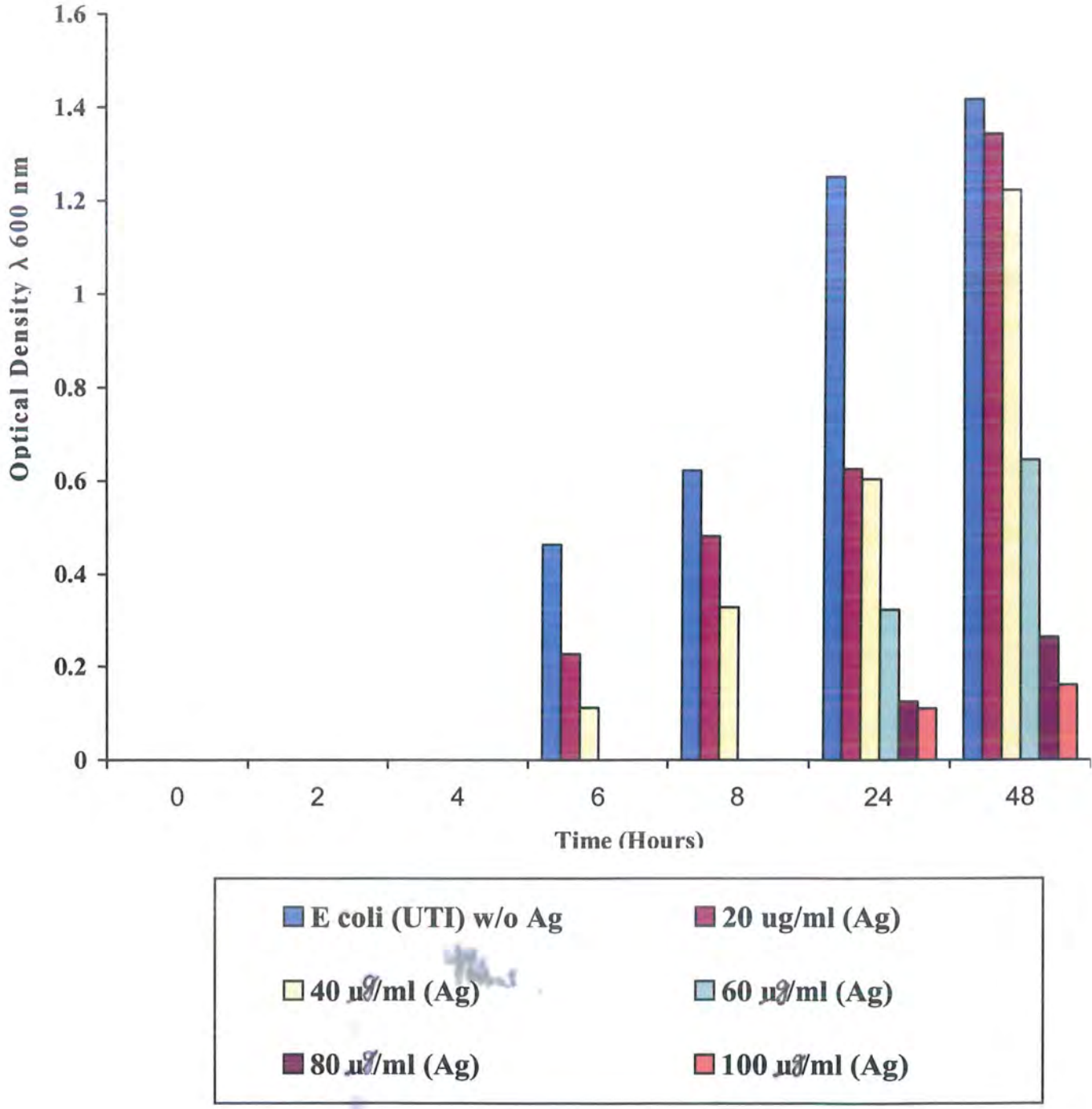


Figure: 4.2.2 b. Optical Density of *E. coli* isolated from UTI solutions with varying concentrations of Ag nanoparticles synthesized by IGC

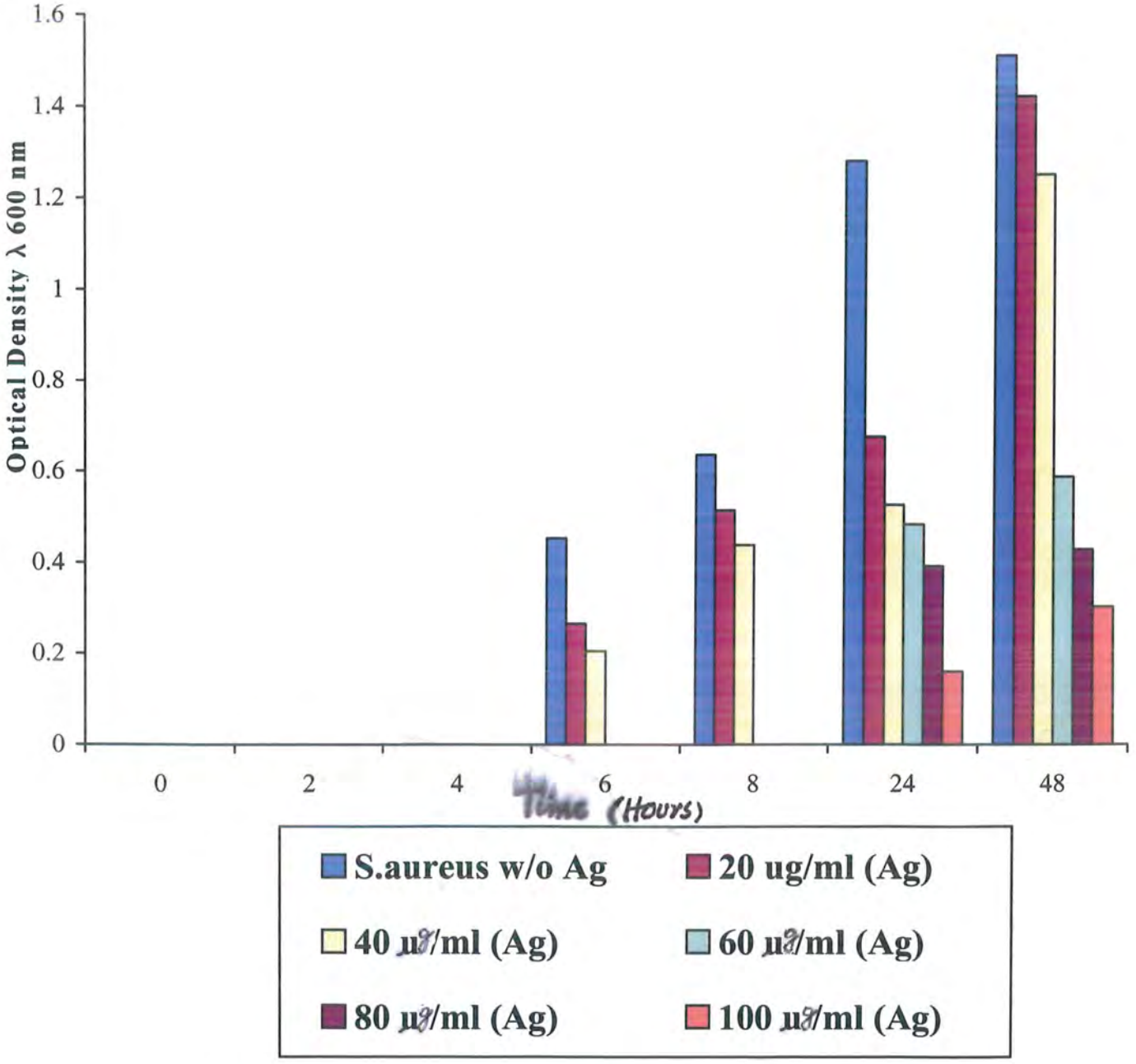


Figure: 4.2.3 b. Optical Density of *S. aureus* ATCC solutions with varying concentrations of Ag nanoparticles synthesized by IGC

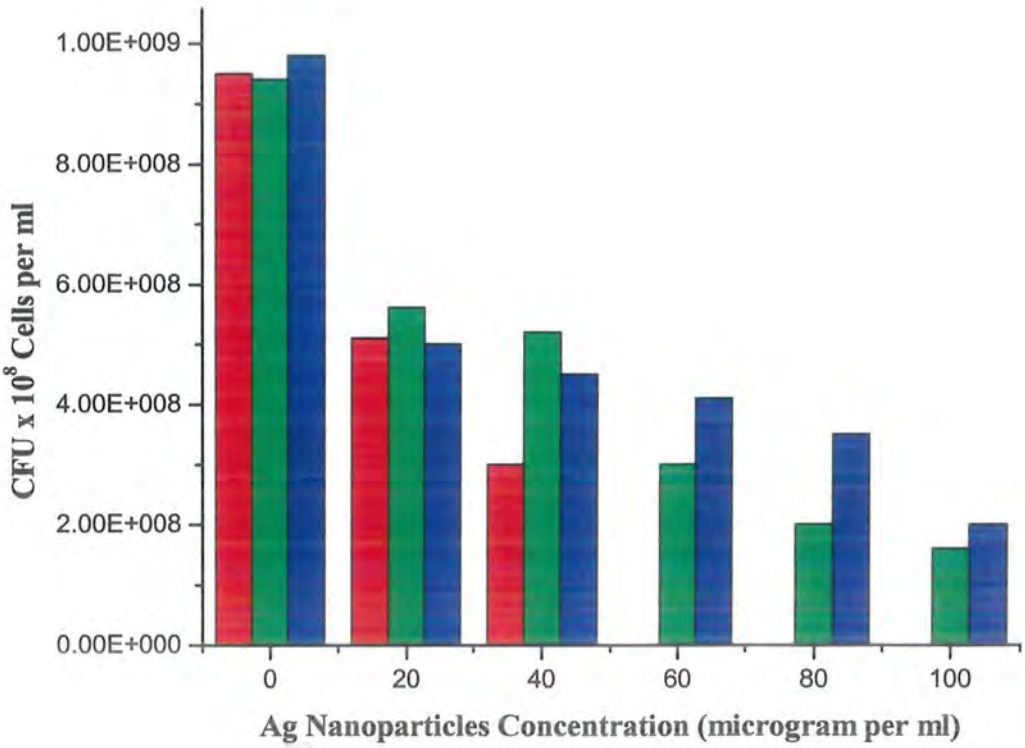


Figure 4.3 b. CFU and Ag nanoparticles concentration (μg/ml) (red) *E. coli* ATCC- 15224 (green) *E. coli* UTI (blue) *S. aureus* ATCC- 6534

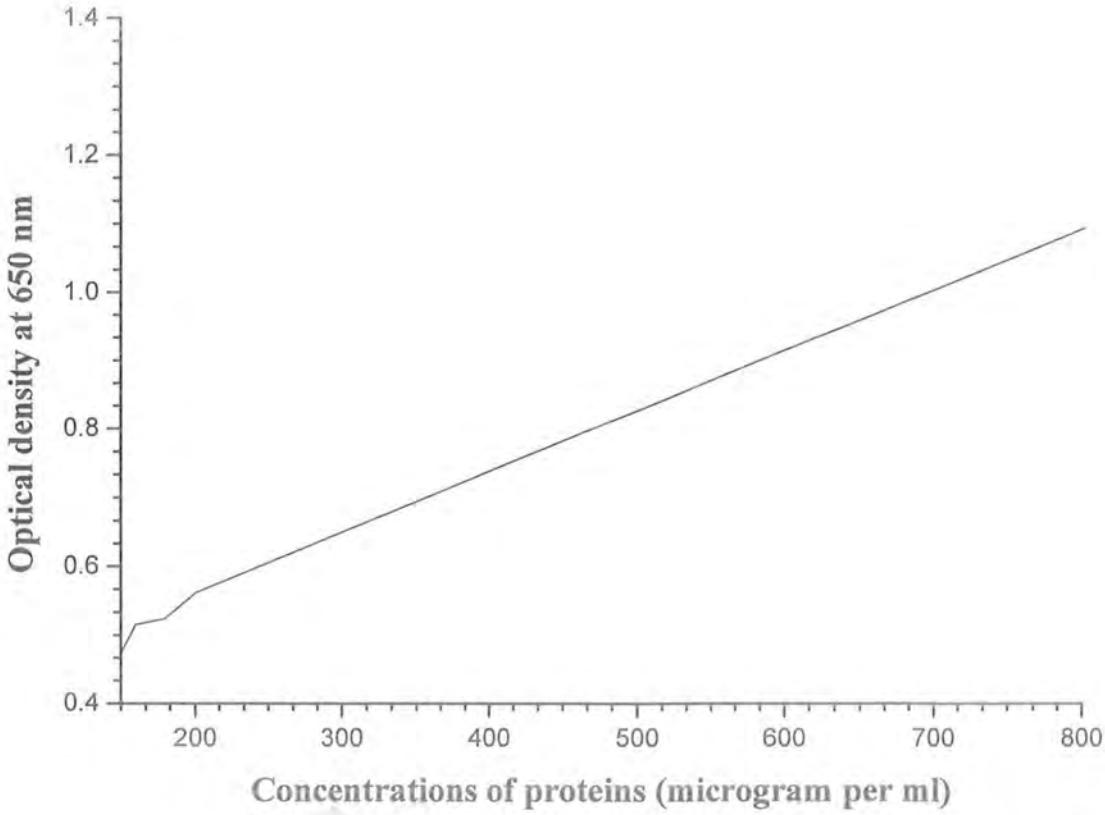


Figure 4.4 b. Standard curve of BSA

Fig. 4.2.1c: Effect of Ag nanoparticles on the growth of *E. coli* ATCC 15224 at 24 hrs. of incubation.

Fig. 4.2.1d: Effect of Ag^0 nanoparticles on the growth of *E. coli* ATCC 15224 at 48 hrs. of incubation.

Fig. 4.2.2c: Effect of Ag^0 nanoparticles on the growth of *E. coli* (UTI) at 24 hrs. of incubation.

Fig. 4.2.3c: Effect of Ag^0 nanoparticles on the growth of *S. aureus* ATCC 6538 at 24 hrs. of incubation.

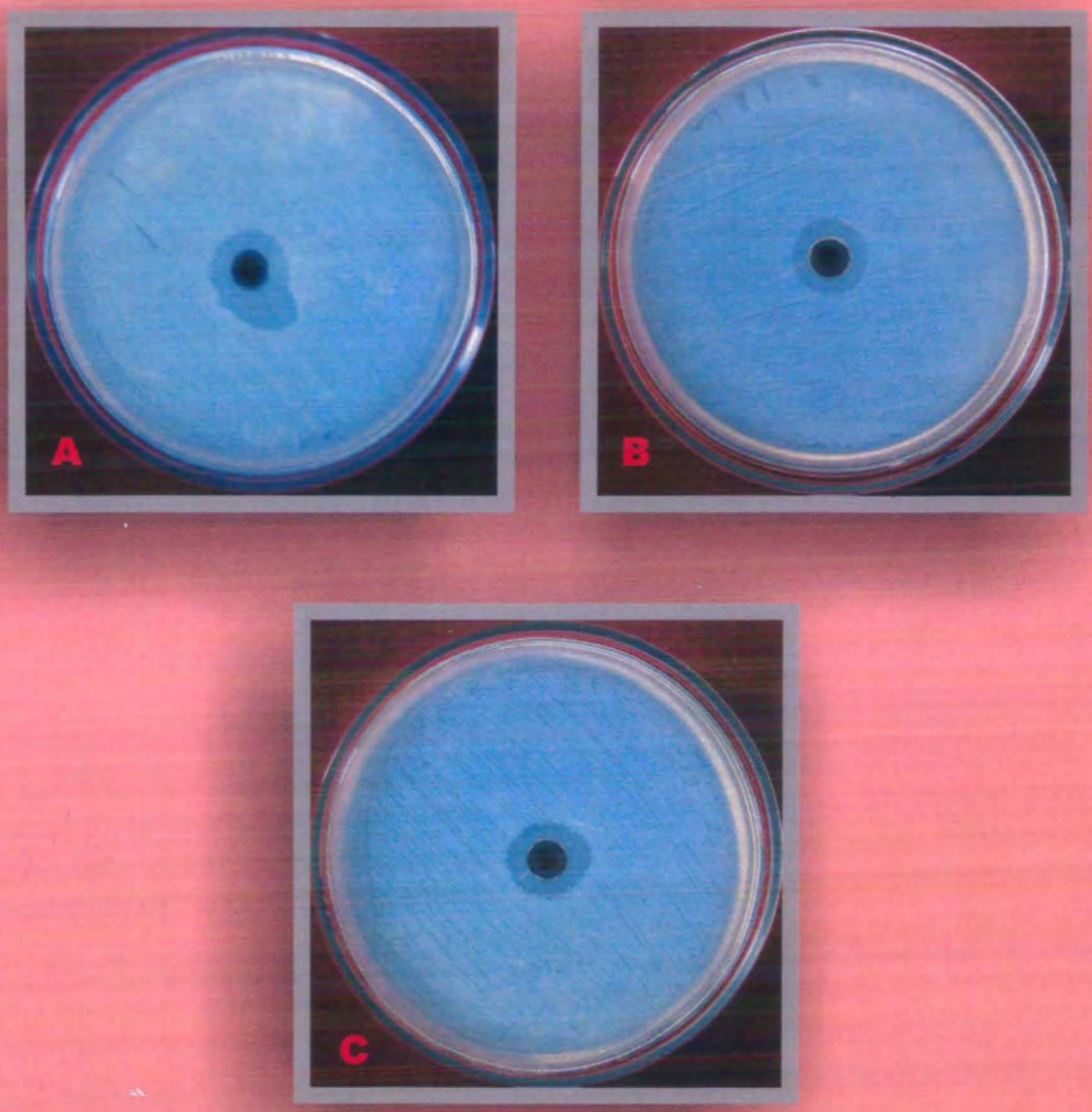
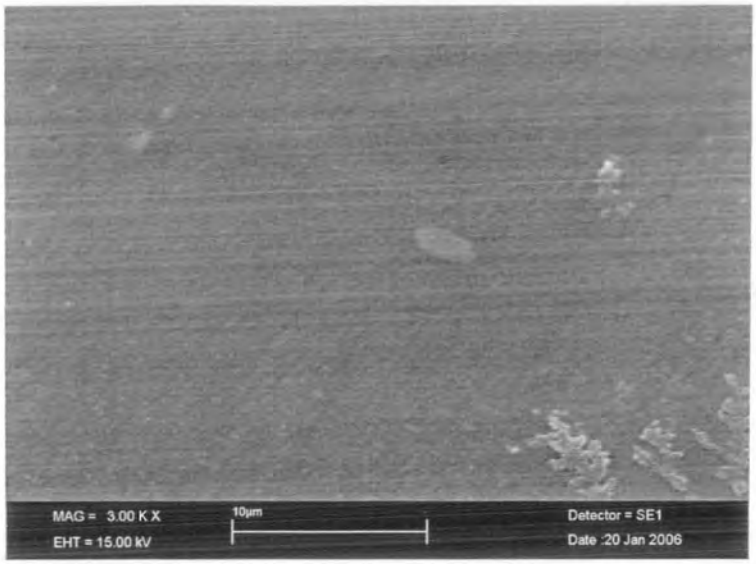
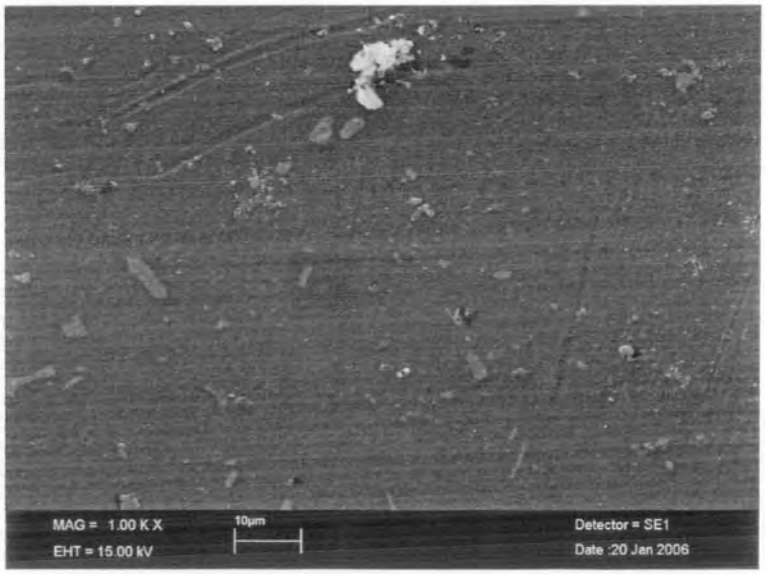


Fig. 4.5b: Antibacterial activity of silver nanoparticles against:
A. *E. coli* **ATCC 15224**
B. *S. aurus* **ATCC 6534**
C. *E. coli* **(UTI)**
Shown by clear zones of inhibition.

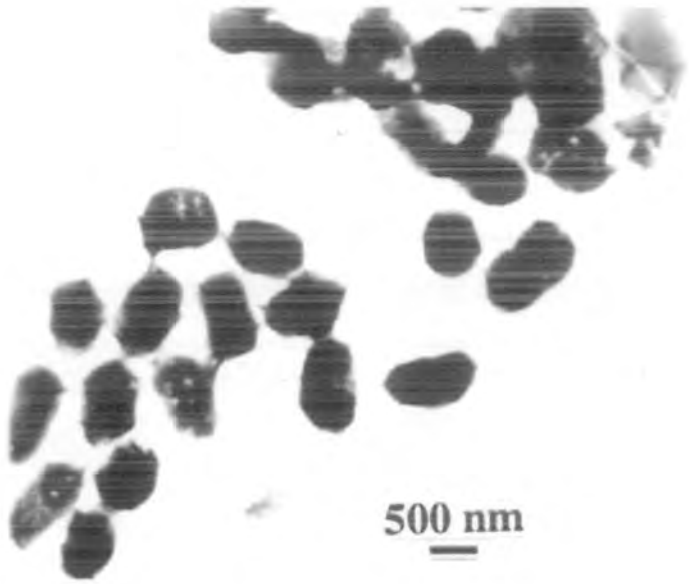


(a)

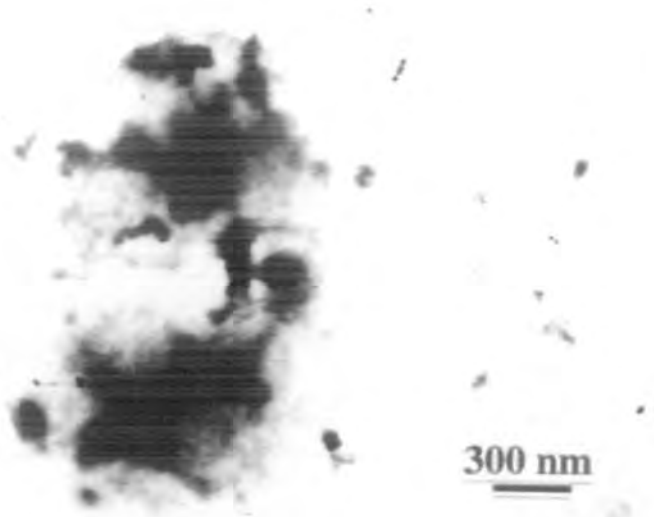


(b)

Figure 4.7 b. SEM micrographs of *E. coli* treated with silver nanoparticles (a) Silver nanoparticles are attached on the surface of cells (b) Damaged *E. coli* cells

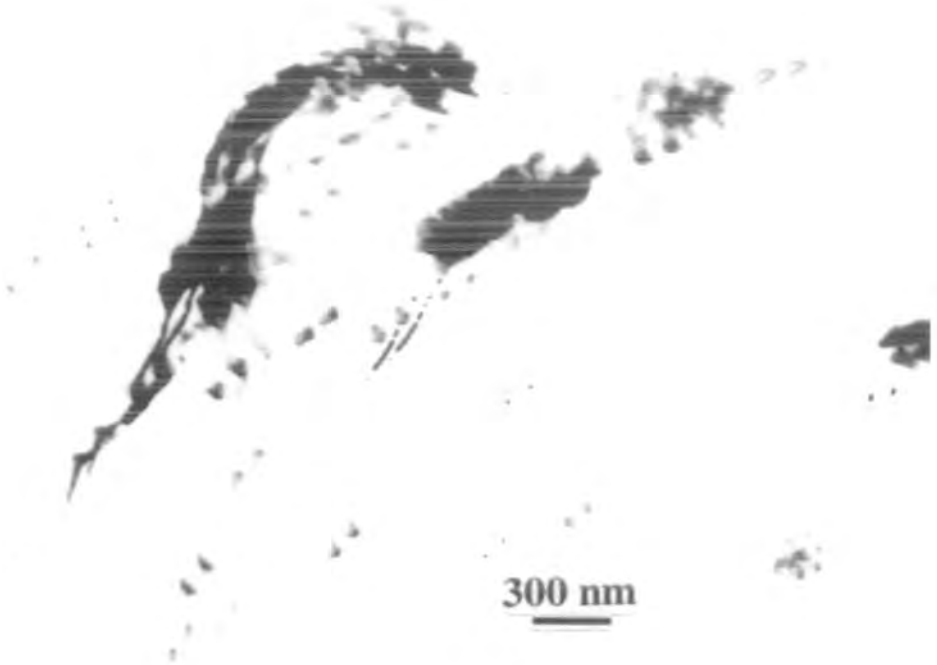


(a)

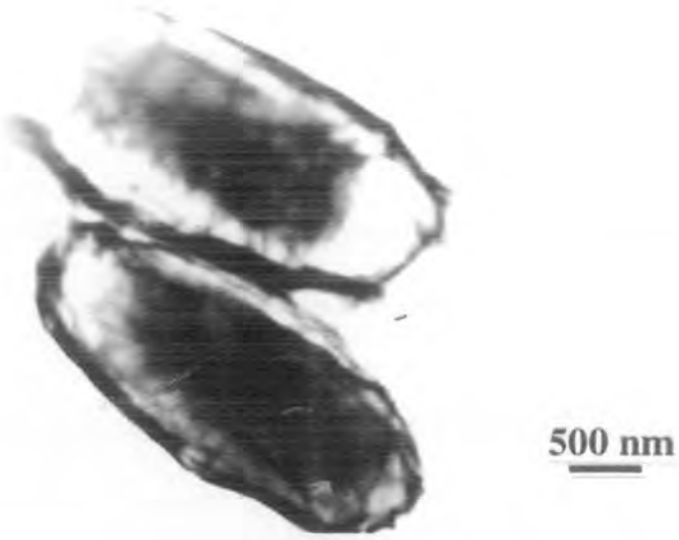


(b)

Figure 4.8 b. Transmission electron micrograph showing (a) untreated *E. coli* (b) *E. coli* treated with silver nanoparticles, the black matter are silver nanoparticles



(c)



(d)

Figure 4.8 b. Transmission electron micrograph showing (c) Cell wall lyses of *E. coli* treated with silver nanoparticles (d) Silver nanoparticles have penetrated inside *E. coli* cell

Discussion

DISCUSSION

The antibacterial properties of silver are known for centuries. Silver ions are highly toxic to bacteria but have low toxicity to the animal cells. Many antibacterial medicines have become less effective due to the emergence of antibiotic resistance but many strains of bacteria failed to develop immunity to Ag^0. Since Klabunde and co workers (as cited in Stoimenov *et al.*, 2002) have demonstrated that reactive metal oxide nanoparticles show excellent bactericidal effects. Sondi (2004) reported that highly concentrated and nonhazardous nanosized silver particles can be prepared easily in a cost effective manner and tested as a new type of bactericidal nanomaterials.

The present study intends to investigate the efficacy of Ag^0 nanoparticles for inhibition of bacterial growth. Nanosized silver powdered is characterized by a large surface to volume ratio, which enhances their reactivity. Three bacterial strains, namely, *E. coli* ATCC, *E. coli* (UTI), *and S. aureus* ATCC, were used as test strains in this study. In the nutrient broth medium, different known concentrations of Ag^0 nanoparticles were used. It was observed that antibacterial efficiency of silver nanoparticles depends on its concentration.

The results of recent study verified the delayed growth of indicator strains. The concentration of silver nanoparticles gradually increased while the OD values gradually decreased. Sondi and Sondi (2004) reported that silver nanoparticles in liquid growth medium, even at high concentration, caused growth delay in *E. coli*. The conc. of the nanoparticles gradually decreases, allowing resumed growth of bacterial cell. This process is governed by the interaction of these particles with intracellular substances of the destroyed cells, causing their coagulation and removal from liquid medium. Kumar and Munstedt (2005) reported that antimicrobial activity of silver cation Ag^0, which binds strongly to electron donor groups in biological molecules containing sulphur, oxygen, or nitrogen. Hence the silver based antimicrobial polymers have to release the Ag^0 to the pathogenic environment in order to be effective. The oxidation of the metallic silver to

the active species Ag^0 is possible through an interaction of the silver with the water molecules.

The zones of inhibition by Agar Well Diffusion method against indicator strains confirmed the antimicrobial activity of the silver nanoparticles, but the zone of inhibition was restricted which can be attributed to the fact that silver is a heavy metal and its particles penetrate in the well while test strains were present on surface of nutrient agar plates, hence little interaction took place between silver nanoparticles and the bacterial cells. This finding is inline with the results obtained by Sondi and Sondi (2004), in which they observed the inhibitory activity of silver nanoparticles on Luria-Bartani agar plates. They concluded that if 10^4 CFU were applied to the plate, a concentration of nanoparticles of $20\mu g\ cm^{-3}$ could completely prevent the bacterial growth.

The protein Estimation showed the less value of protein in highly concentrated nano Ag^0 environment. The sample that was used as control (without nanoparticles) exhibited higher concentration of proteins. The OD values and CFU results showed that the higher the concentrations of silver nano particles, lesser would be the number of the bacterial cells as the bacterial growth was subsided so lower was the enzymatic production, therefore, the estimated protein values were lower at the higher silver particle loading. This finding was supported by the results of Trapalis *et al.*, (2002) that Ag^+ ions penetrate in bacteria and inactive their enzymes, or can generate hydrogen peroxide, thus killing the bacteria. Crawrford (1999) also explained the action of metals on protein. He stated that heavy metals are usually toxic and very reactive with proteins and are believed to bind protein molecules, which results in inhibited cellular metabolism, thus the microorganisms die.

The results of present study showed that inhibitory pattern in *E. coli* ATCC were not consistent. Inhibition was observed within 24 hrs of batch, but after 24 hrs the growth activity of bacteria started once again. In order to check the phenomenon that inhibitory changes are plasmid mediated or not, plasmid isolation was carried out. Its result showed that inhibitory changes were not plasmid mediated. Lee *et al.*, (2003) described that metal

ions when interact with microorganisms cause alteration of chemical structure or by changing the redox states. Sondi and Sondi (2004) explained the mechanism of interaction of silver nanoparticles. It would appear that despite their negative surface charge, they some how interact with the "building elements" of the bacterial membrane, causing structural changes and degradation finally, cell death.

SEM microscopy was used to analyze the surface morphology of microorganisms of both native and treated *E. coli.* Result of present study showed that Ag^0 nanoparticles attach with the cell wall of bacteria and caused the destruction of the cell. According to Sondi and Sondi (2004) the treated bacterial cells were significantly changed and showed major damage, which was characterized by formation of "pits" on their cell walls. They stated that the qualitative analysis of these samples also revealed that silver nanoparticles were incorporated in to the membrane of treated bacterial cells

TEM analysis showed that the nanoparticles were accumulated in the membrane, while some of them successfully penetrated into the cells. It was also observed that nanoparticles had ruptured the cell wall and reached inside the bacteria and resulted in killing cell. According to Stoimenov *et al.*, (2002) that nanoparticles have succeeded in penetrating inside the bacteria cell thus damaging the membranes and the contents leaked out causing death of cell.

CONCLUSIONS

- Nanoparticles in the present study were synthesized by inert gas condensation process these nanoparticles exhibited bactericidal effects.

- Indicator strains *E. coli* ATCC 15224, *S. aureus* ATCC 6538, and *E. coli* UTI, showed delayed growth pattern with different concentrations of silver nanoparticles, with the increasing concentration of silver nanoparticles the number of the bacterial cell decreased.

- After protein estimation it was observed that high concentration of proteins were found in the indictor strains without Ag^0 nanoparticles, as the concentration of Ag^0 nanoparticles increased it gradually decreased the proteins of the indictor strains.

- After plasmid curing it was conformed that the resistance developed by microbes with various concentrations of nanoparticles was not plasmid mediated.

- According to the results showed by transmission electron microscopy it was confirmed that Ag^0 nanoparticles damaged the cell wall and cell membrane of bacteria resulting in the death of cells.

Future Prospects

FUTURE PROSPECTS

- Nanotechnology is a *general-purpose technology* because in its mature form it will have significant impact on almost all industries and all areas of society. It offers to build longer lasting, cleaner, safer, and smarter products for home, communications, medicine, transportation, agriculture, and industry.

- Nanotechnology could be further manipulated to develop materials for environmental cleaning applications such as air and water purification.

- Nanotechnology could further be used in medical research as it has potential for applications in drug development, drug delivery, diagnostics, devices, gene therapy and tissue engineering.

Some of the important applications of nanotechnology for developing countries are as follows:

- Agricultural productivity enhancement.
- Disease diagnosis and screening.
- Water treatment and remediation.
- Drug delivery systems.
- Food processing and storage.
- Air pollution and remediation.
- Health monitoring.
- Energy storage, production, and conversion.

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