

Original Research Article

Biosynthesis of silver nanoparticles from seaweed associated marine bacterium and its antimicrobial activity against UTI pathogens

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A B S T R A C T

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The silver nanoparticles were synthesized using marine bacteria *Bacillus* sp. *NJYRK* was isolated from seaweed *Padina gymnosphora*. Preliminary formation of nanoparticles was confirmed with UV-vis spectrum at the absorbance of 420nm and it is characterized with XRD, FTIR, EDAX, AFM and TEM. The EDAX portraits the presence of silver, the AFM and TEM revealed the sizes of polydispersed (10-30nm) nanoparticles in the medium, the silver nano crystals formation (2θ) was exhibited by XRD spectrum, and FTIR analysis represented the biological compounds involved in nanoparticle synthesis. The antibacterial property of the silver nanoparticles was tested against UTI pathogens. The Antibiotic assay tests (Zone of inhibition, CFU assay and MIC test) reveals the concentration of more than 10µg/ml of silver nanoparticles were inhibited the growth of UTI pathogens. The present investigation states that the marine bacteria synthesized silver nanoparticles have potent antibacterial activity even in minimum concentration against UTI bacteria.

Introduction

Silver is prehistoric metal can be used as broad spectrum antibiotic against several pathogens (Richard *et al.*, 2002; Castellano *et al.*, 2007). It is well known that Ag ions and Ag-based compounds are highly toxic to microorganisms, shows extensive applications in medical fields (Chen *et al.*, 2005; Li *et al.*, 2006). Since ancient, the silver is used as antibacterial agents. In 1700; silver nitrate was used for the treatment of venereal diseases, fistulae

Landsdown, 2002). In the 19th century granulated tissues were removed using silver nitrate to allow epithelization and promote crust formation on the surface of wounds. In 1940s, after penicillin invention, the use of silver against bacterial infections was minimized (Demling and DeSanti, 2001; Chopra, 2007). Now a day's reentry of silver as nanoparticles with more applications and it directly comes in contact with the human

body, such as shampoos (antimicrobial agents), detergent, soaps, shoes, cosmetic products, and toothpaste. Besides it is used in medical and pharmaceutical applications (dental work, catheters, healing burn wound etc.), engineered gloves, face masks, textiles, water filters, HEPA filters etc., (Schierholz *et al.*, 1998; Schierholz *et al.*, 1999; Bosetti *et al.*, 2002; Hillyer *et al.*, 2001). Whilst in fact, the hazards of silver nanoparticles to human health are low. It has been shown that nanosilver in coatings and fabrics can leach out over time but the effect of silver nanoparticles on the environment has less compare to other chemicals (Leaper, 2006; Duran *et al.*, 2007).

Silver based antiseptics have linked to broad-spectrum activity and far lower tendency to induce microbial resistance than antibiotics (Sharkar *et al.*, 2007; Srivasthra *et al.*, 2007). Since some antimicrobial agents are extremely irritant and toxic to host moreover some bacteria that are not effectively killed by the conventional antibiotics these strains were develop the resistant capacity against the normal antibiotics (Lara *et al.*, 2010; Shahverdi *et al.*, 2007; Yoon *et al.*, 2007). Recently scientists are focusing on the silver nanoparticles against multidrug resistant bacteria.

At present, there is a growing need to develop environmentally benign nanoparticle synthesis without using any toxic chemicals and the preparation of uniform nano sized drug particles with specific requirements in terms of size, shape, and physical chemical properties is of great interest in the formulation of new pharmaceutical products. In such the present study was aimed for (i) silver nanoparticle synthesis using seaweed associated bacteria and (ii) application of

synthesized silver nanoparticles against antibiotic resistant bacteria isolated from Urinary tract infection.

Materials and Methods

Chemicals

All analytical reagents and media components were purchased from Commercial and Silver nitrate was procured from Merck (India).

Isolation and characterization of bacteria

The *Padina gymnosphora* was collected from Pudhumadam, Southeast coast of India. The seaweed was identified by the scientist in CSMCRI, Mandapam, India. The sample was transported to our research lab on ice; the sample was washed thrice with sterile filtered seawater. The seaweed was then minced with sterilized seawater. One ml of suspension was inoculated on Zobell Marine agar 2216. Screening was carried out in our lab to isolate the bacteria capable of synthesizing silver nanoparticles. The nanoparticle synthesizing bacteria further identified based on morphological and physiological characterization according to the methods of Bergey's manual of determinative bacteriology followed by nuclear ribosomal DNA sequencing. The amplification of DNA was carried out by colony PCR with FP 5' GGC GTG CTT AAC ACA TGC AAG TCG 3', RP 5' GCG GCT GGC ACG TAG TTA G 3' using standard protocols. The amplified PCR products were sequenced using ABI prism DNA sequencer by BigDye terminator method. The resulting sequence was entered into the BLAST algorithm of National Centre of Biological Information (NCBI) database to obtain closely related phylogenetic sequences.

Biosynthesis of silver nanoparticles

The characterized isolate was inoculated in Zobell marine broth 2216 and incubated for 24h at 30°C. The cell growth was measured as turbidity in spectrophotometer at 660 nm. At the time of 1.0 OD the 3.5 mM aqueous silver nitrate was added in the bacterial culture, at the same time the bacterial culture (without silver nitrate) and Zobell marine broth 2216 with silver nitrate (without bacterial cells) as negative controls were also run simultaneously along with the experimental flask in three replicates. The Erlenmeyer flasks were incubated at 28°C under agitation (200 rpm) without light for 24 h in shaking incubator. The bioreduction of the silver ions in the solution was monitored and the spectrum was confirmed by ultraviolet-visible (UV-vis) spectrophotometer range of 300–600 nm at a resolution of 1 nm.

Recovery of silver nanoparticles

Ultrasonic disruption of cells was carried out with an ultrasonic processor (250w Syclon ultrasonic cell disruptors, USA) over three 15 s periods, and with an interval of 45 s between periods. The sonicated samples were centrifuged at 5,000 rpm for 30 min at 4°C to remove all broth cell-debris. The supernatant was again centrifuged at 20,000 rpm for 30 min and remove the debris. The final supernatant was lyophilized; the lyophilized samples were further used for characterization techniques.

Characterization of nanoparticle

The lyophilized sample was subjected to The X-ray powder diffraction (XRD) patterns were recorded using a Philips PW1710 diffractometer with the Cu KR

radiation (λ) 1.5405Å° at a scanning rate of 0.02 degrees per second in 2θ ranging from 30° to 80°. The samples for XRD were supported on glass substrates.

Lyophilized sample was diluted in milliQ water and make a thin film on a coverslip by dropping 0.1 ml of the sample on the cover slip, and allowed to dry for 30 min. The slides were then scanned with AFM (APE Research-model no: A100SGS). The AFM characterization was carried out in ambient temperature in non-contact mode using silicon nitrate tips with varying resonance frequencies.

For FTIR analysis the powdered sample was ground with KBr pellets, and analyzed on a Thermo Nicolet model 6700 spectrum instrument. A disk of 50 mg of KBr was prepared with mixture of 2 % finely dried samples and then examined under IR-spectrometer. Infrared spectra were recorded in the region of 500–4,500 cm⁻¹.

TEM studies were carried out using Jeol 2100 microscope operating at 120 kV accelerating voltage. Samples were prepared by placing a drop of silver nanoparticles solution (lyophilized sample dissolved in milliQ water) on carbon-coated copper TEM grid. The TEM grid was allowed to dry for 3 h at room temperature then analysis the particle size and structure. The silver nanoparticles and other elemental materials in the experimental solution were confirmed by Phoenix EDX.

Antibacterial activity

The UTI pathogens like *E.coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens* and *Klebsiella* sp. was collected from clinical laboratory and stored in 4°C. The overnight

culture inoculum (100µl) of each bacteria was individually swabbed on Mueller–Hinton agar plates and placed Sterile discs of 5 mm diameter (containing 5,10 and 15µg/ml silver nanoparticles) along with standard antibiotic containing disc (10 mcg/disc) and control disc with sterile milli Q water; were placed in each plate incubated the plates at 37°C for 24 hrs. The zone of inhibition was measured in all plates.

The MIC value was tested using the standard microdilution method, which determines the minimum inhibitory concentration (MIC) leading to the inhibition of bacterial growth. The Silver nanoparticles were serially diluted (5, 10, 15, 20 and 25µg/ml) with LB broth inoculated with the tested bacteria at a concentration of 10⁵CFU/ml. The medium was cultured in a shaking incubator at 37°C for 24 h, and the cultured media (100µl) was spread onto LB agar and incubated at 37°C for 24 h. After incubation, the number of colonies grown on the agar was counted.

The antibacterial activity of silver nanoparticles was studied in liquid nutrient growth medium was studied. The bacterial cells were grown overnight in the nutrient medium at 37°C in 100 rpm. The fresh inoculum (10⁵ cells/ml) of bacterial culture were incubated in the presence of silver nanoparticles loading of 10, 20, 30, 40 and 50 µg/ml added in each flask to observe the bacterial cell growth pattern at 37°C and 100 rpm. The bacterial culture without silver nitrate used as control. Total solution volume used in each flask was 50 ml. Growth of pathogenic bacteria was measured at 620 nm by UV-Vis spectroscopy after every 2 hrs and up to 24 hrs.

Results and Discussion

Among the milieu of natural resources, prokaryotes have been extensively researched for synthesis of metallic nanoparticles. One of the reasons of bacterial preference for nanoparticles synthesis is their relative ease of manipulation. In this study based on biochemical and molecular analysis the seaweed associated bacteria was identified as *Bacillus sp. NJYRK* (Gene bank accession no. JQ029699). Derived from phylogenetic tree construction this bacterium shows 99% similarity the heavy metal tolerant marine sediment bacteria and seaweed associated bacteria from Gulf of mannar (Figure 1).

Silver nanoparticle synthesis, 3.5 mM silver nitrate was incubated with bacterial culture at 28°C for 24 hrs. The silver nanoparticle synthesis was started at 30min of incubation period. The color of the solution turned from yellow to brown. It confirmed the formation of silver nanoparticles (Ahmad *et al.*, 2003) due to the excitation of surface Plasmon vibrations in the metal nanoparticles. It indicates the reduction of the silver ions to nanoparticles. The nanoparticles were further characterization by UV–visible spectrophotometer, EDX, XRD and TEM. UV–visible spectral analysis confirmed the synthesis and stability of synthesized silver nanoparticles. The band was observed in UV visible spectrum (Figure.2) corresponding to the surface Plasmon resonance (SPR) at 430 nm and 441 nm for 24 hrs and 12 hrs incubation respectively, it clearly indicates that on increasing incubation period reduction of silver nano particles increases. The silver nanoparticle was extremely stable without any aggregation and the exact position of absorbance were depends on a size of the

particle. The surface plasmon resonance plays a key role in the determination of optical absorption spectra of metal nanoparticles, which shifts to a smaller wavelength with decrease in particle size (Kowshik *et al.*, 2003).

The Fourier Transform Infrared (FTIR) spectrum obtained from the bacterial synthesized silver nanoparticles (Figure. 3). The presence of the amide groups in the polypeptides provides well known signature in the infrared region of the electromagnetic spectrum. The spectrum shows the appearance of five bands at 1453cm⁻¹, 1542cm⁻¹, 1647cm⁻¹, 3352 cm⁻¹ and 3346 cm⁻¹ for lyophilized silver nanoparticles. The 1453cm⁻¹, 1657cm⁻¹ and 1542cm⁻¹ bands may be assigned –CO stretch and –N–H stretch vibrations to the amide I and II bands, 3352 cm⁻¹ and 3346 cm⁻¹ assigned amine and hydroxyl groups N-H stretching vibration of proteins respectively (Mac Donald *et al.*, 1996). It is well known that proteins can bind to the gold and silver

nanoparticles either through free amine groups or cysteine residues in the proteins (Gole *et al.*, 2001).

According to Energy Dispersive Spectroscopy (EDS) data, was confirmed the presence of elemental silver signal in the sample (Figure 4). The silver nano crystallites display an optical absorption band peaking at 3 keV which is typical of the absorption of metallic silver nanocrystallites (Kohler *et al.*, 2001)The X-ray diffraction pattern (XRD) of silver nanoparticles synthesized using *Bacillus* sp. *NJYRK* is shown in Figure. 5. A number of Bragg reflections with 2θ values of 38.09, 44.98, 64.93, and 77.39 which correspond to the (111), (200), (220), and (311) sets of lattice planes are observed which are indexed to the face-centered cubic structures for silver. The broadening of Bragg’s peaks indicates the formation of nanocrystals (Cullity, 1978; Rita John and Florence, 2010; Theivasanthi and Alagar, 2010; Zargar *et al.*, 2011).

Figure.1 Neighbor-joining phylogenetic tree based on 16s rRNA sequences

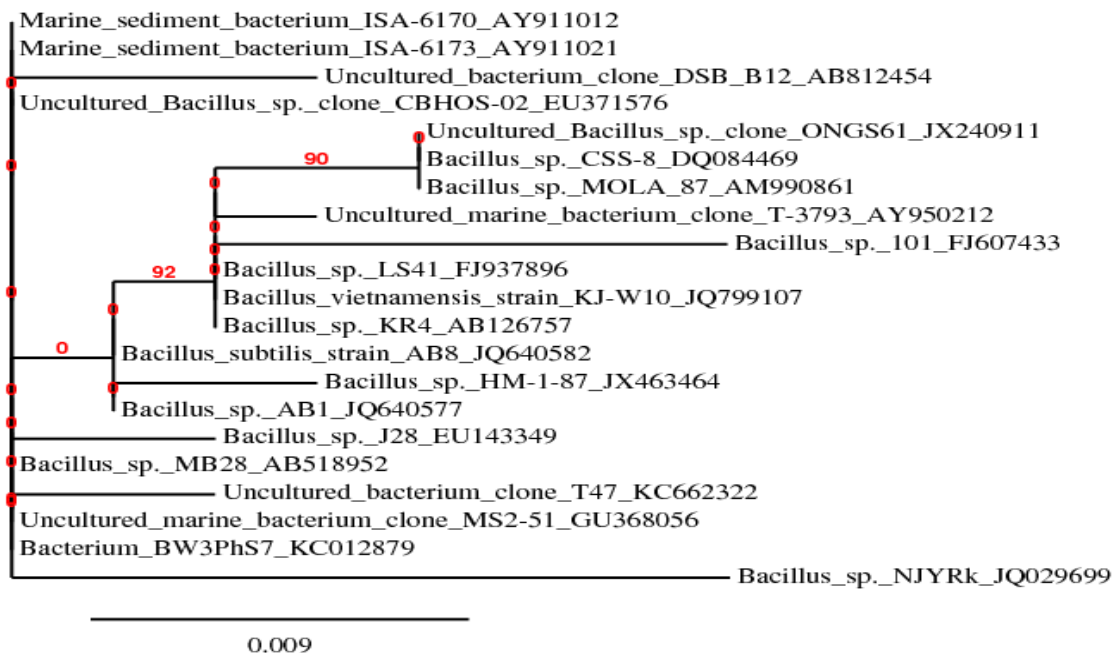


Figure.2 The UV-visible spectra of *Bacillus* sp. *NJYRK* synthesized silver nanoparticles surface plasmon resonance band

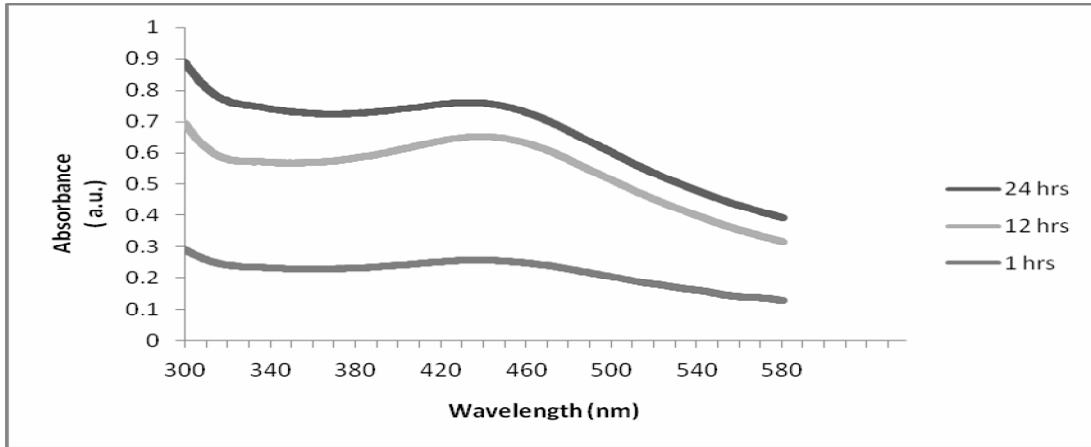


Figure.2 The EDX spectra of *Bacillus* sp. *NJYRK* synthesized silver nanoparticles

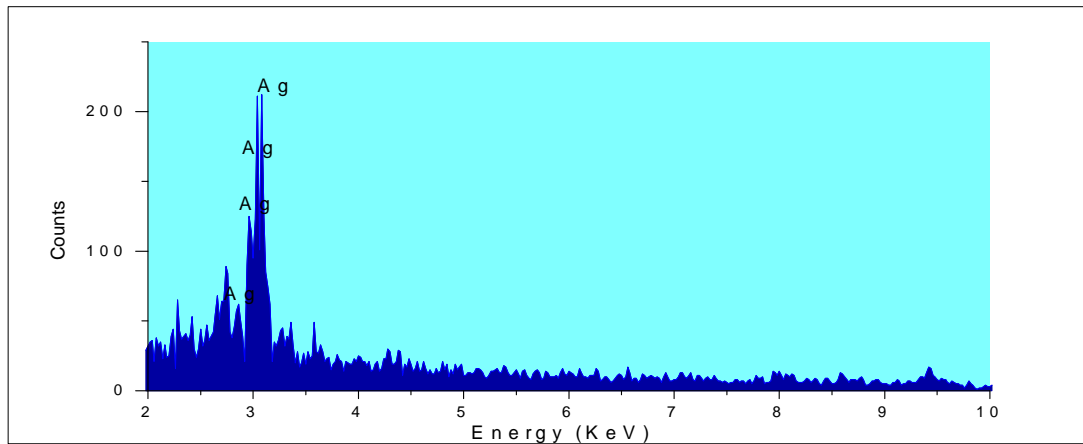


Figure.3 The FTIR spectrum of *Bacillus* sp. *NJYRK* synthesized silver nanoparticles

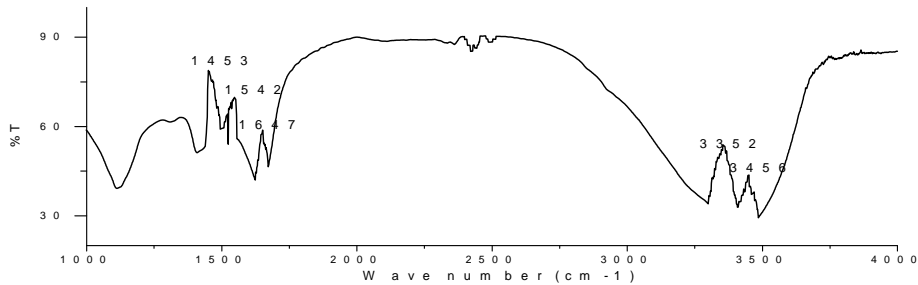


Figure.4 The EDX spectra of *Bacillus sp. NJYRK* synthesized silver nanoparticles

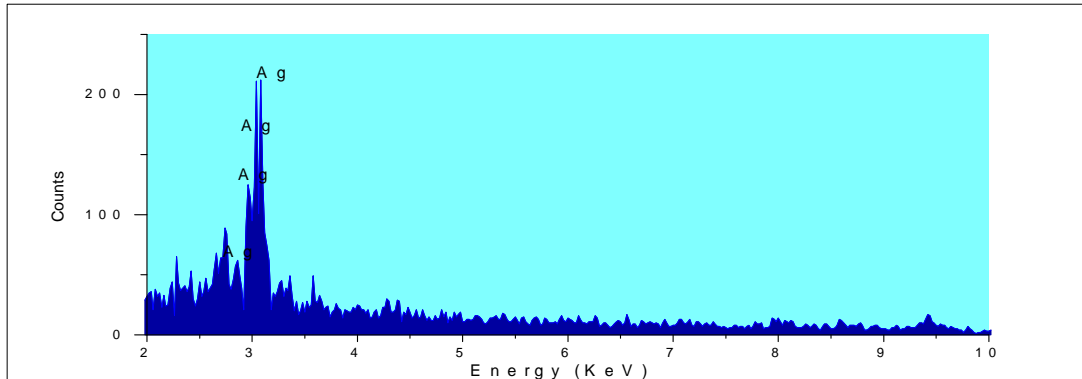


Figure.5 The XRD pattern of *Bacillus sp. NJYRK* synthesized silver nano crystals for 24hrs

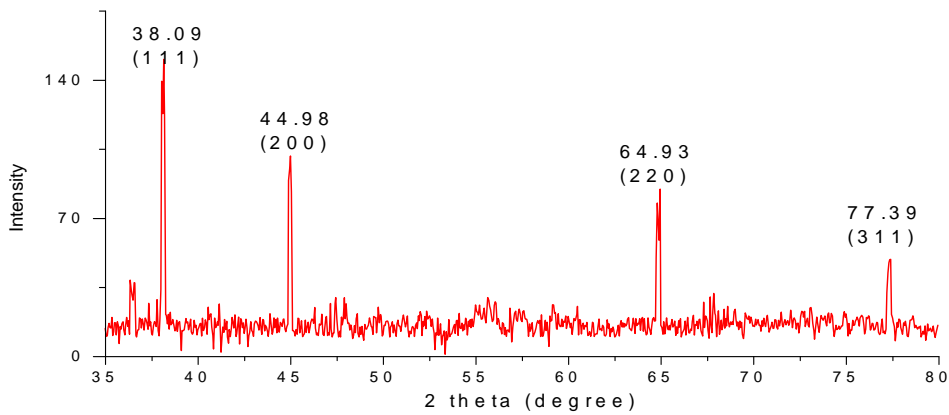


Figure.6 AFM images of lyophilized sample of *Bacillus sp. NJYRK* synthesized silver nanoparticles

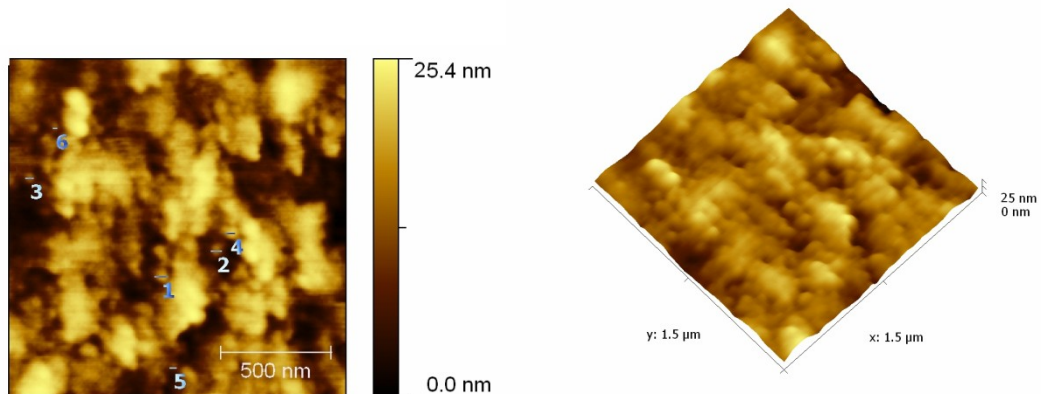
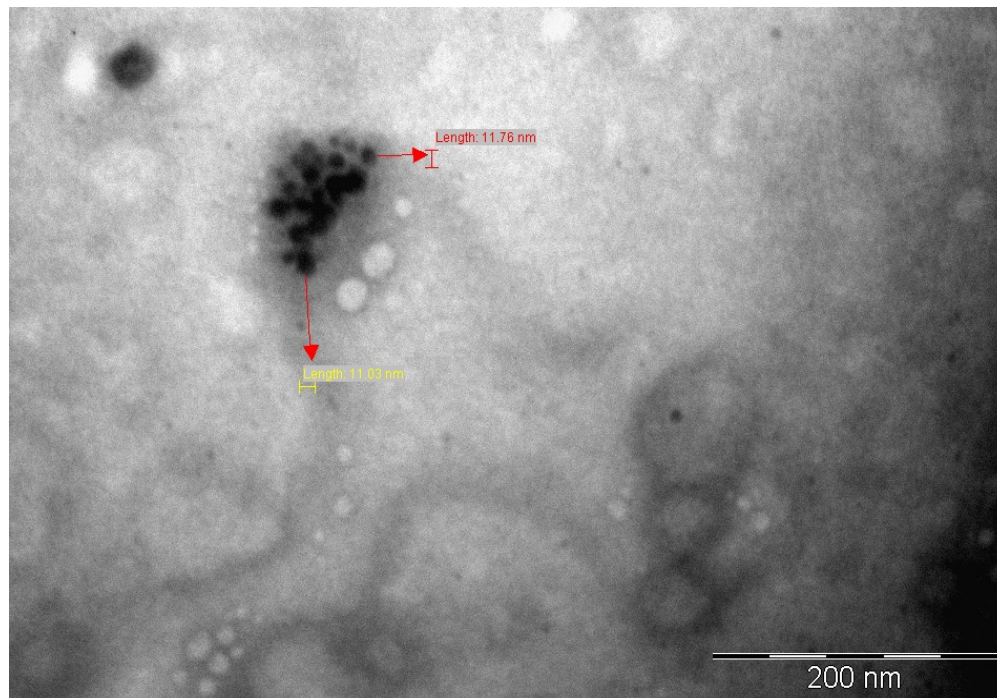


Figure .7 TEM image of silver nanoparticles synthesized by *Bacillus* sp. NJYRK.



The morphology of nanoparticle revealed the fact that the synthesized silver nanoparticles are nearly spherical, as confirmed by absorbance spectrum. The size of the spherical silver nanoparticles obtained from AFM has a wide-ranging distribution in nanometers, which also can be correlated with the observed broad area of the absorbance peak. The particles are polydispersed, without any agglomeration, because this could be due to the presence of some biological compounds in the bacteria can act as a ligand which effectively stabilizes the silver nanoparticles. Figure. 6 shows a three- and two-dimensional view of sample surface over a 3 X 3 μm scan which shows the polydispersed distribution around 25 nm.

The TEM image shows the shape and size distribution (Figure 7), the particles are comparatively spherical, monodispersed with a diameter of 9nm to 25nm.

The synthesized silver nanoparticles exhibited antibacterial activity against *E.coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens* and *Klebsiella* sp. in all concentrations (Table.1). Based on the disc diffusion method the *E.coli* (13.23 ± 0.17 mm) is highly sensitive than compare to *Pseudomonas aeruginosa* (11.46 ± 0.20 mm) and *Serratia marcescens* (11.10 ± 0.12 mm). The *Proteus mirabilis* and *Klebsiella* sp. showed the minimum inhibitory zone (9.42 ± 0.13 and 9.1 ± 0.12 mm) in 15 $\mu\text{g/ml}$.

The bacterial colonies were individually grown in LB broth with various concentrations of synthesized silver nanoparticles (Figure 8). The presence of these particles at a concentration of 5 $\mu\text{g/ml}$ inhibited bacterial growth by 53% (*E.coli*), 47% (*Pseudomonas aeruginosa*), 33% (*Proteus mirabilis*), 55% (*Serratia*

marcescens), and 65% (*Klebsiella* sp.) respectively. The number of bacterial colonies grown on plates with 15µg/ml of silver nanoparticles was significantly reduced and the colonies 81% (*E.coli*), 76% (*Pseudomonas aeruginosa*), 77% (*Proteus mirabilis*), 70% (*Serratia marcescens*) and 76% (*Klebsiella* sp.) respectively on the agar plate. In 15µg/ml concentration mostly inhibit the bacterial growth by > 90 % in all plates. The concentrations of more than 15µg/ml caused 100% inhibition of bacterial growth. Based on this result, more than 15 µg/ml concentration of synthesized silver nanoparticles can be used to completely inhibit UTI pathogens.

During incubation time optical densities were measured periodically up to 24 h at 2 hr interval (Figure 9). It has been observed that absorption in the growth medium decreased with increasing concentration of silver nanoparticles in comparison to the control. This has been accredited to the reduced growth of bacterial cells. Silver nanoparticles at concentration of 10µg/ml and higher were found to be effective against the pathogen, but a complete bacterial growth inhibition has been witnessed at 15µg/ml and above because there was virtually no bacterial growth, as a result insignificant optical absorption. As the number of CFU have reduced

significantly with increasing the concentration of silver nanoparticles, consequently, low number of CFU were observed in the concentration of 15µg/ml and above silver nanoparticles concentration. The bacterial growth inhibition trend found in CFU data has matched well with the results of OD.

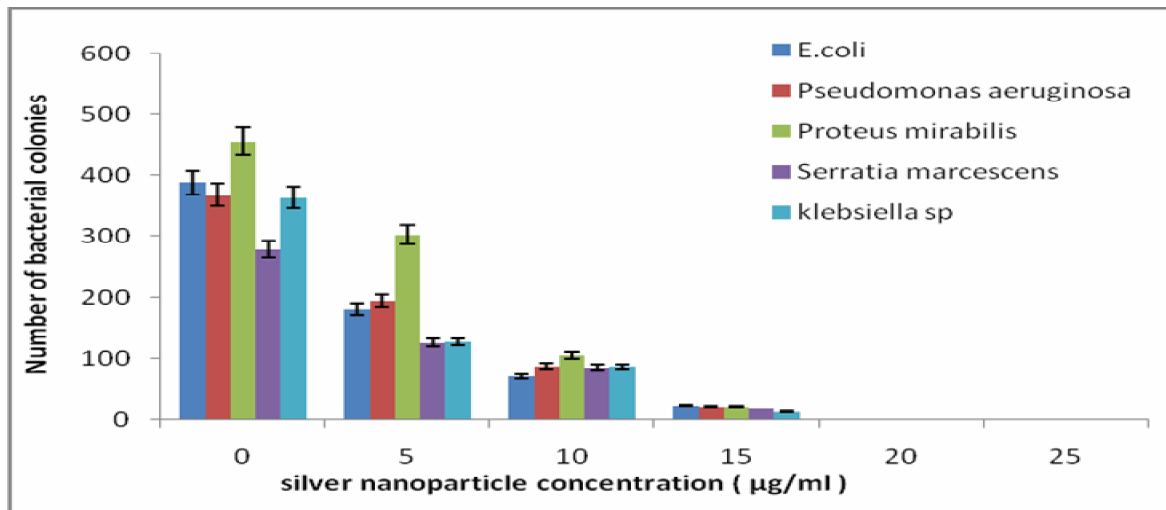
Due to its smaller in size the synthesized nanoparticles may have a higher percentage of interaction than larger particles (Kreibig and Vollmer, 1995; Mulvaney, 1996; Morones *et al.*, 2005; Pal *et al.*, 2007). Since, thenanoparticles smaller than 10 nm interact with bacteria and produce electronic effects, which enhance the reactivity of nanoparticles.

Thus, it is corroborated that the bactericidal effect of silver nanoparticles is size dependent (Raimondi *et al.*, 2005; Morones *et al.*, 2005). The antimicrobial efficacy of the nanoparticle depend on the shapes of the nanoparticles also, this can be confirmed by studying the inhibition of bacterial growth by differentially shaped nanoparticles (Morones *et al.*, 2005). The antibacterial properties of silver nanoparticles of different shape and found that the antibacterial efficacy of silver nanoparticles is shape dependent.

Table.1 Antimicrobial activity of bacterial synthesized silver nanoparticles on different microorganisms (inhibition zone (mm))

Pathogens	5 µg/ml	10 µg/ml	15 µg/ml
<i>E.coli</i>	3.26 ± 0.16	7.37 ± 0.14	13.23 ± 0.17
<i>Pseudomonas aeruginosa</i>	2.63 ± 0.10	4.36 ± 0.14	11.46 ± 0.20
<i>Proteus mirabilis</i>	3.06 ± 0.17	5.16 ± 0.10	9.42 ± 0.13
<i>Serratia marcescens</i>	4.13 ± 0.08	6.13 ± 0.10	11.10 ± 0.12
<i>Klebsiella</i> sp.	3.7 ± 0.12	4.53 ± 0.17	9.1 ± 0.12

Figure.8 Antibacterial Characterization by CFU as a Function of Silver Nanoparticles Concentration on Agar Plates Inoculated with UTI infection causing bacteria after 24 hrs Incubation



The antibacterial properties of silver nanoparticles of different shape and found that the antibacterial efficacy of silver nanoparticles is shape dependent.

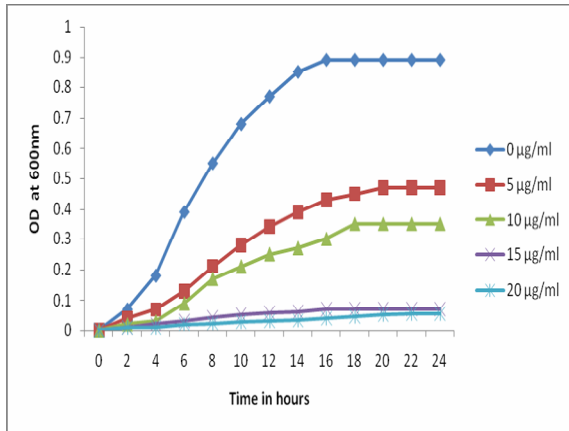
There are several hypotheses to explain the antibacterial activity of silver nanoparticles. The ability of silver nanoparticles to release silver ions is a key to their bactericidal activity. It can interact with functional groups and inactivates the proteins. It can damage bacterial cytoplasmic membranes, reduce the ATP synthesis and inhibit the bacterial DNA replication finally it causes cell death (Lansdown, 2002; Castellano *et al.*, 2007). The high specific surface-to-volume ratio of silver nanoparticles increases their contact with microorganisms, promoting the dissolution of silver ions, thereby improving biocidal effectiveness.

According to Pal *et al.*, (2007) truncated triangular nanoparticles show bacterial inhibition with silver content of 1µg. While, in case of spherical nanoparticles

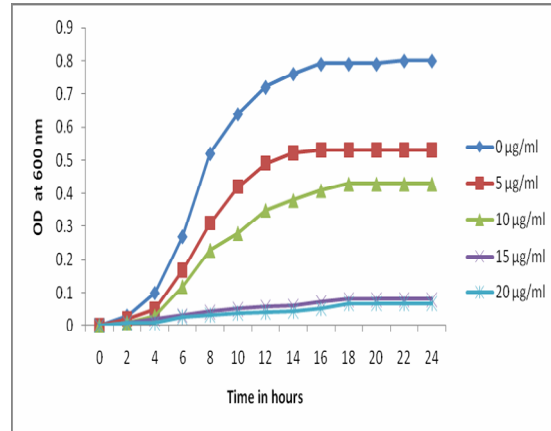
total silver content of 12.5 µg is needed (our study reports 15µg/ml). The rod shaped particles need a total of 50 to 100 µg of silver content.

In this study, the application of silver nanoparticles as an antibacterial agent was investigated by growing *E.coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens* and *Klebsiella* sp. on agar plates and in liquid LB medium, both supplemented with silver nanoparticles. There are various differences between these two methods. When nanoparticles were present on LB agar plates, they could completely inhibit bacterial growth. However, inhibition depends on the concentration of the silver nanoparticles as well as on the number of bacterial colonies used in the experiments. Since the high CFU applied in this study are infrequently found in real-life systems, it appears that these particles could have an excellent biocidal effect and effectiveness in reducing bacterial growth for practical applications such as the formulation of various biocidal materials.

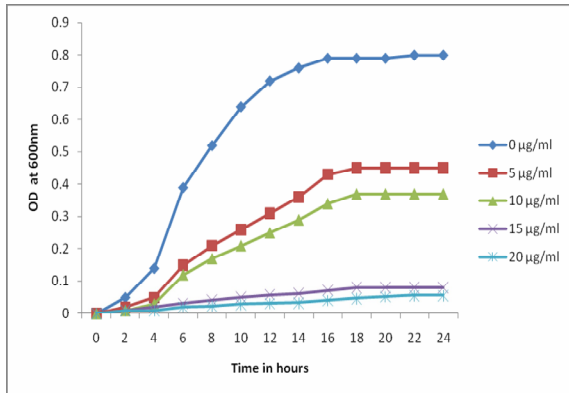
Figure.9 Optical density as a function of time in the solution studies (A) *E.coli*, (B) *Pseudomonas aeruginosa*, (C) *Proteus mirabilis*, (E) *Serratia marcescens* and (E) *klebsiella* sp in LB medium inoculated with 10^5 CFU of bacteria in the presence of different concentrations of silver nanoparticles



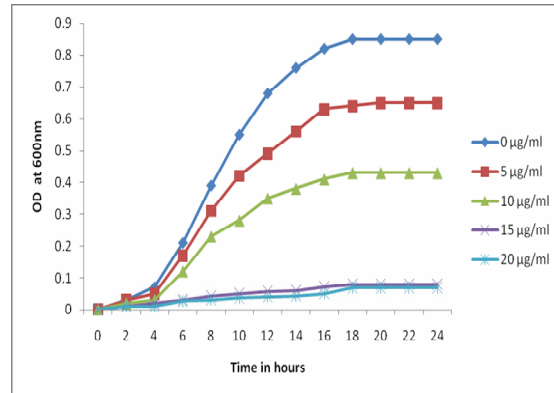
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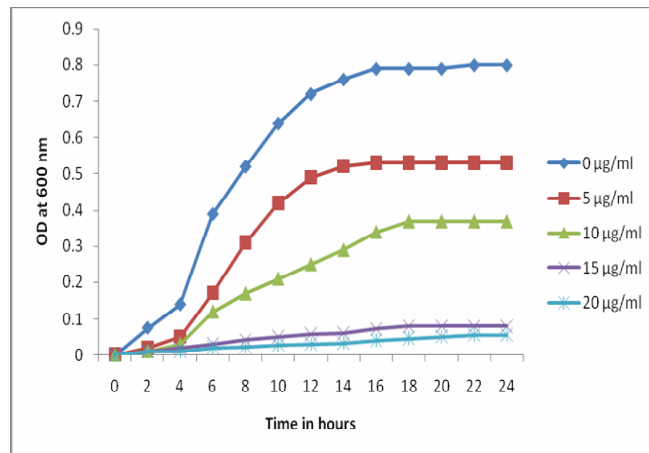
B



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D



E

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In conclusion, this study showed that silver nanoparticles have potent antibacterial activities against *E.coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens* and *Klebsiella* sp.. The growth and multiplication of silver nanoparticle exposed organisms were quickly inhibited. Further more in this biological synthesis didn't used toxic substances like chemical synthesis, so the impact of silver nanoparticles in human being is very less. This study indicates that silver nanoparticles can be applied as effective antibacterial materials against various microorganisms which can cause disease to human beings.

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