CHAPTER-3

Biophysical characterization of silver and gold nanoparticles synthesized by *Pseudomonas veronii* strain AS 41G, *Pseudomonas fluorescens* strain CA 417 and *Aneurinibacillus migulanus* strain 141

GRAPHICAL ABSTRACT
1. INTRODUCTION

Nanoparticle synthesis is one of the prime aspects of nanotechnology due to their unique properties which has traded various applications in the last few years (Baker and Satish, 2012b). It is worth noting that fate of nanoparticles applications remains infancy without accurate and well resolved characterization of nanoparticles (Anumolu and Pease, 2012). Characterization of nanoparticles ensures and determines the application based on their properties. There are various hyphenated techniques accompanied with robust methodologies for the characterization of nanoparticles size, shape, chemical composition, etc. For metallic nanoparticles such as silver and gold initial preliminary confirmation can be achieved by visual observation with change in color (Kavitha et al., 2013d).

a. Biophysical characterization techniques

Biophysical characterization of nanoparticles can be carried out using various hyphenated techniques (Figure 3.1). Characterization of nanoparticles is very essential to reveal its physico-chemical properties such as their chemical nature, size and shape which become one of the important aspects to unveil application part. There are various tools to acquire the properties of nanoparticles for instance preliminary confirmation of nanoparticles can be achieved by UV-Visible spectroscopic technique which is mainly based on the well known phenomenon of light absorption wherein nanoparticles have different optical properties compared to its bulk material that are sensitive due to surface plasmon resonance i.e., collective oscillations of conduction band electrons in response to electromagnetic waves. Nanoparticles especially metal nanoparticles exhibit a specific absorbance bands in their spectra when the light passes through the sample and the obtained results are based on the band energy gap which evaluates mainly the dispersion and aggregation of the nanoparticles (Roy and Fendler, 2004; Daniel and Astruc, 2004; Norman et al., 2006 and Van Dijk et al., 2006).

Whereas microscopic techniques such as scanning electron microscopy, transmission electron microscopy and atomic force microscopy are employed to reveal the size and shape of nanoparticles (Baker et al., 2013b and Kavitha et al., 2013). Accurate measurement of particle size, morphology, surface texture, and
roughness is an important for pre-requisite application of nanoparticles in science and technology (Pletikapic et al., 2012).

Figure 3.1: Important techniques used to characterize nanoparticles

Since particles smaller than 1.0 µm cannot be observed with light due to diffraction effects restrict the resolution of optical microscopy. Hence to observe an object at nanoscale higher resolution is required, electromagnetic radiation of shorter wavelengths must is employed. The basic principle is an electron beam interacts with a sample, many measurable signals are generated and electrons can be transmitted, back scattered and diffracted to give an image. Electron microscopic techniques have been able to routinely employ to achieve magnifications and disclose details with a resolution of up to about 0.1 nm (Herrera and Sakulchaicharoen, 2009).

Further chemical composition and nature of the nanoparticles can be attenuated with Fourier transform infrared (FTIR) spectroscopy which measures infrared intensity versus wavelength of light resulting in predicting the associated biomolecules with nanoparticles. In FTIR interactions between matter and electromagnetic fields in the Infrared region takes place during which
electromagnetic wave mainly couple with molecular vibration resulting in excitation of molecule to higher vibrational state by absorbing Infrared radiation causing vibrational changes and rotational status of the molecules (Kalisz et al., 2008 and Davis and Mauer, 2010).

X-ray diffraction (XRD) is general highly reliable analytical techniques primarily used for phase identification of a crystalline material which can provide information on surface properties and coatings, crystallographic structure or elemental composition. XRD can be used to evaluate single crystal or polycrystalline materials. X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated by a cathode ray tube and directed toward the sample (Figure 3.2).

![XRD Mechanism](image)

**Figure 3.2: XRD Mechanism**

The way beam is scattered by the atoms in the path of the X-ray is studied and scattered X-rays constructively interfere with each other. This interference can be calculated using Bragg’s Law to determine various characteristics of the crystal or polycrystalline material. Bragg's law is used to explain the interference pattern of X-rays scattered by crystals, diffraction has been developed to study the structure of all states of matter.
2. REVIEW OF LITERATURE

According to Prakash et al., 2010, silver nanoparticles were synthesized intracellularly using Bacillus megaterium. The synthesized were accumulated on the surface of the cell wall of bacteria. Bacillus megaterium was grown aerobically and the cultures were challenged with the solutions of silver nitrate. Cell lysate obtained was centrifugation and monitored by UV-Vis Spectrophotometer, X-ray Diffraction (XRD), Transmission Electron Micrography (TEM) and Energy Dispersive Spectroscopy (EDS). TEM showed the production of these metal nanoparticles with particle size in the range of 10-20 nm.

Streptomyces hygroscopicus was evaluated for extracellular synthesis of silver nanoparticles. The synthesized nanoparticles were characterized via. UV-Visible spectrophotometer, X-ray diffraction patterns displayed typical peaks of crystalline silver. The silver nanoparticles were determined to be spherical (20-30 nm) with a purity of 70% as determined by FE-SEM and TEM (Sadasivam et al., 2010).

Culture supernatant of Escherichia coli ATCC 8739, Bacillus subtilis ATCC 6633 and Streptococcus thermophilus ESh1 was evaluated for the synthesis of silver nanoparticles resulted in reduction of silver ion within 5 minutes which was characterized by UV-visible spectrum with peak around 420 nm corresponding to the plasmon absorbance of silver nanoparticles. Transmission electron microscopy (TEM) micrographs showed formation well dispersed silver nanoparticles in the range of 5-25nm. X-ray diffraction (XRD) of silver nanoparticles exhibited 2θ values corresponding to the silver nanocrystal (Shanshoury et al., 2011).

Cell filtrate of Streptomyces sp. ERI-3 when treated with 1mM silver nitrate and incubated at 28°C for 48 h in the dark in an orbital shaker resulted in biosynthesis of silver nanoparticles which were characterized using UV-Visible spectroscopy with maximum absorbance at 430 nm, X-ray Diffraction spectrum exhibited 20 values corresponding to the silver nanocrystals. Electron microscopy micrographs revealed the extracellular formation of spherical nanoparticles in the size range of 10-100 nm. The study provided the evidence that the factors in the cell free culture supernatant facilitate synthesis of silver nanoparticles and was the first report on the biosynthesis of silver nanoparticles using supernatant of Streptomyces sp. ERI-3 bacterium (Zonooz et al., 2011).
According to Silambarasan and Abraham, (2012) *Bacillus cereus* was evaluated for the extracellular synthesis of silver nanoparticles from silver nitrate solution. Silver nanoparticles were characterized by UV-Vis spectroscopy which exhibited peak at 440 nm confirming the nanoparticles formation which corresponds to the surface plasmon resonance. FTIR spectroscopy confirmed the presence of protein as the stabilizing agent surrounding the silver nanoparticles. AFM analysis revealed the irregular shape with 62.8 nm in size. Antimicrobial activity of the silver bio-nanoparticles was performed by well diffusion method against human pathogenic bacteria. The highest activity were recorded against *Staphylococcus aureus* followed by *Klebsiella pneumoniae* and *Salmonella typhi* and *Escherichia coli* showed the least activity.

*Pseudomonas denitrificans* mediated biosynthesis of gold nanoparticles. The optical properties were morphological verified using UV-Vis spectroscopy and High Resolution Transmission electron microscopy. HRTEM micrographs showed the size of the gold nanoparticles ranged from 25-30 nm. The crystalline nature of gold nanoparticles was examined using XRD and was found to be face centered cubic. FTIR analyses of such GNPs possess sulphydryl, amido, carbonyl functional groups which depicts the presence of peptides involved in capping the nanoparticle leading to its stability. Moreover, the stability of GNPs synthesized from *Pseudomonas denitrificans* exudates was compared with chemically synthesized GNPs with the help of 5M NaCl (Mewada *et al.*, 2012).

An eco-friendly biosynthesis of gold nanoparticles was achieved by nine different bacteria from the Enterobacteriaceae family. Their respective supernatants were examined for ability to produce gold nanoparticles. The reaction was performed in a dark environment at 37º C. After 24 hours, it was observed that the colour of the solutions turned to dark purple from light yellow. The gold nanoparticles were characterized by UV-Visible spectroscopy, dynamic light scattering, scanning electron microscopy and Fourier transform infrared spectroscopy (FTIR) for yield, particle size, shape and presence of different functional groups, respectively (Honary *et al.*, 2012).

As similar to silver nanoparticles, many scientific literatures report the synthesis of gold nanoparticles using various microbial species. An efficient
environment friendly approach for the biosynthesis of rapid and stable gold nanoparticles was achieved by using whole cells of *Geotrichum candidum*. The enzymes/proteins secreted by the microbe mediated the reduction of metal salts to nanoparticles. The study also reflects the importance of parameters such as culture age, temperature, pH, metal salt, and cell mass concentrations. Biologically synthesized gold nanoparticles were characterized by using UV-Visible spectroscopy, dynamic light scattering, energy dispersive spectroscopy, scanning electron microscope and Fourier transform infrared spectroscopy (Mittal *et al.*, 2013).

The use *Geobacillus* sp. strain ID17, a thermophilic bacterium was reported to mediate gold nanoparticles synthesis. Cells exposed to Au\(^{3+}\) turned from colourless into an intense purple colour. This change of colour indicates the accumulation of intracellular gold nanoparticles. Gold nanoparticles characterized by using TEM which showed two different shapes of particles were predominant with quasi-hexagonal shape and size ranging from 5-50 nm. FTIR was utilized to protein type of compound on the surface of biosynthesized gold nanoparticles. The study also highlights that reductase activity involved in the synthesis of gold nanoparticles which has been previously reported to be present in others microorganisms (Correa-Llanten *et al.*, 2013).

3. MATERIALS AND METHODS

a. **Synthesis of silver and gold nanoparticles**

Actively growing colonies of the selected endophytic bacterial isolates were cultured in nutrient broth and incubated for 72 hours. Later the culture broth was centrifuged at 8000 rpm at 4\(^{\circ}\)C for 20 minutes to obtain cell free supernatant and was challenged with 1mM of silver nitrate and incubated on rotary incubator with 180 rpm under optimized conditions for each isolate based on the earlier results obtained in chapter 2. Similarly for gold nanoparticles synthesis, cell free supernatant was treated with \(10^{-3}\) mM gold chloroaurate and incubated on rotary incubator with 180 rpm under optimized conditions.

b. **Biophysical characterization of nanoparticles**

Samples were drawn periodically and monitored with UV-visible spectroscopy by recording the spectra between 200 to 700 nm using Shimadzu double beam spectrophotometer. FTIR spectroscopy analysis conferred functional group of biomolecules responsible to mediate the synthesis on a JASCO FT-IR 4100
instrument at room temperature with a resolution of 4 cm\(^{-1}\). For XRD studies nanoparticles were coated on XRD grid and spectra were recorded by Rigaku Miniflex-II Desktop X-ray diffractometer instrument operating at a voltage of 30 kV and average size was calculated based on Scherrer equation. \(N = \frac{K \lambda}{\beta \cos \theta}\). Where \(K\) is the Scherrer constant with value from 0.9 to 1 (shape factor), where \(\lambda\) is the X-ray wavelength (1.5418 Å), \(\beta 1/2\) is the width of the XRD peak at half height and \(\theta\) is the Bragg angle. Size and morphology of nanoparticles was analyzed by using Transmission Electron Microscopy, an aliquot of nanoparticles was transferred on to a carbon-coated copper TEM grids. The films on the TEM grids were allowed to stand for 2 minutes, then extra solution was removed and the grid was allowed to dry prior to measurement and scanned using a TECHNAI-T12 JEOL JEM-2100 Transmission electron microscope operated at a voltage of 120 kV with Bioten objective lens. Subsequently, the particle size was ascertained using a Gatan ccd Camera.

c. Antimicrobial activity of nanoparticles

Antimicrobial activity of synthesized nanoparticles was evaluated against important human and phytopathogens via disc diffusion assay, well diffusion assay, micro dilution assay, CFU plate method, food poison technique and minimal inhibitory concentration. Mode of action of nanoparticles was evaluated with DNA damage study.

i. Well and disc diffusion assay

In brief pre-warmed MHA (Mueller-Hinton agar) plates were seeded with \(10^6\) CFU (colony forming unit) suspensions of test organism was swabbed uniformly, by using sterile cork borer 10 mm diameter of agar was punctured and 50 µl of 1 mg/ml nanoparticles were added into each well and incubated at 37° C for 24 hours. After incubation, the zone of inhibition were measured and interpreted with gentamicin. Disc diffusion assay was performed similar to that of well diffusion assay, instead of puncturing well the disc was placed and impregnated with 50 µl of 1 mg/ml nanoparticles and incubated as mentioned above (Baker et al., 2014).

Statistical analyses of results were performed using IBM SPSS version 20 (2011). One way ANOVA (analysis of variance) at value \(p<0.001\) followed by Tukey’s Post Hoc test with \(p \leq 0.05\) was used to determine the significant differences between the results obtained in each experiment.
ii. **Broth dilution assay**

Micro dilution assay was performed where in 50 µL of different concentration of nanoparticles varying from 25-100 µg/ml was suspended in sterile saline and each aliquot was suspended in different test tubes with 10 ml of Muller Hinton broth seeded with 150 µL of test bacterial cells (5 x 10^6 CFU/mL). Incubate the plate at 37° C on a shaker (150 rpm) for 20 to 24 hours and later record the absorbance at 600 nm. One positive and negative control was maintained to distinguish the activity of the nanoparticles based on optical density of control compared with nanoparticles treated test pathogens.

iii. **CFU plate method**

Test organism was added in to the media and poured onto the sterile Petri plate and allowed to solidify. Different concentration of nanoparticles varying from 10-100 µg/mL was added on to the surface of the media in sterile condition and spread. All the plates were incubated at 37° C for 24 hours and results were observed one control plate was kept without adding nanoparticles (Sondi and Salopek-Sondi, 2004).

iv. **Minimal inhibitory concentration**

Minimal inhibitory concentration was carried out based on the protocol described by Sarkar et al., 2007. The resazurin solution was prepared by dissolving a 270 mg tablet in 40 mL of sterile distilled water. Plates were prepared under aseptic conditions. A sterile 96 well plate was labelled. A volume of 100 µL of test material (nanoparticles) was dissolved in sterile saline and pipette into the first row of the plate. To all other wells 50 µL of nutrient broth was added. Serial dilutions were performed using a multichannel pipette. To each well 10 µL of resazurin indicator solution was added. Later 30 µL of isosensitised broth was added to each well to ensure that the final volume was single strength of the nutrient broth. Finally, 10 µL of bacterial suspension (5 × 10^6 CFU/mL) was added to each well to achieve a concentration of 5 × 10^5 CFU/mL. Plate was wrapped with cling film. Each plate was prepared with a set of controls. Gentamicin served as positive control and placed in an incubator at 37° C for 18 to 24 hours. The color change was then assessed visually. Any colour changes from purple to pink or colourless were recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value.
v. DNA damage study

DNA was extracted from *Staphylococcus aureus* using alkaline lysis based on the protocol described by Sambrook and Russell, 2001. Purity of the DNA was measured at optical density 260/280. Biosynthesized silver nanoparticles were treated with 10 ng of DNA and incubated at 37° C. Later the mixture was evaluated electrophoretically using 1.2% agarose gel (Vahdati and Sadeghi, 2013).

4. RESULTS

The progress of the reaction between metal ions and supernatant were monitored by UV-Visible spectra of silver and gold nanoparticles. UV-Visible spectrometry ascertains the formation of nanoparticles. The absorption spectrum of silver nanoparticles was observed between 200 to 600 nm which confirmed the formation of silver nanoparticles. In case of gold nanoparticles, change in the colour from colour of the reaction mixture to pink or ruby red depending on the intensity indicated the synthesis of gold nanoparticles. UV-Visible spectrum resulted in absorption peaks between 500 to 700 nm which confirmed the formation of gold nanoparticles. The reduction of metal ions and the formation of stable nanoparticles occurred rapidly within a shorter duration of time under optimized parameters. FTIR analysis predicted the possible interaction metal salts and biomolecules which would reduce the ions into nanoparticles and stabilize the nanoparticles by capping it. The FTIR analysis of the nanoparticles resulted in different vibrational stretches which correspond to various functional groups. FTIR analysis predicted the possible interaction metal salts and biomolecules which would reduce the ions into nanoparticles and stabilize the nanoparticles by capping it. The FTIR analysis of the nanoparticles resulted in absorption different bands which correspond to various functional groups. FTIR analysis of nanoparticles synthesized was obtained in different vibrational stretching whose functional groups are predicted.

a. Nanoparticle synthesis from *Pseudomonas veronii* strain AS 41G

i. UV-Visible analysis of synthesized nanoparticles

Supernatant secreted by *Pseudomonas veronii* strain AS 41G mediated synthesis of silver nanoparticles within 15 minutes of time duration under optimized condition. Samples were monitored every minute which was analyzed using UV-Visible spectrophotometer as shown in the figure 3.3(a) with various peak
conferring between 150 to 300 nm. During the analysis it was observed that there was no further synthesis occurred and peaks were overlapping which indicated that the raw material in the silver nitrate (i.e., silver ions) was no more available for the crystal growth and formation of nanoparticles. The lines with different color represent the spectra recorded at time interval.

![UV-Visible spectrum of silver nanoparticles synthesized by Pseudomonas veronii strain AS 41G](image)

**Figure 3.3(a): UV-Visible spectrum of silver nanoparticles synthesized by *Pseudomonas veronii* strain AS 41G**

When supernatant of *Pseudomonas veronii* strain AS 41G was treated with gold chloroaurate resulted in formation of gold nanoparticles causing a colour change from the colour of reaction mixture to ruby red in colour which was further confirmed with UV-Visible spectrophotometer with a peak conferring between 500 to 650 nm (Figure 3.3b).
ii. Fourier transform infrared spectroscopic analysis of synthesized nanoparticles

Fourier transform infrared spectroscopy analysis of silver nanoparticles synthesized by using supernatant of *Pseudomonas veronii* strain AS 41G resulted in vibrational stretch at different frequencies as shown in the figure 3.4(a). Major peaks were conferred at 3406 which corresponds to hydroxyl group, 1629 corresponds to carbonyl group and peak at 1400 corresponds to carboxyl group. Similar kind of result was obtained with respect to gold nanoparticles devoid of major changes as shown in the figure 3.4(b).
Figure 3.4(b): FTIR analysis of gold nanoparticles synthesized by
*Pseudomonas veronii* strain AS 41G

### iii. X-ray diffraction analysis of synthesized nanoparticles

Crystalline nature of the silver and gold nanoparticles was further confirmed by the XRD analysis. The XRD patterns of silver and gold nanoparticles using supernatant *Pseudomonas veronii* strain AS 41G as shown in the figure 3.5(a) represents the diffraction peak at 2θ values 38°, 44°, 65° and 77° assigned to the (111), (200), (220) and (311) of lattice plane of face centered cubic (fcc) of silver nanoparticles. Similarly diffraction peak at 2θ values 38.29°, 44.42°, 64.83°, 77.94° and 82.7° assigned to (111), (200), (220), (311) and (222) sets of lattice plane of face centered cubic (fcc) for gold nanoparticles respectively (Figure 3.5b). The XRD pattern clearly showed that the synthesized silver and gold nanoparticles formed were composed of pure crystalline and formed by the reduction of Ag⁺ and Au³⁺ ions.
Figure 3.5(a): XRD analysis of silver nanoparticles synthesized by *Pseudomonas veronii* strain AS 41G.

Figure 3.5(b): XRD analysis of gold nanoparticles synthesized by *Pseudomonas veronii* strain AS 41G.
iv. *Transmission electron microscopic analysis of nanoparticles*

The morphological characteristics of silver and gold nanoparticles were investigated by Transmission electron microscopy. The TEM analysis revealed the size of the nanoparticles to be in the range between 5 to 50 nm as shown in the figure 3.6. Synthesized nanoparticles were polydisperse in nature with myriad shapes spherical, near to spherical, hexagonal and triangular. Whereas as majority of the nanoparticles are predominantly spherical in shape.
Figure 3.6: TEM microgram of nanoparticles synthesis by *Pseudomonas veronii* strain AS 41G
b. Nanoparticles synthesis by *Pseudomonas fluorescens* strain CA 417

i. UV-Visible analysis of synthesized nanoparticles

Under the optimized condition the reaction was completed within 15 minutes of incubation with elevated temperature at 80°C and alkaline pH 8. After 15 minutes there was no further synthesis occurred. The lines with different colour represent the spectra recorded at time interval. The synthesis of silver nanoparticles from *Pseudomonas fluorescens* strain CA 417 was analyzed using UV-Visible spectra which resulted in peaks ascertaining between 350 to 550 nm (Figure 3.7a).

![Graph showing UV-Visible spectrum of silver nanoparticles synthesized by Pseudomonas fluorescens strain CA 417.](image)

**Figure 3.7(a):** UV-Visible spectrum of silver nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417

Similarly, supernatant of *Pseudomonas fluorescens* strain CA 417 resulted in synthesis of gold nanoparticles which was confirmed with UV-Visible spectra between 450 to 550 nm as shown in the figure 3.7(b). Even in the case of gold nanoparticles the reduction of metal ions and formation of stable nanoparticles occurred within shorter duration of time under optimized parameters.
ii. Fourier transform infrared spectroscopic analysis of synthesized nanoparticles

FTIR analysis of silver nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417 resulted in predominant peaks occurring at 3346 which corresponds to hydroxyl group, 1635 corresponds to carbonyl group and 680 may correspond to aromatic group (Figure 3.8a). Even in this case there was no much change with respect to FTIR analysis of gold nanoparticles except the minor peaks observed at 1365 and 1217 which may corresponds to alkanes and aliphatic amines (Figure 3.8b).
Figure 3.8(a): FTIR analysis of silver nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417

Figure 3.8(b): FTIR analysis of gold nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417
iii. X-ray diffraction analysis of synthesized nanoparticles

XRD pattern obtained for silver nanoparticles (Figure 3.9a) and gold nanoparticles (Figure 3.9b) showed Braggs reflections that have been indexed on the basis of the face centered cubic structure of silver. The distinct peaks at $38^\circ$, $44^\circ$, $64^\circ$ and $78^\circ$ can be assigned to face-centered cubic (fcc) metallic crystal corresponding to the (111), (200), (220) and (311) facets of the crystal planes at 20 angle.

![Figure 3.9(a): XRD analysis of silver nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417](image-url)
iv. *Transmission electron microscopic analysis of nanoparticles*

The morphological characteristics of silver and gold nanoparticles were investigated by Transmission electron microscopy (TEM). The TEM analysis revealed the size of the nanoparticles to be in the range between 5 to 50 nm as shown in the figure 3.10. Synthesized nanoparticles were polydisperse in nature with myriad shapes spherical, near to spherical, hexagonal and triangular. Whereas as majority of the nanoparticles are predominantly spherical in shape.
Figure 3.10: TEM microgram of nanoparticles synthesis by
*Pseudomonas fluorescens* strain CA 417
c. **Nanoparticles synthesis by *Aneurinibacillus migulanus* strain 141**

Extracellular synthesis of nanoparticles was carried out using cell free supernatant secreted by *Aneurinibacillus migulanus* strain 141 under the optimized parameters the reaction was completed within 25 minutes of incubation time which was monitored using UV-Visible spectra with peaks conferring in between 350 to 550 nm (Figure 3.11a) indicating the synthesis of silver nanoparticles and peaks between 450 to 650 nm confer the synthesis of gold nanoparticles (Figure 3.11b). The lines in the figure represents represent the spectra recorded at different time.

**i. UV-Visible analysis of synthesized nanoparticles**

![UV-Visible spectrum of silver nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141](image)

Figure 3.11(a): UV-Visible spectrum of silver nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141
Figure 3.11(b): UV-Visible spectrum of gold nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141

**ii. Fourier transform infrared spectroscopic analysis of synthesized nanoparticles**

Similarly FTIR analysis of silver nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141 resulted in predominant peaks occurring at 3339 which corresponds to hydroxyl group, 1634 corresponds to carbonyl group and 669 may correspond to aromatic group (Figure 3.12a). Similar results were conferred in case of gold nanoparticles without any major changes as shown in the figure 3.12(b).
Figure 3.12(a): FTIR analysis of silver nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141

FTIR Analysis of silver nanoparticles

Figure 3.12(b): FTIR analysis of gold nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141

FTIR Analysis of gold nanoparticles
iii. X-ray diffraction analysis of synthesized nanoparticles

The XRD pattern exhibited the crystalline nature of the silver and gold nanoparticles with diffraction intensities occurring at 38°, 44°, 65° and 77° which corresponds to (111), (200), (220) and (311) of lattice plane of face centered cubic (fcc) of silver nanoparticles (Figure 3.13a). Whereas diffraction peak at 2θ values 38.29°, 44.42°, 64.83°, 77.94° and 82.7° assigned to (111), (200), (220), (311) and (222) sets of lattice plane of face centered cubic (fcc) for gold nanoparticles respectively (Figure 3.13b).

Figure 3.13(a): XRD analysis of silver nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141
iv. **Transmission electron microscopic analysis of nanoparticles**

Morphological characterization of nanoparticles using TEM analysis revealed different size ranging from 5 to 25 nm with polydispersity of gold nanoparticles with myriad shapes such as spherical, near to spherical, hexagonal and triangular (Figure 3.14). Whereas majority of the nanoparticles are predominantly spherical in shape similar to the results of silver nanoparticles. Average size revealed the 30 nm average size of nanoparticles. The study revealed that the average size of the nanoparticles was within the range of 30 nm. It was also observed that the nanoparticles were stabilized with capping agent surrounding the nanoparticles.
In the present investigation, moderate antibacterial activity against the test pathogenic bacteria but antifungal activity was not observed using silver and gold nanoparticles synthesized from *Pseudomonas veronii* strain AS 41G, *Pseudomonas fluorescens* strain CA 417 and *Aneurinibacillus migulanus* strain 141.
d. Antibacterial activity of silver nanoparticles

i. Well and disc diffusion assay

Well diffusion and disc diffusion assay exhibited clear zone of inhibition across the well and the disc. It was observed that well diffusion assay served better results compared to disc diffusion assay as shown in the figure 3.15. Among the test organisms evaluated, *Klebsiella pneumoniae* (MTCC 7407) was more sensitive to silver nanoparticles which showed significant zone of inhibition than standard gentamicin followed by *Pseudomonas aeruginosa* (MTCC 7903), *Staphylococcus aureus* (MTCC 7443), *Xanthomonas oryzae*, *Xanthomonas axonopodis*, *Escherichia coli* (MTCC 7410) and least activity was observed with *Bacillus subtilis* (MTCC 121). Similar results were achieved with the nanoparticles synthesized with all the three isolates. This might be due to the fact that size of the nanoparticles was almost the same when observed in TEM analysis.

![Figure 3.15: Antibacterial activity of nanoparticles via disc and well diffusion assay](image-url)
Table 3.1: Antibacterial activity of silver and gold nanoparticles against human and phytopathogenic bacteria at 10 mg/ml concentration

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Test Bacteria</th>
<th>Endophytic bacteria</th>
<th>Standards</th>
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<tbody>
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<td>Pseudomonas veronii</td>
<td>Pseudomonas fluorescens</td>
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<tr>
<td></td>
<td></td>
<td>strain AS 41G</td>
<td>strain CA 417</td>
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<tr>
<td></td>
<td></td>
<td>GNP</td>
<td>SNP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zone of inhibition in mm</td>
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Human pathogenic bacteria

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<th>SNP</th>
<th>GNP</th>
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<th>GNP</th>
<th>SNP</th>
<th>GNP</th>
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<td><em>Bacillus subtilis</em> (MTCC 121)</td>
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Phytopathogenic bacteria

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<th>GNP</th>
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<tbody>
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<td>12.33±0.33&lt;sup&gt;de&lt;/sup&gt;</td>
<td>16.00±0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.33±0.57&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>15.33±0.33&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td><em>Xanthomonas oryzae</em></td>
<td>14.00±0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.66±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.00±0.57&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>11.33±0.33&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td><em>Xanthomonas vesicatoria</em></td>
<td>14.66±0.33&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>12.66±0.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.00±0.57&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>11.00±0.57&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**F-Value for One Way ANOVA**

|                  |                     |                     |                     |                     |                     |
|------------------|---------------------|---------------------|---------------------|---------------------|
|                  | 58.76               | 25.44               | 52.85               | 79.89               | 73.60               | 93.24               | 911.40              |

**Note:** Values given are mean of triplicates ± S. E. M (Standard Error of the Mean) (significant p<0.001), by one way ANOVA.

Values followed by same superscript letter(s) are significantly different at (p<0.05) by Tukey’s Post Hoc Test.

GNP-Gold nanoparticles, SNP-Silver nanoparticles
ii. Broth dilution assay

Broth dilution assay resulted in drastic decrease in optical density of the broth seeded with different test pathogens against the increase in the concentration of silver nanoparticles as shown in the figure 3.16(a,b&c) representing the optical density at Y-axis and different concentration of silver nanoparticles at X-axis. Significant activity was conferred with 100 µg/ml of silver nanoparticles concentration against all the test organisms. Even in this case *Klebsiella pneumoniae* (MTCC 7407) was more sensitive to silver nanoparticles.

![Figure 3.16(a): Antibacterial activity of silver nanoparticles synthesized by *Pseudomonas veronii* strain AS 41G using broth dilution](image)

Figure 3.16(a): Antibacterial activity of silver nanoparticles synthesized by *Pseudomonas veronii* strain AS 41G using broth dilution
Figure 3.16(b): Antibacterial activity of silver nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417 using broth dilution

Figure 3.16(c): Antibacterial activity of silver nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141 using broth dilution
iii. **CFU plate method**

Colony forming assay results showed increased number of colonies in control plate and the numbers of colonies were gradually started reducing as the concentration of nanoparticles increased from 0 to 100 µg/ml which can be clearly observed in the figure 3.17(a,b&c). Different colored bar graph represents different pathogens and the variation among the bar graph represents the activity of silver nanoparticles. Even in the case of colony forming assay it was observed that the *Klebsiella pneumoniae* (MTCC 7407) was more sensitive compared to other test pathogens followed by *Staphylococcus aureus* (MTCC 7443), *Bacillus subtilis* (MTCC 121), *Pseudomonas aeruginosa* (MTCC 7903) and least activity was observed against *Escherichia coli* (MTCC 7410).

![Figure 3.17(a): Antibacterial activity of silver nanoparticles synthesized by Pseudomonas veronii strain AS 41G using CFU](image)

Figure 3.17(a): Antibacterial activity of silver nanoparticles synthesized by *Pseudomonas veronii* strain AS 41G using CFU
Figure 3.17(b): Antibacterial activity of silver nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417 using CFU.

Figure 3.17(c): Antibacterial activity of silver nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141 using CFU.
iv. **Minimal inhibitory concentration of silver nanoparticles**

Minimal inhibitory concentration (MIC) resulted in lowest concentration of the gold nanoparticles to inhibit the test pathogens which varied from 31.25-250 µg/ml with significant activity against *Klebsiella pneumoniae* (MTCC 7407).

e. **Antibacterial activity of gold nanoparticles**

i. **Well and Disc diffusion assay**

Gold nanoparticles exhibited antibacterial activity using well diffusion assay (Figure 3.15) with significant zone of inhibition was observed against *Pseudomonas aeruginosa* (MTCC 7903) followed by *Escherichia coli* (MTCC 7410), *Staphylococcus aureus* (MTCC 7443), *Xanthomonas oryzae*, *Bacillus subtilis* (MTCC 121) and *Klebsiella pneumoniae* (MTCC 7407).

ii. **Broth Dilution**

Broth dilution assay resulted in drastic decrease in optical density of the broth seeded with different test pathogens against the increase in the concentration of gold nanoparticles as shown in the figure 3.27(a, b & c) which represents the optical density at Y-axis and different concentration of silver nanoparticles at X-axis.

![Figure 3.18(a): Antibacterial activity of gold nanoparticles synthesized by *Pseudomonas veronii* strain AS 41G using broth dilution](image)
Figure 3.18(b): Antibacterial activity of gold nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417 using broth dilution

Figure 3.18(c): Antibacterial activity of gold nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141 using broth dilution
iii. **CFU plate method**

Significant activity was conferred with 100 µg/ml of gold nanoparticles concentration against all the test organisms. Similarly *Pseudomonas aeruginosa* (MTCC 7903) was more sensitive to gold nanoparticles. Colony forming assay results showed increased number of colonies in control plate and the colonies number gradually decreased as the concentration of gold nanoparticles increased from 0 to 100 µg/ml as represented in figure 3.19 (a, b & c) varied colour bar graph represents different pathogens and the variation among the bar graph represents the activity of gold nanoparticles. Even in the case of colony forming assay it was observed that the *Pseudomonas aeruginosa* (MTCC 7903) was more sensitive compared to other test pathogens followed by *Escherichia coli* (MTCC 7410), *Staphylococcus aureus* (MTCC 7443), *Bacillus subtilis* (MTCC 121), *Pseudomonas aeruginosa* (MTCC 7903) and least activity was observed against *Klebsiella pneumoniae* (MTCC 7407).

![Figure 3.19(a): Antibacterial activity of gold nanoparticles synthesized by *Pseudomonas veronii* strain AS 41G using CFU](image)
Figure 3.19(b): Antibacterial activity of gold nanoparticles synthesized by *Pseudomonas fluorescens* strain CA 417 using CFU

Figure 3.19(c): Antibacterial activity of gold nanoparticles synthesized by *Aneurinibacillus migulanus* strain 141 using CFU
iv. **Minimal inhibitory concentration of gold nanoparticles**

Minimal inhibitory concentration resulted in lowest concentration of the gold nanoparticles to inhibit the test pathogens which varied from 31.25- 250 µg/ml with significant activity against *Pseudomonas aeruginosa* (MTCC 7903).

f. **DNA damage study of silver and gold nanoparticles**

The gel electrophoresis showed intact band with the control DNA of *Staphylococcus aureus* (MTCC 7443) without treatment of nanoparticles. Whereas DNA treated with silver and gold nanoparticles showed deformed and damage of DNA which indicated the action of nanoparticles on DNA as shown in the figure 3.20. 10 µl of nanoparticles treatment was sufficient to break the DNA strand.

![Figure 3.20: DNA Damage of nanoparticles](image)

5. **DISCUSSION**

The results obtained in the present investigation contribute to the growing scientific knowledge on endophytes playing an emerging role in synthesis of nanoparticles. Majority of reports of endophytes for synthesis of nanoparticles are being reported with fungal endophytes. Whereas the present study attributes the evaluation of bacterial endophytes for the synthesis of nanoparticles by using three potent isolates *Pseudomonas veronii* strain AS 41G, *Pseudomonas fluorescens* strain CA 417 and *Aneurinibacillus migulanus* strain 141. Among the three isolates two isolates *Pseudomonas veronii* strain AS 41G and *Aneurinibacillus migulanus* strain 141 were novel species with very rare reports available on their applications.
Interestingly the present findings is the first report on *Pseudomonas veronii* strain AS 41G and *Aneurinibacillus migulanus* strain 141 as an endophyte and their evaluation for synthesis of nanoparticles. In the present investigation an attempt was made and successfully achieved to envision their role in reducing the metal salts to synthesize nanoparticles at shorter time duration. These findings forms one of the important investigation and provides a new platform for highlighting their roles in coming decades.

The results obtained were promising enough and reproducible efficiently. Upon survey of similar results from endophytes we could find the endophytic fungus *Penicillium* species isolated from healthy leaves of *Curcuma longa* was capable of synthesizing silver nanoparticles extracellularly which were confirmed with maximum absorbance between 420 to 425 nm. Synthesized nanoparticles showed antibacterial activity against multi drug resistant *E. coli* and *S. aureus* (Singh *et al.*, 2014). These results were in accordance with results obtained in the present investigation with maximum absorption ranging between 410 to 420 nm and nanoparticles showed antibacterial activity against *S. aureus* and *E. coli*.

Extracellular synthesis of silver nanoparticles was achieved by using culture supernatant of *Epicoccum nigrum*, an endophytic fungus isolated from *Phellodendron amurense*. The synthesized nanoparticles were characterized via UV-spectrophotometry which showed maximum absorption intensity between 420 to 425 nm. The study revealed that different variables such as temperature, pH, and concentration were very important in mediating and influencing the nanoparticle formation. According to the study, alkaline pH, 1mM silver nitrate concentration and elevated temperature influenced the nanoparticle synthesis. The synthesized nanoparticles showed antifungal activity against pathogenic fungi (Qian *et al.*, 2013). The results obtained in the present study are consistent with similar findings observed with alkaline pH, elevated temperature influencing the nanoparticle synthesis.

Gold nanoparticles synthesized using cell free extract of endophytic *Saccharomonospora* sp., isolated from *Azadirachta indica* A. Juss. showed synthesis of nanoparticles with 6 hours of incubation time which continued till 24 hours. The crystalline nature of the nanoparticles was analyzed via XRD exhibiting the diffraction pattern of four distinct peaks corresponding to the (111), (200), (220), and
(311) appearing at 2θ angle 38.2°, 44.5°, 65.6°, and 78.6°. The FTIR analysis revealed the presence of protein molecule in the supernatant responsible for mediating the nanoparticle synthesis. The synthesized nanoparticles were triangular in shape as observed by TEM analysis. These observations correlate with the findings of present investigation except the presence of protein in the supernatant responsible for the synthesis (Verma et al., 2013). In our observation nanoparticle synthesis was rapid within 15 minutes of incubation time and completed within 30 minutes.

Silver nanoparticles synthesized from endophytic Bacillus sp., isolated from Garcinia xanthochymus. The nanoparticles were characterized with UV-Visible spectrophotometer with maximum absorption at 432 nm whose size ranged from 2 to 100 nm with polydispersed in nature as recorded by TEM analysis. The nanoparticles synthesized exhibited antibacterial activity against series of human pathogens (Sunkar and Nachiyar, 2012). These results justifies with findings of the present doctoral studies with synthesized nanoparticles polydispersed in nature and exhibiting antibacterial activity against significant human pathogens including MRSA strain.

Extracellular synthesis of gold nanoparticles was observed using endophytes isolated from the medicinal plant Bauhinia variegata L. among the isolates screened, Pencillium citrinum was capable of synthesizing nanoparticles with the reduction time in minutes compared to the other isolates. The gold nanoparticles showed characteristic absorbance range of 510 to 560 nm with maximum absorbance at 544 nm (Alappat et al., 2012). This result is in accordance with the findings of present investigation wherein the synthesis was rapid and achieved within minutes and showed the absorbance was in the range of 510 to 560 nm.

Silver nanoparticles were synthesized using endophytic Aspergillus terreus isolated from Rhizophora annamalayanna. The synthesis of nanoparticles was confirmed using XRD, SEM with EDX, TEM and FTIR. The study reports the nanoparticles with size of 100 nm and predominantly with spherical in shape. Antidermatophytic activity of silver nanoparticles was evaluated against Trichophyton rubrum, Epidermophyton floccosum and Trichophyton mentagrophytes with 15 mm zone of inhibition against all the test pathogens (Rathna et al., 2013). But in the present investigation antifungal activity was not recorded with synthesized nanoparticle. This might be due to various reasons, as maximum reports confer silver
nanoparticles as potent antibacterial agent compared to antifungal. In our findings prokaryotic system was employed in the synthesis of nanoparticles which showed activity against prokaryotic pathogens.

It has been well reported that nanoparticles are known to improve the remedial efficacy with potent antibacterial activity against various pathogenic bacteria with their different mode of action such as by interacting with cytoplasmic components, nucleic acids, inhibit vital enzymes, and damage the DNA causing subsequently affect the replication (Baker and Satish, 2012b). Keeping this in view mode of action of synthesized nanoparticles on DNA was determined by protocol of Vahdati and Sadeghi, (2013) resulting in subsequent reduction of DNA band intensity causing the DNA to damage with the treatment of nanoparticles compared with the control DNA. Both silver and gold nanoparticles showed DNA damage activity. These findings correlate with the earlier reports of DNA damage study using nanoparticles (Prasad et al., 2014).

These results envisaged in the present study have significantly contributed to the growing interest of biogenic based principle for synthesis of nanoparticles especially with the use of endophytes ascertains their new emerging role towards synthesizing nanoparticles. As endophytes is untapped reservoir but scanty reports available towards nanoparticle synthesis. An attempt made in the present study was successful in reporting 3 potent endophytic bacteria for rapid synthesis of nanoparticles bearing antibacterial activity. With the emergence of drug-resistant microorganisms, a new approaches and different strategies are rapidly reported one such recent approach is use of nanoparticles in combating drug resistant microorganisms. In the present findings silver nanoparticles showed significant activity against *Klebsiella pneumoniae* (MTCC 7407) compared to standard gentamicin.