SYNERGISTIC ANTIBACTERIAL AND ANTIBIOFILM ACTIVITY OF CHEMICALLY SYNTHESIZED SILVER NANOPARTICLES AGAINST PSEUDOMONAS AERUGINOSA

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ABSTRACT
The biofilm are complex structures which undergo various stages of transformation to form the mature biofilm. The bacteria inside the mature stage are capable to be the causative agent for various infections. In order to eradicate and prevent the biofilm on medical devices implants nowadays the silver nanoparticles are utilized. The new antibiofilm technologies target various stages of biofilm formation silver nanoparticles possess potential antibacterial and antibiofilm properties. The silver nanoparticles characterized by spectral analysis where the surface Plasmon resonance at 420 nm, XRD average size 26.5nm, the SEM & TEM micrographs reveals the nature of the silver nanoparticles to be spherical in nature. The antibacterial activity of silver nanoparticles showed the potential activity. The visualization of the antibiofilm was observed with the help of optical microscope, crystal violet staining, titre plate method, congo red agar plate and SEM micrographs. From all the observed results it was proved that the silver nanoparticles would be the powerful weapon to eradicate the biofilm over the surgical instruments.

KEYWORDS: Silver nanoparticles, XRD, antibacterial, antibiofilm effect, SEM.

INTRODUCTION
Infectious disease is the second most common cause of death; the majority of these deaths are due to bacterial-related infections (Jones et al., 2008) Several studies have emerged on antimicrobial therapy lead to failure owing to the growing bacterial resistance to multiple antibiotics (Simoes et al., 2011). Biofilms are microbial consortia embedded in self produced exopolymer matrices composed mainly of exopolysaccharides (EPS). Microbes living in these matrices benefit from nutrient and water supplies (Goller et al., 2008) improved lateral gene transfer (Maha et al., 2001) and protection against adverse environmental conditions, such as desiccation and chemicals, including detergents, disinfectants and antibiotics (Stewart and Costerton 2001 and Costerton 2007). Biofilms can also function as reservoirs for pathogenic organisms and sources for disease outbreaks. For instance, biofilms are implicated in otitis media (Bakaletz, et al., 2007), otolaryngologic infections (Post et al., 2007), osteomyelitis (Brady et al., 2008), bacterial endocarditis, cystic fibrosis (Hoiby et al., 2002) and non-healing wounds (James et al., 2008).

Planktonic microorganisms can adhere to surfaces and initiate biofilm formation. These surfaces include medical devices such as urinary and central venous catheters (Jacobsen et al., 2008), contact lenses (Imamura et al., 2008) as well as mechanical heart valves, and endotracheal tubes (Lynch et al., 2008). These devices are particularly susceptible to biofilm formation because immune responses are markedly reduced in proximity to foreign bodies. In addition; biofilm prevent the phagocytosis of bacteria by phagocytes and limit the access for antibodies (Donlan et al., 2002; Cerca et al., 2006).

The formation of biofilms is also a major cause of implant failure and often limits the lifetime of many indwelling medical devices. Treatment of an infection with current remedies is frequently futile after the biofilm is established. Often, the only solution is physical removal of the biofilm or implant, which is both costly and traumatic to the patient (Lynch & Robertson 2008).

In addition, several antibiotics are inefficient in destroying stationary phase cells and biofilm cells, which often require low nutrition to survive (Fernández et al., 2011). Therefore, new approaches have emerged for reduction in deaths associated with bacterial infections using multiple antibiotic therapies, which can be additive or synergistic, or through the discovery of new drugs with broad-spectrum activity.
**Pseudomonas aeruginosa**, a gram negative bacterium, is an important opportunistic pathogen. It has been reported to be resistant to commonly used empirical antibiotic treatment and has been documented to be responsible to high rates of morbidity and mortality (Goossens et al., 2003). *P. aeruginosa* is a causative pathogen for several infections which includes urinary tract infection, septicemia, osteomyelitis and endocarditis. Thus, it possesses new challenges as the emergence of multidrug resistance strains is at alarming rates.

Among metal nanoparticles with proven antimicrobial activity, those made of silver are particularly effective bactericidal agents (Seil and Webster 2012). The antibacterial properties of silver have long been known and nanoparticles of this metal (AgNPs) are believed to be less toxic than silver ions.

In recent years, the application of AgNPs in various fields has expanded considerably. AgNPs have been successfully used in medical and pharmaceutical nanotechnology for the delivery of therapeutic agents, in chronic disease diagnostics, and as part of sensors (Wong and Liu, 2010; Thiwawong et al., 2013).

Now days AgNPs are developed as a new generation of antimicrobial agents may be an attractive and cost-effective means to overcome the drug resistance problem seen with Gram-negative and Gram positive bacteria. This need has led to the resurgence of silver (Ag)-based compounds due to Ag’s broad activity and possibly far lower inclination to induce bacterial resistance using Ag compared to current antibiotic therapies. It is believed that the probability of bacteria acquiring resistance against Ag is low because Ag+ ions simultaneously act on multiple sites within bacterial cells (Feng et al., 2000).

Therefore, the aim of the present study was to evaluate the antibiofilm potential of AgNPs against biofilms forming *Pseudomonas aeruginosa* by crystal violet staining, tube method, Congo red agar and scanning electron microscopy (SEM).

**MATERIALS AND METHODOLOGY**

**Synthesis and characterisation of silver nanoparticles**

The silver nanoparticles were prepared by using chemical reduction method suggested by Fang et al., 2005. All ingredients of reacting materials were prepared in double distilled water. In typical experiment, 50 ml of 1 × 10^{-3} M AgNO₃ was heated to boiling. To this solution, 5 ml of 1% trisodium citrate was included drop by drop. Solution was mixed vigorously during this process and heated until the colour change was noticeable (pale brown). Finally, it was removed from the heating element and stirred until cooled to room temperature.

**Characterization of metallic nanoparticles**

**UV-visible absorbance spectroscopy (Shankar et al., 2004)**

UV-Visible spectroscopy analysis was carried out on a Systronic UV-Visible absorption spectrophotometer 117 with a resolution of ±1nm between 200-1000nm processing a scanning speed of 200nm/sec. Maximum absorption of the UV-Visible spectra of metallic nanoparticles in aqueous solution with different wavelength in nanometers from 340 to 800nm.

**X-ray diffraction (XRD) (Prema et al., 2010)**

A thin film of the metallic nanoparticle was made by dipping a glass plate in a solution and carried out for X-ray diffraction studies. The crystalline silver nanoparticle was calculated from the width of the XRD peaks and the average size of the nanoparticles can be estimated using the Debye–Scherrer formula (Rau et al., 1962).

\[ D = \frac{k \lambda}{\beta \cos \theta} \]

Where \( D \) = Thickness of the nanocrystal, \( k \) = Constant; \( \lambda \) = Wavelength of X-rays.; \( \beta \) = Width at half maxima of (111) reflection at Bragg’s angle 2θ, \( \theta \) = Bragg’s angle; The size of the silver nanoparticle was made from the line broadening of the (111) reflection using the Debye–Scherrer formula. Where, Constant (K) = 0.94; Wave length (\( \lambda \)) = 1.5406 x 10^{-10} m.

**Zeta potential analyser**

Zeta potential is a physical property which is given the net surface charge of the nanoparticles, when these particles inside the solution repelling each other since produced Coulomb explosion between the charges of the nanoparticles giving rise to no tendency for the particles to agglomerate. The criteria of stability of NPs are measured when the values of zeta potential ranged from +30 mV to -30 mV (Zhang et al., 2008). Surface zeta potentials were measured using the laser zeta meter (Malvern zeta seizer 2000, Malvern). Liquid samples of the nanoparticles (5ml) were diluted with double distilled water (50 ml) using NaCl as suspending electrolyte solution (2 x10^{-3} M NaCl). The pH was then adjusted to the required value. The samples were shaken for 30 minutes. After shaking, the equilibrium pH was recorded and the zeta potential of the metallic particles was measured. A zeta potential was used to determine the surface potential of the silver nanoparticles. In each case, an average of three separate measurements was reported. The criteria of stability of NPs are measured when the values of zeta potential ranged from higher than +30 mV to lower than -30 mV (Akman et al., 2011).

**DLS particle size analyser (Garima Singhal et al., 2011)**

Size distribution of reduced nanoparticles was measured using DLS (Zetasizer Nano zs zen 3600, Malvern, UK). From this measurement, the mean size of particles inside the sample is obtained along with the correlation between the numbers of particles of a particular size versus the size of the nanoparticles. It was based on Mie-
scattering theory. In order to find out the particles size distribution of the samples were dispersed in water by horn type ultrasonic. Then experiment was carried out in computer controlled particle size analyzer to find out the particles size distribution.

**Scanning electron microscopy (SEM) and EDAX (Savithramma et al., 2011)**
The obtained pellet was subjected for SEM analysis. Thin films of the sample was prepared on a carbon coated glass slide by just dropping a very small amount of the sample on the glass slide. extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry for analysis. In EDAX profile analysis a small region of the sample was taken for the elemental composition analysis.

**Transmission electron microscope (TEM)**
The size and morphology of the nanoparticles was studied via TEM (Jeol, Japan). The microscope was operating at an accelerating voltage of 80 kV. The silver samples were first diluted (1:10) in acetone and an aliquot (20 μl) was applied onto a carbon coated grid. The solution was then left for 5 minutes and the excess was removed from the grid by blotting with a filter paper. The grids were placed in the grid box for two hours to dry before imaging.

**Sampling, Screening and isolation of biofilm forming bacteria**

**Sample collection**
The sea water samples were collected from Rameshwaram and they were transferred aseptically to the laboratory condition. Then serially diluted with sterile saline solution were used for bacterial isolation. 0.1 ml suspensions were spread on the nutrient agar plates. Plates were incubated at 48 hrs at room temperature. Mucoid colonies were selected and re-streaked on another nutrient agar plate to obtain pure culture. For further studies the isolates were maintained on nutrient agar plates.

**Screening for biofilm formation**

**A) In situ microscopic observation of biofilm – Light microscopic analysis**
Briefly, 1% overnight culture (0.5 OD at 600nm) For visualization of biofilms by light microscopy, the biofilms were allowed to grow on glass pieces (1x1 cm) placed in 24-well polystyrene plates(Tarson Pvt Ltd) supplemented with nanoparticles (10 μg/ml) and incubated for 24 h at 37°C. Crystalline violet staining was performed as described above. Stained glass pieces were placed on slides with the biofilm pointing up and were inspected by light microscopy at magnification of 40x. Visible biofilms were documented with an attached digital camera (Nikon, Eclipse, Ti 100).

**B) Crystal violet assay**
The biofilm assay was carried in a 96-well-flat bottom tissue culture plate. Briefly, The bacterial suspension was adjusted to be equivalent to 0.5 McFarland’s standard. A serial dilution was then prepared from 10^6 cfu/ml till 10^1 cfu/ml. Each well of the microtiter plate was filled with 150 µl of bacterial suspension. The plate was incubated for 24 h at 37°C. After incubation, the bacterial suspension of each well was gently removed. The wells were washed three times with 0.2 ml of phosphate buffer saline (PBS pH7.2) to remove free-floating ‘planktonic’ bacteria. Adherence of bacteria to the culture plate was stained with crystal violet (0.1%, w/v). Excess stain was rinsed off by washing with deionized water and plates were kept for drying. After drying, 95% ethanol was added to the wells and the optical densities (OD) of stained adherent bacteria were determined with a microplate reader (Spectra-Max 190, USA) at 590 nm. The optical density value was considered as the formation of biofilm on the surface of the culture plate. The experiment was performed in triplicates.

**C) Tube method**
Qualitative assessment of biofilm formation was determined as previously described by Christensen et al., (1982). To 10 milliliters TSB with 1% glucose was inoculated with a loopful of microorganism from overnight culture plates and incubated for 24 h at 37°C. The tubes were decanted and washed with PBS 0.1% (pH 7.3) and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were then dried in inverted position and observed for biofilm formation. The experiment was performed in triplicates.

**D) Congo red agar method (CRA)**
The determination of biofilm formation was carried out by the method described by Freeman et al., 1989 had described an alternative protocol for screening of the biofilm formation which utilizes a specially prepared solid medium – brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red for screening the formation of biofilm Pseudomonas aeruginosa MTCC (2453) and other isolated strains of Pseudomonas aeruginosa. The medium composes of BHI (37 g/l), sucrose (50 g/l), agar No.1 (10 g/l) and Congo red stain (0.8 g/l). Congo red was prepared in the form of concentrated aqueous solution and it was autoclaved at 121°C for 15 min, separately from other medium constituents. Following autoclave, the concentrated solution was added to agar which was previously cooled to 55°C. Plates were inoculated and incubated aerobically for 24–48 h at 37°C. The experiment was performed in triplicates.

**E) SEM micrograph**
Biofilms were assessed as previously described to check nature of the isolated culture which was cultured for overnight reaching the confluency of (~1 × 10^7 CFU ml^-1). Briefly, the cells were washed with PBS twice, fixed with 2.5% glutaraldehyde, then fixed samples were subsequently washed again with PBS and dehydrated gently by washing in a series of ethanol alcohol (30, 50, 70, 90% for 10 min each step). The samples were then dried on a critical point dryer and mounted on SEM grid. The grids were allowed to dry for analysis. In EDAX the nanoparticles size distribution was determined by the method described by Freeman et al., (2011) and it was determined as previously described as previously described by Freeman et al., (1989).
70, 80, 95 and 100% for 10 min) at room temperature and Critical point drying was performed. Afterwards, cells were then oriented, mounted on the aluminum stubs and sputted with gold before imaging. The topographic features of the biofilms were visualized with a SEM. The experiment was performed in triplicates.

Morphological, biochemical and molecular characterization of isolated strains

The selected isolates were identified by morphological and biochemical methods based on the characteristics described in Bergey’s Manual of Systematic Bacteriology (Holt et al., 1994).

DNA isolation from isolated bacteria

Take overnight saturated culture for DNA isolation was done by CTAB method initially 2.0 ml of overnight culture was taken and centrifuged at 12,000 rpm for 3 minutes at 4°C. Supernatant was discarded and cell pellet was resuspended in 200µl of lysis buffer and mixed well. 60µl of 5M NaCl was added to remove the protein and cell debris and centrifuged at 12000 rpm for 10 minutes at 4°C. Clear supernatant was transferred to new tube and 100% ethanol was added to precipitate the DNA. The tube was incubated at -20°C for 1 hr. After incubation, centrifuged for 10 minutes at 4°C. Supernatant was discarded and pellet was washed with 70% ethanol twice. Pellet was resuspended in 50µl of ddH2O and DNA was run in gel electrophoresis.

PCR amplification of DNA

PCR amplified for 16S rRNA genes using the universal bacterial primers were purchased from Xcleris Pvt Ltd 27F (5’- AGA GTT TGA TCM TGG CTC AG- 3’) and 1492R (5’- GGT TAC CTT GTT ACG ACT T-3’). This primer combination amplifies a 1500bp 16S rDNA fragment (Weisburg et al., 1991).

Amplification reaction was performed in a 0.2 ml optical-grade PCR tube (Tarsons, India). 50 nanogram of DNA extract was added to a final volume of 50µl of PCR reaction mixture containing 1.5mM MgCl2, 1X Reaction buffer (without MgCl2) (Fermentas), 200µM of each dNTPs (Fermentas), 100pM of each primer and 1.5U Taq DNA polymerase (Fermentas). PCR was performed in an automated thermal cycler with an initial denaturation at 95°C for 5min. followed by 30 cycles of 95°C for 30sec. (denaturation), 52°C for 45sec (annealing), 72°C for 90 sec. (extension) and 72°C for 10min, (final extension). PCR product was run on 1% agarose in TAE buffer (40mM Tris, 20mM Acetic acid, 1mM EDTA (pH8.0) to confirm that the right product (1500bp) was formed. The PCR product was purified using the QIAGEN PCR purification kit for sequencing and further analysis (Weisburg et al., 1991).

After PCR amplification 16s rRNA followed by 16s rRNA sequencing and BLAST analysis to construct a phylogeny with CLUSTAL W multiple sequence alignment and phylogenetic tree construction by Mega 6.0 software.

Growth Medium and Culture Conditions

*Pseudomonas aeruginosa* MTCC (2453) was procured from MTCC and the 3 isolated strains of *Pseudomonas aeruginosa* were used. In the present study, the cultures were grown in LB (Luria-Bertani) broth medium at 30°C for different length of time as per the requirement of the experiment. For the preparation of the LB medium, 10 g Sodium Chloride, 10 g Casein and 5 g Yeast Extract were dissolved in sterilized 1000 ml MilliQ water and pH was then adjusted at 7.4 before sterilization by autoclaving.

Antibiotic susceptibility testing

Individual isolates were tested, based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2006), by the Kirby–Bauer disc diffusion method for susceptibility to the following antibiotics:IMPM –Imipenem (30µg); CAZ - Cefazidime (30 µg); CFP - Cefoperazone (30 µg); GM – Gentamicin (30 µg); CIP - Ciprofloxacin (30 µg). Antibiotic discs -used were procured by Hi Media (Mumbai, India).

Determination of Minimum Inhibitory Concentration (MIC) by Broth Dilution Shaking (BDS) and MBC

MIC of silver nanoparticles against was measured using standard broth dilution shaking assay *Pseudomonas aeruginosa* MTCC (2453) and other isolated strains of *Pseudomonas aeruginosa* as described previously. To do the experiment, an aliquot (10 µl) of an overnight saturated culture of *Pseudomonas aeruginosa* MTCC (2453) and other isolated strains of *Pseudomonas aeruginosa* (~1 × 10^7 CFU ml^{-1}) were separately added to 5 ml of sterile LB broth in each tube. After that, different concentrations of silver nanoparticles (1to15μ/ml) were separately added to each tube and incubated them at 30°C for 48 h. In the control set, only the organism was grown in absence of silver nanoparticles. The MIC was considered as the lowest concentration of silver nanoparticles in which there would be no visible bacterial growth after 48 hrs of incubation at 37°C.

Antibiofilm efficacy of the silver nanoparticles

To determine the antibiofilm efficacy of the chemically synthesized silver nanoparticles were tested with biofilm forming culture by using the same above listed methods such as light microscopy (Nithya and Pandian 2010) crystal violet assay, tube method Christensen et al., (1982), congo red agar(Freeman et al., 1989) and further by SEM micrograph analysis.

Determination of exopolysaccharide was carried out by total carbohydrate assay and total protein assay. The total carbohydrate content was estimated using phenol sulphuric acid method by Dubios et al., 1956 and the
total protein present in the culture was estimated by the Lowry et al., 1951.

Glass slides were washed in 0.9% NaCl (0.5 ml) and incubated in an equal volume of 5% phenol (0.5 ml) to which 5 volumes of concentrated H₂SO₄ containing 0.2% of hydrazine sulphate was added. The mixture was incubated for 1 h in the dark and absorbance was measured at 490 nm (Favre-Bonté et al., 2003).

The protein content was also measured by the method of Lowry et al., 1951. The procedure is based on quantitating the color obtained from the reaction of Folin–Ciocalteau phenol reagent to which 0.1N Sodium Hydroxide and equal volume of alkaline copper reagent was added to all tubes and the mixture was incubated for 15 min at room temperature. Folin’s reagent (Sigma, USA) was added at a concentration of 50µl/ml. The entire mixture was mixed well and incubated for another 30min at room temperature. Optical density was measured at 690nm. The protein concentration of the sample was obtained from the graph plotted for the standard BSA concentrations as optical density values.

RESULTS AND DISCUSSION
Synthesis and characterization of silver nanoparticles
Silver nanoparticles were synthesized according to the method described by Fang et al., 2005 where the colloidal solution colorless solution turned pale brown and then to black indicating that the silver nanoparticles were formed.

The visual observation of color change was noticed in Figure 1 (A) and taken for the characterization Figure 1 (B) UV-Vis spectroscopy shows the surface Plasmon peak of silver nanoparticles 420 nm. The controlled synthesis mechanism proposed on the basis of Folin-Ciocaltceau phenol reagent was validated through silver nanoparticles (AgNPs) synthesis. AgNPs synthesis was taken as model system for validating the hypothesis, since it is a major challenge to synthesize stable monodispersed silver nanostructures via chemical synthesis route. Silver nitrate (AgNO₃) was used as precursor, whereas trisodium citrate not only behaved as reducing agent but also helps in stabilizing the high surface energy particles from agglomeration. The color of the reaction mixture changed from colorless to yellow and finally to black depicting the presence of uniform facet nanostructures. The absorption spectrum between 400-450 nm is usually the characteristic of the silver nanoparticles. Further, (Shankar et al., 2004) reported that Silver nanoparticles exhibited Yellowish brown color in aqueous solution due to excitation of surface Plasmon vibrations in silver nanoparticles.

In Figure 1 (C) shows the XRD spectrum was analyzed from that using Debye scherrer’s formula the calculated average size was found to be 28.5nm, XRD analysis showed intense peaks corresponding to (111), (200), (220) and (311) Bragg’s reflection which were in good agreement with reference to the unit cell of FCC structure of metallic silver (Joint Committee for Powder Diffraction Standards, JCPDS File No. 01-089-3722). The Debye–Scherrer’s equation was used for calculating the mean size of silver nanoparticles with respect to (111) of Bragg’s reflection, which gave an estimation of about ~28 nm of silver nanoparticles.

In Figure 1 (D) shows the EDAX profile which confirms the presences of silver (26.87%), was confirmed in the EDS intensity graph and the other elements such as Sodium (Na), Nitrogen (N), Carbon (C) are also found due to the glass slides and the precursor salts in water, further no other elemental traces were identified which indicates the purity nature of the nanoparticles.

In Figure 1 (E) SEM micrograph was observed and the shape was found to be round in nature The scanning electron micrograph of silver nanoparticles is depicted and the micrograph shows that the particles have a spherical nature and size ranges from 21.22 ± 5.17, (Prema, 2010) and this is in accordance to results observed. SEM image of synthesized silver NPs by glucose which are spherical in shape and have a smooth surface morphology. It is also apparent that resulting NPs are more and less uniform in size and shape (Kheybari et al., 2010). Figure 1 (F) shows the TEM image of the synthesised silver nanoparticles.

Further in figure 1 (G) shows the zeta potential nature of the prepared silver nanoparticles in the colloidal state was maintained at room temperature condition for a period of 2 years and the zeta potential measurement was analyzed where the average zeta potential measurements was found to be -18.64± 0.56 mV from which the surface charge of the synthesized material was found to be negative and the value indicates that the particles remain stable without getting aggregated or precipitated in the colloidal form and the order of the stability was found to be potentially good.

In Figure 1 (H) shows the average particle size of the same sample were subjected for the DLS particle size analyzer was found to be 28.75±0.32 nm where the size observed where in accordance with the average size determined by XRD, SEM and TEM analysis.

Sample collection, characterisation of the bacterial isolates
Collection of samples
The sea water samples were collected from the area Palk Bay (lat. 9° 17’ long. 79° 7’) of Rameswaram coast of Tamil Nadu, India (Figure 2 A). The collected water samples were taken to the laboratory in an aseptic condition and serially diluted and the spread plate technique was done (Figure 2B). The single colonies which exhibited mucoid and viscous nature were selected for biofilm screening assay. In (Figure 2 C) the pure lines of the single colonies were streaked in Luria Bertani plates for further studies.
A) Screening for biofilm forming bacteria

The selected 16 colonies were subjected for the screening assay for the biofilm forming bacteria, using crystal violet staining, tube method and Congo red agar plates. Out of the 16 isolates 6 isolates showed the positive results are tabulated in Table 1. They were screened for the Pseudomonas sp., by using cetrimide agar plate in which three gave a strong positive by producing green pigmented colonies shown in Figure 3.1 and they were compared with the standard Pseudomonas aeruginosa MTCC 2453. The isolates and the MTCC cultures were subjected to morphological, biochemical and molecular characterization. In morphological characterization of the all the 4 strains exhibited greenish pigment on the cetrimide agar plate, colonies were round and mucoidal in nature, microscopic examination revealed as rod shaped, gram negative bacteria shown in Figure 3.2 a and the corresponding SEM micrograph are shown in Figure 2.3 b at a magnification of 5.00Kx.

C) Biochemical & molecular characterisation of the bacterial strains

Biochemical characterization as tabulated in Table 2 where the positive for voges proskauer, citrate utilization, glucose fermentation, catalase, oxidase, urease and nitrate reduction and negative for indole ring and methyl red test which confirms the isolated strains as Pseudomonas sp. The confirmation of the ability to form biofilm was examined by tube method and Congo red agar plate and shown in Figure 3.4.

Further the isolates where taken for molecular characterization, initially the genomic DNA was isolated and the band was viewed in the 0.8% agarose gel electrophoresis shown in Figure 3.5 A Then the samples were subjected to 16s rRNA PCR amplification under the designed condition and the amplicons were analyzed in the 1.2% agarose electrophoresis and the gel was documented using gel documentation. The ampiclon bands were viewed in the range of ~1.5 kb size in Figure 3.5 B.

The band was eluted from the gel and it was given for sequencing using Big dye terminator and the sequences were taken and phylogenetic tree was constructed shown in Figure 3.6 was drawn using Neighbor-joining phylogenetic dendrogram illustrating the clustering of partial 16S rRNA gene sequences of the isolates. The tree topology was analyzed by neighbor-joining and parsimony analysis with bootstrapping of 1000 replicates using the Mega 6.0 software.

Antibiotic susceptibility of the isolated Pseudomonas aeruginosa strains

The isolated strains were taken to for the analysis of antibiotic susceptibility where then analyzed against the various antibiotics and silver nanoparticles at a constant concentration of 30µg/ml. the zone of inhibition observed for the various antibiotics and silver nanoparticles are shown in Table 3.

Antibacterial and antibiofilm effect of silver nanoparticles

Biofilm and multidrug resistance have been identified as virulence factors of great magnitude in microbial infections P. aeruginosa are medically considered to be major organism associated which are associated with the vast variety of diseases; some strains can cause chronic infections and gain increased resistance to antimicrobial agents through biofilm formation(Bit et al., 2011).

Biofilm development is an intense area of research and the components involved in the development have been considered as possible targets for therapy. To update, there exist a few known compounds which exhibit non toxic antibiofilm compound, no clear evidence of mechanism of resistance of biofilm formation has been elucidated but the important factor for glyocalyx which helps the cells to grow within the biofilm evades the host defense activity and certain drugs, antibacterial agents must have the ability to invade the polysaccharide matrix so that they can penetrated and to kill or remove the biofilms. Nowdays for the easy penetration inside the glyocalyx nanoparticles proves to be the best tool to penetrate such biofilms and reduce biofilm formation by the use of nanofunctionalization surface techniques to prevent the biofilm formation.

A) MIC and MBC of silver nanoparticles against the isolated Pseudomonas aeruginosa

The MIC of Pseudomonas aeruginosa, against the AgNPs was found to be in the range 9-11 µg/ml for MIC for were observed. From the results of MIC and MBC of AgNPs against Pseudomonas aeruginosa are tabulated in the Table 4, where the least inhibitory concentration are shown as MIC values and minimum bactericidal concentration are shown as MBC.

B) Antibacterial activity of the silver nanoparticles by well diffusion method

From the stock solution of silver nanoparticles 50µg/ml was taken and various concentrations ranging from 1 to 15 µg/ ml was prepared and studied by well diffusion method and the zone of inhibition was measured in mm and shown in Figure 4.

C) Antibiofilm effect of silver nanoparticles against the isolated Pseudomonas aeruginosa

The antibiofilm efficacy of silver nanoparticles were determined using various assays such as optical microscopy, crystal violet, tube method Congo red agar plate and SEM analysis.

The biofilm-producing bacteria secrete certain chemicals that protect them from disinfectants and antimicrobials, and phagocytic host immune systems. Several conventional methods of detecting biofilm production have been established, such as the standard Tube method (Christensen et al., 1982), plate method (Freeman et al., 1989). Because these methods can give indecisive
results, we attempted to detect the localization of biofilm with respect to the bacterial cell wall by SEM.

A) Optical microscopic analysis
The light microscopic analysis confirmed the antibiofilm activity of the silver nanoparticles at the concentration of 10 µg/ml at their bacterial inhibitory concentration (BIC). The images show the reduction in the surface area covered by the biofilm resulting in the inhibition of the biofilm formation and maturation. Initially before the treatment of AgNPs the surface of the glass slide was completely covered with the bacterial growth in densely cluster and when treated with silver nanoparticles at the concentration of 10 µg/ml the clustering nature of the bacterial cells were not visualized they were stained with 0.4% crystal violet staining and visually observed with 40x magnification and shown in Figure 5.1.

B) Crystal violet staining method
Qualitative MTP method of biofilm screening of bacterial isolates showed absorbance at wavelength 570nm where O.D stands to be more than 0.25 indicates to be good biofilm formers when the O.D value is between 0.12 – 0.24 indicated to be moderate and O.D below 0.12 seem to be weak / non biofilm formers.

The experiments were performed in the presence and absence of silver nanoparticles. In the control experiments the observed values suggested that the Pseudomonas aeruginosa strains which showed the O.D value (0.34, 0.32, 0.29 and 0.32) which indicates the strains are strong biofilm formers when they were treated with silver nanoparticles at the concentration of 10 µg/ml. The biofilm formation was decreased which was observed by the decrease in the O.D value (0.06, 0.05, 0.07 and 0.09) which suggest that the biofilm inhibition was observed. The observed results are tabulated in Table 6 and Figure 5.2.

C) Tube method
Qualitative Tube Method of biofilm screening of bacterial isolates showed thick blue color ring that were observed as the positive result for the biofilm production. Most of the isolates showed thick blue ring at the liquid–air interface. When the tubes were treated with AgNPs the thick blue ring was not observed. The observed results are tabulated in Table 6.

D) Congo red agar
Biofilm formation was detected in many organisms when they secrete exopolysaccharides. The isolated strains were tested by growing the organism in Brain heart infusion agar supplemented with Congo red (BHIC). When the colonies were grown without AgNPs in the medium, the strains of Pseudomonas aeruginosa produced black colonies with dry crystalline consistency, which is considered as positive result for the biofilm formation.

When the strains of Pseudomonas aeruginosa were grown on BHIC with AgNPs at concentration of 10 µg/ml, the organisms survives, but no black crystalline colonies were seen which reveals the control of exopolysaccharide production by AgNPs treatment. It was found that at a higher concentration of AgNPs inhibited bacterial growth by more than 98%. When the exopolysaccharide synthesis is arrested, the organism cannot form biofilm. It was also observed and confirmed that 10 µg/ml of AgNPs significantly arrested biofilm formation without affecting viability, where as the biofilm forming ability was completely blocked. The observations of the Congo red agar plate method without and with silver nanoparticles are shown in Figure 5.3.

From earlier reports it is clearly known that nanoparticles have potential antimicrobial property, in the current study, we have examined the antimicrobial activity of silver against Pseudomonas aeruginosa strains. The result showed that the silver nanoparticles exhibited strong bactericidal activity against strains. It is clearly observed that the zone of inhibition increase with the increase in concentration of the silver nanoparticles.

E) SEM image analysis
Existing literature documented that biofilm formation happens to be a very important strategy for microbial sustainability as well as the progression of the disease (Donlan et al., 2002). Some populations of biofilm associated bacteria exhibit antibiotic resistance (Vandeputte et al., 2013), reduced growth rate, secretion of different surface molecules and virulence factors (Hall-Stoodley et al., 2009). The matrix of a biofilm is mainly made up of EPS consisting of polysaccharides and proteinaceous substances. Polymers like glycopeptides, lipids and lipo polysaccharides form a scaffold and hold the biofilm together (Flemming et al., 2010).

The attenuation in biofilm formation could be considered as a potential way to make the microbial population more susceptible to antibiotics so that they can be removed from the target site comfortably.

The biofilm grown on the glass slides in 24 well plate for 24 hrs were fixed with the help of glutaraldehyde and observed in the SEM where the presence of the bacteria a clustered nature indicate biofilm forming ability of the microorganism when the bacterial culture was treated with silver nanoparticles at the concentration of 10µg/ml the number of cells decrease and which indicated the inhibition of the biofilm forming ability of the bacteria and the SEM microscopic images are given in magnification of 5.00Kx and shown in Figure 5.4.

From the values observed in Table 6 shows the extent of microbial biofilm formation on glass slides in presence and absence of silver nanoparticles was also measured by determining the amount of total protein and carbohydrate as an alternative tool to address the extent of microbial
population. Over there, because protein and carbohydrate can only come from the biotic source in the bacteria and not from the abiotic source like glass slides. It was clearly observed that when the content of protein and carbohydrate was found to be more in the non treated cells when compared with the silver nanoparticles treated slides. Thus the results reveals that when the microorganisms were treated with silver nanoparticles, then microorganism attaches less to the glass surface that may not be able to form biofilm over the glass surface, which evidently proved that the biofilm forming ability of the bacteria was reduced.

Owing to its known ability to express bactericidal/bacteriostatic activity even at very low concentrations, silver is certainly the metal most used to confer anti-infective properties to biomedical devices. The use of silver as a bulk material has been dropped over time, while it’s utilization for coating or doping solid or hydrogel materials, or for alloying with other metals, has been more and more thrived. Nowadays, silver has become one of the most largely used anti-infective materials. Silver is considered able to exert its antimicrobial activity by several mechanisms, of which those till now recognized have been surveyed.

Briefly, Ag ions interact with sulphur- or phosphorus-containing groups belonging to proteins of bacterial cell wall or plasma membrane, create membrane holes by which cytoplasmic content flows out of the cell and then cause bacterial cell death. Inside the microbial cell, Ag ions inhibit cytochromes of the electron transport chain, bind to and damage DNA, RNA and ribosomes and also lead to the formation of reactive oxygen species (ROS), which are toxic to both bacterial cells and eukaryotic host cells. Interestingly, bacterial resistance to silver is rare and develops slowly in comparison to the resistance to antibiotics. This is presumably due to the multiple antimicrobial mechanisms mediating silver bactericidal activity, whereas antibiotics have usually only one mechanism of action. AgNPs have been designed and introduced as a new generation of antimicrobials, especially devoted to be used as coatings (Chaloupka et al., 2010).

Nanosilver is presumed to be more active than bulk silver materials as it should be able to reach bacteria in tightest proximity at a highest surface/mass ratio, thus producing higher local concentrations of AgNp and consistently, higher bactericidal effects (Wigginton et al., 2010). A major criticism related to the use of AgNPs in preventing/combating biomaterial-associated infections is the potential risk of cytotoxic effects on eukaryotic cells. Intriguingly, in a quite different context, beneficial effects related to the cytotoxicity of AgNPs on eukaryotic cells have recently emerged.

Mechanisms by which AgNPs exert their antibacterial effect have been for long debated, dealing with the question whether the AgNPs themselves can directly damage bacteria, by a “particle specific” action, besides silver ions. Xiu et al., 2012 showed that AgNPs are devoid of bactericidal effects when synthesized and tested under strictly anaerobic conditions precluding Ag0 oxidation and Ag release. They concluded that the antibacterial activity of AgNPs has studied.

Figure 1: Shows A) Visual observation of colour change from yellow to red indicate the reduction of Ag+ to Ag0, B) UV visible spectrum, C) XRD spectrum, D) EDAX, E) SEM micrograph and F) TEM micrograph of silver nanoparticles.
Figure 1.2: G) Zeta potential of silver nanoparticles and H) DLS Particle size in diameter (nm).

Figure 2: A) Shows the sample collected for bacterial isolation B) Spreading plate technique for the collected sample C) Shows the Streak plate and colony morphology of the 16 isolated samples in Luria bertani agar plates.

Figure 3.1: Shows the screening the positive results for the Pseudomonas sp., Figure 3.2 shows the A) Gram staining and B) SEM micrograph of the isolates at the magnification of 5.00Kx.
**Confirmation of the biofilm forming *Pseudomonas sp.***

Figure 3.3: Shows the confirmatory test for the biofilm forming *Pseudomonas sp.*

![Image of biofilm test](image)

Figure 3.4: A) Shows the isolated genomic DNA of the selected samples (in lane 1 – isolate K1, lane 2 – isolate K3, lane 3 – isolate K14. B) Shows the PCR amplified products of the isolated K1, K3 and K14 in Lane 1 - 1Kb ladder, Lane 2 - isolate K1, Lane 3 – isolate K3, Lane 4 – negative control and Lane 5 isolate K14.

![Image of PCR results](image)

Figure 3.5: Shows the phylogenetic tree of the isolated *Pseudomonas aeruginosa* strains K1, K3 and K14 drawn using Neighbor-joining phylogenetic dendrogram illustrating the clustering of partial 16S rRNA gene sequences of the isolates. The tree topology was analyzed by neighbor-joining and parsimony analysis with bootstrapping of 1000 replicates using the Mega 6.0 software.

![Phylogenetic tree image](image)
Figure 4: Shows antibacterial activity of the Ag Nps against the selected strains by well diffusion method.

Figure 5.1: Shows the crystal violet assay staining without silver nanoparticles A) *Pseudomonas aeruginosa* MTCC 2453, B) *Pseudomonas aeruginosa* K 1, C) *Pseudomonas aeruginosa* K 3 and D) *Pseudomonas aeruginosa* K 14, With silver nanoparticles E) *Pseudomonas aeruginosa* MTCC 2453, F) *Pseudomonas aeruginosa* K 1, G) *Pseudomonas aeruginosa* K 3 and H) *Pseudomonas aeruginosa* K 14.

Figure 5.2: Shows the crystal violet assay in titre plate without silver nanoparticles A) *Pseudomonas aeruginosa* MTCC 2453, B) *Pseudomonas aeruginosa* K 1, C) *Pseudomonas aeruginosa* K 3 and D) *Pseudomonas aeruginosa* K 14, with silver nanoparticles E) *Pseudomonas aeruginosa* MTCC 2453, F) *Pseudomonas aeruginosa* K 1, G) *Pseudomonas aeruginosa* K 3 and H) *Pseudomonas aeruginosa* K 14.
Figure 5.3: Shows the Congo red assay without silver nanoparticles A) *Pseudomonas aeruginosa* MTCC 2453, B) *Pseudomonas aeruginosa* K1, C) *Pseudomonas aeruginosa* K3 and D) *Pseudomonas aeruginosa* K14. with silver nanoparticles E) *Pseudomonas aeruginosa* MTCC 2453, F) *Pseudomonas aeruginosa* K1, G) *Pseudomonas aeruginosa* K3 and H) *Pseudomonas aeruginosa* K14.

Figure 5.4: Shows the SEM image without silver nanoparticles A) *Pseudomonas aeruginosa* MTCC 2453, B) *Pseudomonas aeruginosa* K1, C) *Pseudomonas aeruginosa* K3 and D) *Pseudomonas aeruginosa* K14. With silver nanoparticles E) *Pseudomonas aeruginosa* MTCC 2453, F) *Pseudomonas aeruginosa* K1, G) *Pseudomonas aeruginosa* K3 and H) *Pseudomonas aeruginosa* K14.

Table 1: Screening assay for biofilm formation.

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Crystal violet assay</th>
<th>Tube method</th>
<th>Congo red agar plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>K2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K3</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>K4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K5</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>K6</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>K7</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>K8</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 1: Shows the screening for biofilm forming bacteria.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>K9</th>
<th>K10</th>
<th>K11</th>
<th>K12</th>
<th>K13</th>
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<tbody>
<tr>
<td>Colony Morphology</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Gram staining and shape</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole ring test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilisation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cetrimide agar plate</td>
<td>+ greenish pigment</td>
<td>+ greenish pigment</td>
<td>+ greenish pigment</td>
<td>+ greenish pigment</td>
<td>+ greenish pigment</td>
</tr>
<tr>
<td>Crystal violet assay</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Congo red agar plate</td>
<td>Black crystalline colonies</td>
<td>Black crystalline colonies</td>
<td>Black crystalline colonies</td>
<td>Black crystalline colonies</td>
<td>Black crystalline colonies</td>
</tr>
</tbody>
</table>

Table 2: Biochemical characterisation of the isolate bacterial strains.

<table>
<thead>
<tr>
<th>Name of the test</th>
<th>Pseudomonas aeruginosa (MTCC 2453)</th>
<th>Isolate K1</th>
<th>Isolate K3</th>
<th>Isolate K14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony Morphology</td>
<td>Round shape, mucoid</td>
<td>Round shape, mucoid</td>
<td>Round shape, mucoid</td>
<td>Round shape, mucoid</td>
</tr>
<tr>
<td>Gram staining and shape</td>
<td>Gram negative, rod</td>
<td>Gram negative, rod</td>
<td>Gram negative, rod</td>
<td>Gram negative, rod</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole ring test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilisation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cetrimide agar plate</td>
<td>+ greenish pigment</td>
<td>+ greenish pigment</td>
<td>+ greenish pigment</td>
<td>+ greenish pigment</td>
</tr>
<tr>
<td>Crystal violet assay</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Congo red agar plate</td>
<td>Black crystalline colonies</td>
<td>Black crystalline colonies</td>
<td>Black crystalline colonies</td>
<td>Black crystalline colonies</td>
</tr>
</tbody>
</table>

Table 3: Antibiotic susceptibility pattern of the isolated strains.

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Antibiotics (zone of inhibition in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa (MTCC 2453)</td>
<td>15 16 14 11 17</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K 1</td>
<td>17 15 13 17 11</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K 3</td>
<td>15 11 7 9 10</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K 14</td>
<td>11 16 17 11 12</td>
</tr>
</tbody>
</table>

Table 2.3: The interpretation were done according to CLSI, where R resistant, I – intermediate, S- sensitive. For IPM: R<14mm, I=14-15mm, S>16mm; CFP R<16; I 16-20; S>20; CAZ R<15, I 15–17, S > 17; GM: R <13, I: 13–14, S > 14; CIP: R < 16, I: 16–20, S > 20. IPM - Imipenem; CAZ- Cefazidime; CFP- Cefoperazone; GM – Gentamicin; CIP- Ciprofloxacin.
Table 4: MIC and MBC of silver nanoparticles.

<table>
<thead>
<tr>
<th>Test microorganism</th>
<th>MIC (µg/ml)</th>
<th>MBC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa (MTCC 2453)</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K1</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K2</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K14</td>
<td>9</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 4: MIC and MBC of silver nanoparticles against the selected microorganisms.

Table 5: Shows various methods for determining the antibiofilm effect of silver nanoparticles.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Without AgNPs</th>
<th>With AgNPs</th>
<th>Biofilm formation by CRA method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Without AgNPs</td>
</tr>
<tr>
<td></td>
<td>++ (0.34)</td>
<td>-- (0.06)</td>
<td>+++</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (MTCC 2453)</td>
<td></td>
<td></td>
<td>(Black crystalline)</td>
</tr>
<tr>
<td></td>
<td>++ (0.32)</td>
<td>- (0.05)</td>
<td>++</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K1</td>
<td></td>
<td></td>
<td>Dark brown</td>
</tr>
<tr>
<td></td>
<td>++ (0.29)</td>
<td>- (0.07)</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K3</td>
<td></td>
<td></td>
<td>Blackish brown</td>
</tr>
<tr>
<td></td>
<td>++ (0.30)</td>
<td>- (0.09)</td>
<td>++ black</td>
</tr>
</tbody>
</table>

Table 5: Shows the various methods for determining the antibiofilm effect of silver nanoparticles.

Table 6: Total carbohydrate and protein content.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Carbohydrate content (Dubios et al.,) (mg/dl)</th>
<th>Protein content (Lowry’s et al.) (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without AgNP treatment</td>
<td>With Ag NP treatment</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (MTCC 2453)</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K1</td>
<td>11</td>
<td>5.4</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K3</td>
<td>10.5</td>
<td>5.8</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa K14</td>
<td>15</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Table 6: Shows the total carbohydrate and protein content analyzed in the Pseudomonas aeruginosa MTCC 2453, K1, K3 and K14 isolates.

SUMMARY AND CONCLUSION

According to the report of the National Institutes of Health and Center of Disease control, about ~65–80% infections occurred by biofilm formation microbes, in which the Gram-negative bacterium P. aeruginosa, E. coli and the Gram-positive S. aureus are the most common ones. For this purpose, in order to prevent biofilm formation in the environment water logging sites. From those sites the water samples were collected and screened for the biofilm forming bacteria the main area focused was Pseudomonas sp., for that purpose the strains were screened with cetrimide agar plate further they were subjected to biochemical & molecular characterization was performed and proved to be Pseudomonas aeruginosa isolates K1, K3 and K14. Then the phylogentic tree was constructed with Mega 6.0 version. Further, the synthesized silver nanoparticles by chemical method were used as a potent antibiofilm agent and its ability was studies with various methods and it was concluded that at the concentration of 10µg/ml of silver nanoparticles.

However, the exact mechanism of action of AgNPs in biofilm-related studies is yet to be demonstrated. Kostenko et al., 2010 reported that coat nanocrystalline silver has the highest antibiofilm efficacy compared to aqueous silver and silver ion and the silver concentration alone cannot account for the antibiofilm efficacy of the silver dressings. The type of silver species present also plays a role. The reduction of the silver particle to the nanoscale level increases the relative surface area, which provides higher Ag release rates than for elemental silver
particles. Moreover, nanoparticles have a higher capacity to attach to and to penetrate bacterial membranes and accumulate inside cells, providing a continuous release of silver ions inside the cell (Rai et al., 2009; Kostenko et al., 2010). These studies confirmed the exclusive ability of silver nanoparticles to disrupt biofilm of pathogenic bacteria. It suggests and proves that silver nanoparticles have potential antibiofilm and therapeutic ability.

Conflict of interest
The authors declare no conflict of interest.

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