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Original Research Article

Optimisation of reactant concentration in Biosynthesis of Silver nanoparticles using pathogenic bacteria isolated from clinical sources and their characterisation

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ABSTRACT

Introduction: The all-around attraction towards Silver Nanoparticles (AgNPs) is fairly due to its biocompatibility, so that it can be used in therapeutics and diagnostics. In order, to harvest them with such unique properties, there is this pressing urge to develop a cheaper and an eco-friendly process without the use of toxic chemicals. In lieu of this, this study was taken up to optimise the reactant parameters required to obtain a maximum yield of AgNPs using cell-free culture extract of bacteria obtained from clinical sources.

Materials and Methods: Bacterial strains were procured from routine clinical samples. Bacterial biomass production was done in liquid media which was further harvested and lysed. The supernatent(E) in varying concentrations was then mixed with varying concentrations of 1mM AgNO3(M) (E:M ratio). Visual examination for colour change and confirmation by UV-Spectrophotometry (UV-S) was done and AgNPs were separated by Ultra-centrifugation. The synthesised NanoParticles(NPs) were characterized by Scanning Electron Microscopy(SEM) and X-Ray Diffraction(XRD) studies for confirmation.

Results: The UV-S showed an absorption value at 450nm as 1.3 in 1E:1M for Escherichia coli followed by Abs: 1.0 for Staphylococcus aureus in 1E:1M ratio. The Dynamic Light Scattering study showed that the particles obtained in the study were predominantly in the acceptable range of 127.35nm & 90.96nm for Staphylococcus aureus & Escherichia coli respectively. When observed under SEM, the synthesised particles were found to be agglomerated, but polydispersed and crystalline in nature. In XRD study a maximum peak was obtained at 38.22 which confirmed the crystalline structure synthesized from bacterial extract as AgNPs when compared with JCPD standards.

1. Introduction

Among several nanoparticles, attraction towards Silver Nanoparticles (AgNPs) is mainly because of its bio-compatibility in therapeutics and also as antimicrobial agent biosensors, antibacterial agents, cancer treatment, gene therapy, and DNA analysis, enhancing reaction rates and magnetic resonance imaging.[1–4] History of synthesis of nanoparticles dates back to when Klaus and co-workers have shown that the bacterium Pseudomonas stutzeri AG259, which was isolated in a silver mine, when placed in a concentrated aqueous silver nitrate solution, played a major role in the reduction of the Ag+ ions and the formation of Silver Nanoparticles (AgNPs) of a well-defined structure and distinct size and within the bacterial periplasmic space.[5]

Many bacteria, fungi, and plants have shown the ability to synthesize metallic nanoparticles and all have their own advantages and disadvantages. The use of microbes to synthesis nanoparticles is a very eco-friendly process and has enormous advantages over the other known physical and chemical processes since it takes place at ambient pressure and temperature.[6]

Saifuddin et al in 2009, demonstrated the extracellular synthesis of AgNPs (∼ 5–50 nm) using a cell-free extract of B. subtilis and microwave irradiation.[7] In 2007,

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Shahverdi et al reported the rapid green synthesis of AgNPs (within 5 mins) using the cell-free culture supernatants of gram Negative bacteria like Klebsiella pneumoniae, Escherichia coli and Enterobacter cloacae.[8]

In the studies, two strains of Bacillus subtilis (denoted T'1 and I'1a) were used. The biological synthesis of AgNPs was performed using supernatants obtained from cultures of bacteria growing on brewery effluents, molasses, and Luria–Bertani (LB) medium.[9]

The nanoparticles displayed free radical scavenging activities. This paper thus highlights nanoparticle synthesis by a hitherto unreported Actinomycetes culture, identifies the biomolecule involved in the process and describes the associated antioxidant activity.[10]

Hosseini-Abari et al, proposed a cost-effective and environmental-friendly biotechnological process for the synthesis of silver nanoparticles extracellularly using the bacterial spores.[11]

Furthermore, in 2009, the Susceptibility of MRSA, methicillin-resistant Staphylococcus epidermidis and Streptococcus pyogenes to AgNPs was demonstrated.[12]

Extracellular and intracellular synthesis of AgNPs using several bacterial strains like B. amyloliquefaciens, B. flexus, B. megaterium, and S. aureus has also been demonstrated.[13–15]

Interestingly, the antibacterial activity was seen to be exhibited by microbial GLP-capped AgNPs against V. cholerae comparable to ciprofloxacin.[16]

Few researchers also suggested the water-soluble fraction of the extracellular polysaccharides (EPS)/matrix of Nostoc commune is a potent capping agent and a reducing for the biosynthesis of AgNPs. Morsy FM et al, also suggested that AgNPS can be used as an effective surface sterilizing agent on seed crops against phytopathogenic fungi.[17] The multidrug-resistant organisms like P. aeruginosa and K. pneumonia were seen to be more susceptible to the AgNPs when compared to foodborne pathogen L. monocytogenes. Aspergillus spp. showed a maximum susceptibility compared to Penicillium spp. Researchers also suggested that exopolysaccharide-stabilized AgNPs can be used in various biomedical applications as antimicrobial agents.[18]

The nanocomposite material obtained by Liu C et al showed desirable activity in inhibiting both Gram-positive Staphylococcus aureus and Gram-negative Escherichia coli on an agar culture plate and also in liquid culture, showing the potential of the material to be used in wound dressings as an antimicrobial.[19]

Moreover, the synthesized AgNPs inhibited many medically important pathogenic bacteria like Bacillus subtilis, Enterococcus faecalis, Escherichia coli and Salmonella typhimurium and yeast-like Candida albicans.[1] Drug delivery systems which are Nano-scaled can be used efficiently into a porous 3D scaffold in grafts to enhance the tissue regeneration capacity. In conclusion, Nano-structured biomaterials are a very rapidly expanding research area and are providing newer enabling technologies in the field of regenerative medicine.[3]

To achieve the AgNPs with their unique properties, there is a pressing need to develop a cheaper and eco-friendly method for the synthesis of AgNPs which eliminates the use of toxic chemicals during their synthesis. In this pursuit, this study was taken up to study to find out the optimum parameters required to achieve silver nanoparticles using a cell-free extract of bacterial strains from clinical sources.

2. Materials and Methods

2.1. Bacterial biomass production

A Gram-Positive Bacteria (Staphylococcus aureus) & a Gram-Negative Bacteria (Escherichia coli) were obtained from clinical samples in pure form and were used for the production of the bacterial biomass. The isolated bacteria were inoculated in culture flasks with Luria Bertani Broth and incubated for 48 hours to give maximum yield. A media free culture of the bacteria was obtained by centrifuging at 5000rpm for 10mins followed by washing with distilled water. The media free bacteria obtained were kept in distilled water for 24hrs, to lyse the bacteria. This was then centrifuged at 12000 rpm for 15 minutes (Figure 1). The supernatant was then collected for further processes to synthesis nanoparticles.

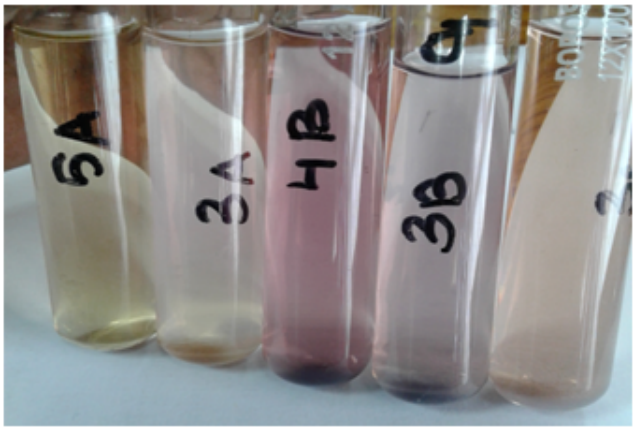

Fig. 1: Visible observation of colour change

2.2. Preparation of AgNO3 solution

Silver nitrate was chosen as the metal salt for the biosynthesis of AgNPs. AgNO3 (HiMedia). A volume of 500 ml of 1mM solution of silver nitrate required for the study was prepared using double distilled water and was stored in an amber-colored bottle.

2.3. Synthesis of AgNPs

2.3.1. Phase I

3 ratios were put up; 9:1(9ml supernatant and 1ml metal salt solution){9E:1M}, 1:1 (5ml supernatant and 5ml metal salt solution {1E:1M}), and 1:9 (1ml supernatant and 9ml metal salt solution) {1E:9M}, for each organism. The mixtures were left at room temperature to react. Hourly sub-samples were taken out of the reaction mixtures and were subjected to UV-visible Spectrophotometer analysis to fix the suitable reaction time and the appropriate ratio to obtain maximum AgNP production. After 24 hrs, the initial detection of AgNPs was carried out by proper visual observation of the characteristic colour change in the filtrate (Figure 2). The reacted samples after the colour change were subjected to UV spectrum analysis from 200-800 wavelengths at 1 nm resolution.

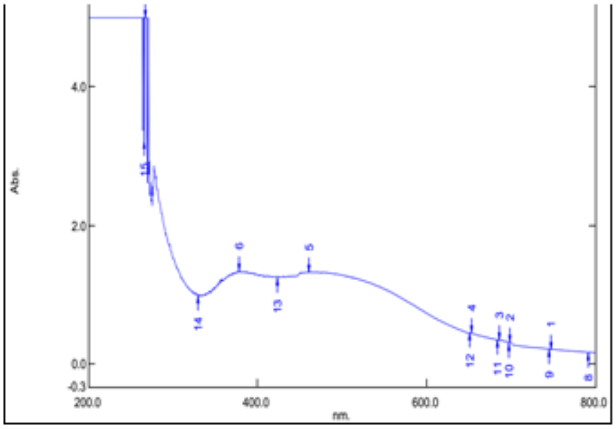

Fig. 2: E. coli- 1:1-460nm-Abs: 1.3

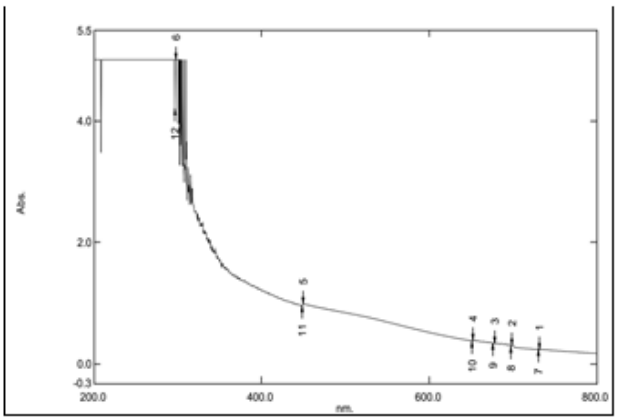

Fig. 3: Staphylococcus aureus -1:1-450nm-Abs: 1.0

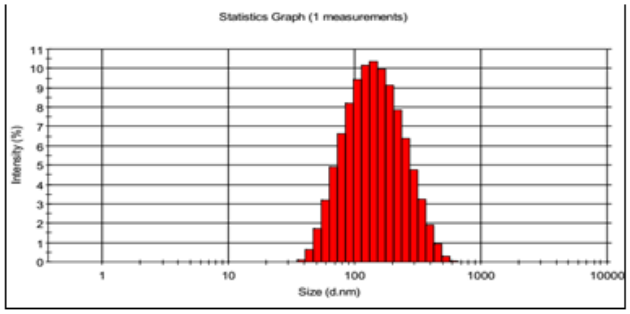

Fig. 4: Average particle size of AgNP by Staphylococcus aureus by DLS

2.3.2. Phase II

From the UV spectra obtained for three reaction samples, the best out of the three ratios of metal salt to supernatant was selected and the synthesis was done for further studies. AgNP pellets were recovered by centrifuging (15,000 rpm) for 15 min. The residue settled at the bottom of the tubes are washed in distilled water repeatedly to remove any culture media. Further it was transferred to petri dish and dried at 60 and then in muffle furnace at 750oC to burn out all organic matter to get nanoparticles alone. Thus prepared nanoparticles are used for further characterization study.

2.4. Characterization

SEM is used for morphological characterization at the nm to mm scale. The dried powder of nanoparticles are viewed under SEM (Model JSM-6610LV.D). Images on surface morphology are obtained. For XRD analysis (Rigaku Ultima IV), the liquid phase nanoparticle solution was dried in an oven at 60°C and then in a muffle furnace at 750°C to form a powder. The dried powder was collected for characterization by an X'pert Pro x-ray diffractometer operating at 40 kV and a current of 30mA with Cu Ka radiation in θ-2θ configuration.

2.5. Antibiotic susceptibility testing

The dried powder of AgNPs thus generated were challenged with 0.5 Mc Farland standard of E.coli & Staphylococcus aureus in varying concentrations of 10μg, 20μg, 30μg, 40μg/ml, 50μg/ml, 60μg/ml, 70μg/ml, 80μg/ml, 90μg/ml & 100μg/ml in Muller Hilton agar broth. At periodic intervals of 4 hrs, and their absorbance was plotted.

3. Results

3.1. UV spectra

In the reaction tubes, reduction of silver nitrate in to silver has taken place in 6 hours exhibiting change of colour of the reaction solution to brown. Change of colour from milky

white to brown is the characteristic indicator of formation of silver. Besides, the UV-Vis spectrum showed a peak at 460nm (normal range is 380-520nm). UV spectra are given as Figures 3 and 4. Maximum absorption is shown in 1:3 (1E:1M) for Escherichia coli Abs: 1.0 at 450nm for Staphylococcus aureus in 1E:1M ratio, (Table 1). From this phase I study it is demonstrated that ratio of 1:1 of bacterial extract to 1mM AgNO3 solution is ascertained as the optimum ratio for synthesizing smaller nanoparticles. Hence, the same ratio is taken for phase-II and further synthesis and characterization.

3.2. *Dynamic light scattering*

Particle size obtained by DLS is tabulated in Table 2 and shown in Figures 5 and 6 confirming the size of the Silver particle obtained in the study was predominantly in the acceptable range (90-120nm).

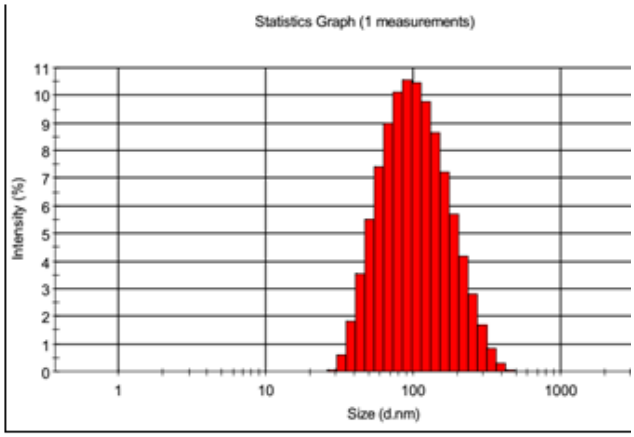

Fig. 5: Average particle size of AgNP by Escherichia

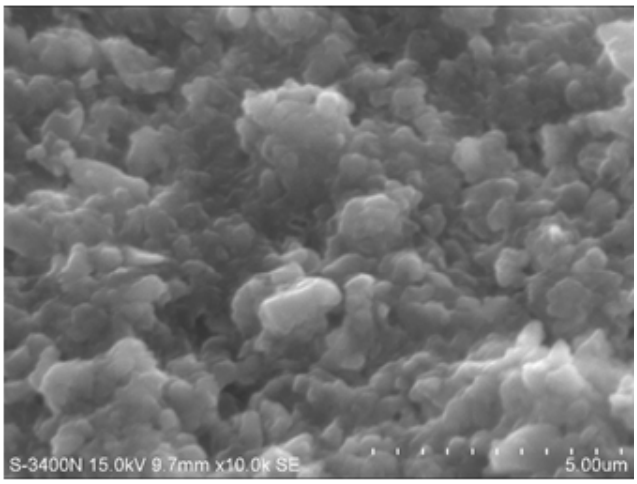

Fig. 6: SEM image of AgNPs

3.3. *Scanning electron microscopy*

The SEM image of AgNPs are given in Figure 7. The images revealed agglomeration of particles but polydispersed.

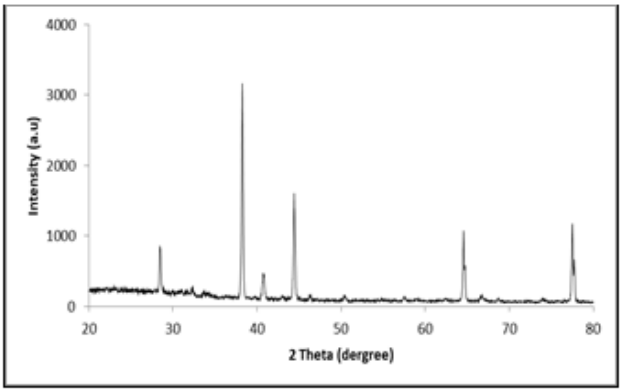

Fig. 7: XRD for AgNPs synthesized in the study

3.4. *X-ray diffraction*

The results are presented inFigure 8 and 2 theta values are given in Figure 9 showing the 2 theta values for the silver particles: 38.22, 44.45.64.56 and 77.50 and the braggs diffraction at 111,200,220 and 311 which closely matching with the international standard given by Joint Commission on Powder Diffraction standard (JCPD standard).

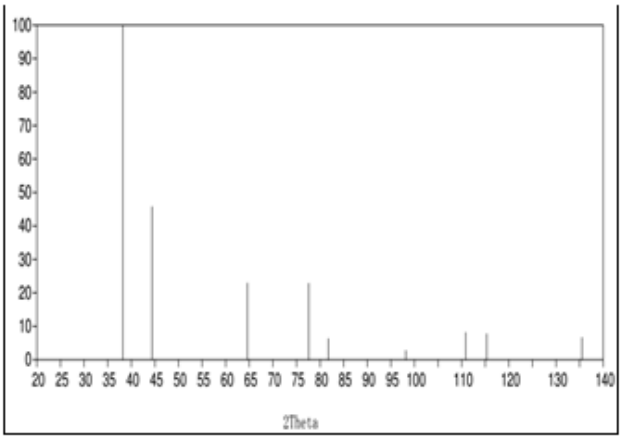

Fig. 8: Standard XRD for AgNPs

3.5. *Antibiotic susceptibility testing*

Figure 10 show the anti-bactericidal activity of AgNPs against Escherichia coli & Staphylococcus aureus, with MIC of 30 μg/ml and 60 μg/ml respectively.

Table 1: Appropriate Wavelength (W) & Maximum absorbance (A) by UV spectrophotometry

Organism/Ratio	9E:1M	1E:1 M	1M:9E
Staphylococcus aureus	W: 450nm A : 0.7	W: 450nm A : 1.0	W: 450nm A : 0.6
Escherichia coli	W: 450nm A : 0.7	W: 460nm A : 1.3	W: 450nm A : 0.6

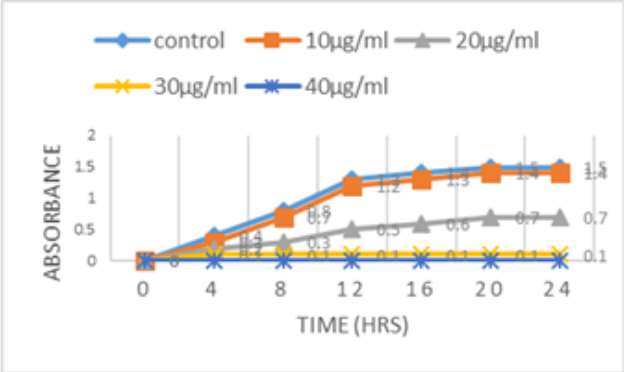

Fig. 9: Absorbance spectra of AgNPs against E.coli

Table 2: Particle size by DLS

Organism	Average
Staphylococcus aureus	127.35nm
Escherichia coli	90.96nm

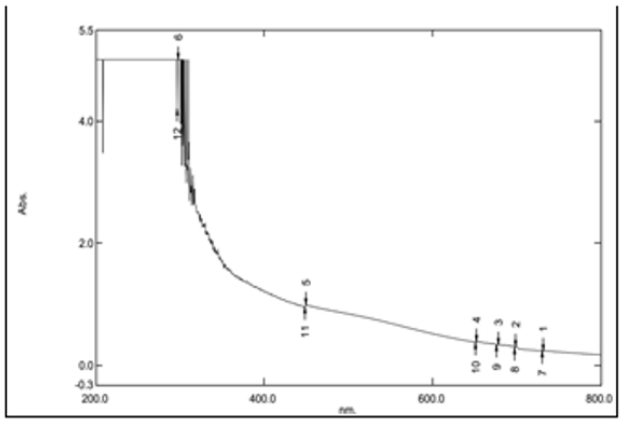

Fig. 10: Staphylococcus aureus -1:1-450nm-Abs: 1.0

4. Discussion

AgNPs are reported to possess unique properties such as good conductivity, chemical stability, and catalytic properties than its bulk counterpart. These NPs also have antifungal, anti-viral and anti-inflammatory properties also.[5,20] They showed effective antimicrobial activity against highly multi-resistant strains such as methicillin-resistant Staphylococcus aureus.[21] In view of such potency of AgNPs, in the recent past, they have made efforts to make use of microorganisms as possible eco-friendly nanofactories for the synthesis of silver nanoparticles.

The formation of silver NPs is mediated by the organic substances present in the cell free extract of the selected bacteria. The metal salt is reduced to metal i.e. silver nitrate is reduced to silver NPs. The formation of such particles is indicated by the change in the colour of the reaction solution to brown and this colour is the characteristics of silver nanoparticles.[22] The colour change takes place due to the Plasmon vibrations exhibited the nanoparticles formed in the solutions. Similar observation was also reported earlier in Bacillus megaterium, where a pale yellow to brown colour was formed due to the reduction of aqueous silver ions to silver nanoparticles.[15] This supports the fact that change in colour as observed in the experiment can be considered as an indication of silver nanoparticles formation. Further such inference is also confirmed by UV spectra recording a peak at 460nm (within the characteristic band range-380-520nm). Therefore, as reported 23 earlier, UV spectral studies is also could be an effective tool in nanoparticle studies.[23]

Optimisation of extract to metal salt is a crucial factor in standardising inputs. Moreover, as the synthesis involves chemical reactions, contraction of both reactants viz. cell free extract of given bacterium and the metal salts. Therefore, in the present study, the chemical reduction was more efficient in synthesising Nano sized particles at the ration of 1:1 for both bacterial extracts at 10 mMol silver nitrate concentration. The ratio has showed an average size of the particles as 85 nm.

The nature of chemical reduction or the mechanism behind metal reduction by extract and organic molecules involved in this reduction process, have been discussed widely without arriving any concrete explanation. However, presently it is explained that intracellular enzymes like nitrate reductase present in the microbes are responsible for the reduction of these metal ions to metal nanoparticles.[24] This was also reported in the bacteria Bacillus licheniformis, where this enzyme secreted by the bacterium was responsible for the reduction of Ag metal to metal nanoparticles.[22,25] Spectroscopically analysed the organic compounds present in the cell free bacterial extract and suggested the strong interaction of aromatics, lipids, amino acids as well as ketones. Besides, various bacteria are reported to possess a variety of bio agents that reduce metal salt to metal nanoparticles such as Rhamnolipids in Pseudomonas aeruginosa;[26,27] URAK –a fibrinolytic enzyme in Bacillus cereus.[27] Cellulose in Gluconacetobacter with Cellulose[28]-Actinorhodin pigment In Streptomyces coelicolor;[29] Flagellin in Salmonella typhimurium[30] - Presence and binding of protein, carbohydrates and aromatics and fatty

acids with AgNPs might have behaved as reducing agent. Biomolecules like proteins, bio-surfactants and enzymes, present in the microorganisms act as reducing agents. Also, in many bacterial strains, bio-surfactants can be used as stabilizing agents or capping agents.[31]

Extracellular synthesis of AgNPs happens by of the trammelling of metal ions on the outer surface of the microbial cells and in the presence of the enzymes or biomolecules they are reduced, while intracellular synthesis happens inside the microbial cells. Researchers also suggested that the extracellular synthesis of nanoparticles favours large-scale, is cheap, and requires simpler downstream processing and production. Hence the synthesis of AgNPs extracellularly is preferred,[24] when compared to intracellular method[32] as it involves more of fine chemicals and processing techniques.

The exact mechanisms of toxicity activity and antimicrobial activity by AgNPs are a still unclear subject matter among biotechnologists and microbiologists world-wide. However, quoting the biophysical properties of them in general, the mechanism might could be because of more than one cellular kinetics**.** There are reports indicating, the electrostatic attraction between AgNPS which are positively charged and bacterial cells which are negatively charged.[32] Other probable mechanisms involve interaction between biological macromolecules such as enzymes and DNA and silver molecules through an electron-release mechanism[33] or a probable free radical production.[34] Both silver ions and AgNPs can actually work by changing the three dimensional structure of proteins by altering the disulphide bonds and by blocking the functional operations of the microbes.[35–37] Park et al proposed that the inhibition of protein synthesis and cell wall synthesis has been also been caused by AgNPs.[38] The growth of microbes is inhibited by the nanoparticles which modulated the phosphotyrosine profile of the bacterial peptide affects signal transduction.[39]

Therefore, from the results obtained from the current microbial assay, it could be reported that AgNPs are more potent in treating diseases caused by Gram positive bacteria rather than Gram negative bacteria. It is to be noted that besides such wide range of research undertaken globally to find the ways and means of utilizing the AgNPs in the field of medicine and diagnostic, an attempt was also made and reported that AgNPs are preliminarily found to possess ability to suppress cancer cell proliferation.[40] Therefore, it is hopefully conceived that AgNPs can be used as a magic Nano robot that could be employed search and kill the target cell and/or pick and place the drug in the specified site precisely.

5. Acknowledgements

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6. Source of Funding

None.

7. Conflict of Interest

None.

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