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Research Article

Novel Approach towards Synthesis of Silver Nanoparticles from *Myxococcus virescens* and their Lethality on Pathogenic Bacterial Cells

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Abstract

Aim: We report extracellular synthesis of silver nanoparticles by cell filtrate of *Myxococcus virescens*. Moreover, the effect of silver nanoparticles on survivability or lethality of human pathogenic bacteria viz. *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* subsp. *spizizenii* (ATCC 6633) and *Pseudomonas aeruginosa* (ATCC 10145) has been studied.

Methods and Results: The silver nanoparticles were synthesized from *M. virescens* cell filtrate, and characterized by UV-Vis Spectroscopy, Nanoparticle Tracking Analysis (NTA) by LM 20, Zeta Potential analysis, Fourier Transform Infra-Red Spectroscopy (FTIR) and Transmission Electron Microscopy (TEM). Antibacterial efficacy of silver nanoparticles was tested which comprised the effect of survivability or lethality of human pathogenic bacteria. Biosynthesized silver nanoparticles demonstrated promising cell lethality for human pathogenic bacteria.

Conclusion: *M. virescens* mediated synthesized silver nanoparticles are quite stable, and capped with proteins. This is a sustainable, eco-friendly and simple process for synthesis of desirable silver nanoparticles with lethal properties against clinical bacterial cells.

Significance and Impact of Study: *M. virescens* mediated synthesized nanoparticles could make a starting point to obtain new antibacterial substances towards multiple antibiotic-resistant pathogenic bacteria.

Keywords: *Myxococcus virescens*, Silver Nanoparticles, Human pathogens, Cell lethality, Antibacterial activity

Introduction

Nanobiotechnology is amalgamation between nanotechnology and biology, which refers to the ability to create and manipulate biological and biochemical materials, devices, and systems at nano level [1]. In nanotechnology, for development of new nanostructure materials and devices it requires nanoparticles with unique properties. Silver has been used widely since ancient time to treat infection and prevent spoilage [2]. When the era of antibiotic began the use of silver for its antimicrobial properties decreased [3]. Since antibiotic resistant microorganisms emerged the interest to use silver as antimicrobial agent is rising again. Zero valent silver (Ag^0) nanoparticles are a valuable alternative for ionic silver. Due to their large specific surface to volume ratio nanoparticles have different properties than ionic silver. Silver nanoparticles are active against broad spectrum Gram-negative and Gram-positive bacteria. Furthermore, silver nanoparticles showed antifungal [4] and antiviral activity [5].

Biosynthesis of silver nanoparticles is an environmental-friendly method without the use of toxic and expensive chemicals. The ability of microorganisms in production of metal nanoparticles has opened a new exciting approach toward the development of these natural nano–factories [6]. The important aspect in the process of producing highly stable, well-characterized and and highly active nanoparticles are selection of the best organisms and optimization of conditions for growth and enzyme activity. These factors could control morphology, size, stability, aggregation and other properties of bionanoparticles. Microorganisms demonstrated more advantages over other biological systems because of their high tolerance towards the heavy metals [7].

Due to physicochemical properties, silver nanoparticles have been extensively utilized and are currently used as antibacterial agents in fruit storage [8], textile and health industries [9], also for labeling and as biosensors [10]. To achieve the nanoparticles with their unique properties, there is a pressing need to develop a cheaper and eco-friendly method for synthesis of nanoparticles which eliminates the use of toxic chemicals during their synthesis process. Metal nanoparticles like silver, gold and platinum are extensively exploited for the welfare of human beings [11]. The synthesis of silver nanoparticles is broadly studied by chemical and physical methods [12]. Biological methods using plants [13,14], fungi [15], and bacteria [16] have superior option for chemical and physical synthesis of silver nanoparticles.

In recent times microorganisms and plants are found as environment-friendly nanofactories. A wide range of microbes including bacteria like, *Pseudomonas aeruginosa* [16], *Bacillus* sp. [17], *Bacillus cereus* [18], cyanobacteria-*spirulina platensis* [19],

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actinobacteria-*streptomyces* sps. [20], *Bacillus subtilis* [21] and fungi like *Fusarium oxysporum* [22], *Fusarium acuminatum* [15], *Fusarium solani* [23], *Phoma glomerata* [24], *Alternaria alternata* [25], *Fusarium culmorum* [26], *Neurospora crassa* [27], *Trichoderma* [28], *Fusarium graminearum*, *Fusarium scirpi* [29] were exploited for the synthesis of silver nanoparticles. Plants like *Gliricidia sepium* (Jacq.) [30], *Carica papaya* [31], *Opuntia ficus-indica* [13], *Allium cepa* [32], Weed [33], *Foeniculum vulgare* [34], *Ocimum sanctum* [35], *Cassia auriculata* [36], *Phyllostachys* sp. [37], Soap nuts [38], *Murraya koenigii* [39], *Lawsonia inermis* [40] were also found to be important source for green synthesis of silver nanoparticles. Microorganisms demonstrated more advantages over other biological systems because of their high tolerance towards the heavy metals like Fe, Co, Ni, Cu, and Zn, As, Cd, Hg, Pb or U [41-43].

Myxobacteria (slime bacteria) are included among the Delta group of Proteobacteria. They are found in the topsoil where they feed on organic matter and prey on other microorganisms by secreting hydrolytic enzymes and antimicrobials [44]. Antibiotic and enzymes produced by myxobacteria kill microorganisms and lyse cells. Reduction of silver ions is catalyzed by enzymes or by nonenzymatic interaction of Ag+ with cell wall functional groups. In the current studies we focused on synthesis of silver nanoparticles from cell supernatant of *M. virescens* and the influence of silver nanoparticles on the growth of human pathogenic bacteria.

Materials and Methods

Test myxobacterium

Myxococcus virescens was isolated from top level of the forest soil (depth 10 cm) under Scots pine (*Pinus sylvestris* L.) trees of Torun, Poland. The chemical properties of the soil were as follows: pH H_2O-4.22, pH KCl-3.44, C org. % 1.78, and N % 0,075, C/N24. Soil samples were moisturized using water with dissolved antibiotics: actidione (100 mg/dm^3), nystatine (100 mg/dm^3) and penicillin (50 mg/dm^3). For inoculation of myxobacteria, lumps of soil (weighing ca. 0.1 g) as a inoculums were used, which were put onto petri dishes containing solidified Vy/2 myxobacteria-semi selective medium (Baker's yeast 0.5%, $CaCl_2 2H_2O$ 0.1%, cyanocobalamin 0.5ug ml^{-1}, agar 1.5%, pH7.2) (Reichenbach and Dworkin 1981). Inoculated plates were incubated at 30^{O}C for 30 days. Myxobacteria were subcultured and stored on Vy/2 agar medium.

Preliminary identification of myxobacterial strain was carried out on the basis of diagnostic keys by Reichenbach and Dworkin [45]. The strain was identified by 16s RNA analysis. Genomic DNA was extracted and amplified following the procedures given by [46]. The isolated DNA was amplified by PCR technique, using the following primers: 10-30F and 1500R. The samples were subjected to the preliminary denaturation (98^{O}C, 3 min.) in the thermocycler. Amplification (28 cycles) was carried out using the following thermal profile: 93^{O}C for 1 minute (denaturation), 52^{O}C for 1 minute (joining the starters), and 72^{O}C for 2 minutes (elongation of the starters). The final elongation of the starters was carried out at temperature 52^{O}C (1 min.) and 72^{O}C (10 min.). To obtain the specific products of PCR (size – 1584bp), Q/Aquick Gel Extraction Kit (Qiagen) was used. The length of amplified fragment of DNA was estimated on the basis of molecular weight standard. In our studies, we used DNA Marker III (Roche) containing DNA fragments of length from 564 to 21226bp. The replicated and purified genetic material was sequenced using ABI Prism TM Big Bye TM Terminator Cycle Sequencing Kit. The sequence consensus was compared with NCBI Gene Bank data base.

Extracellular synthesis of silver nanoparticles

For the synthesis of silver nanoparticles, 100 ml CAS broth [47] was prepared in flask, sterilized and inoculated with fresh growth of *Myxococcus virescens*. The inoculated flasks were incubated at 30^{O}C for 72 hours. The culture was then centrifuged at 12,000 rpm for 15 minutes and supernatant was discarded. Bacterial cells settled at bottom were washed 2-3 by sterile distilled water to remove media traces. Afterwards, bacterial cells were suspended in sterile distilled water and incubated for 48 hours. After incubation, suspension was centrifuged at 12,000 rpm and cell free filtrate which contains osmotically lysed bacterial cell content was treated with 1mM $AgNO_3$ and incubated for 48 hours. Only cell filtrate and broth with 1mM $AgNO_3$ were also maintained as control. The experiment was set up in triplicate.

Detection of silver nanoparticles

After the 48 hr incubation of cell filtrate and $AgNO_3$ (mixture), the preliminary detection of silver nanoparticles was carried out by visual observation of colour change in filtrate. All the above reaction mixtures were then subjected to UV-visible Spectrophotometer analysis (Shimadzu UV-1700, Japan). The spectrum was scanned from 200-800 wavelengths at 1 nm resolution.

Characterisation of silver nanoparticles

Nanoparticle tracking analysis (NTA) by LM 20

Liquid samples of silver nanoparticles were used to perform NTA analysis, in which laser beam (approximately 40mW at *k*=635 nm) was passed through a scattering cell. Particles present within the path of the laser beam were observed via a dedicated non-microscope optical instrument (LM-20, NanoSight Pvt. Ltd., UK) having Charge Coupled Device (CCD) camera. The motion of the particles in the field of view (approximately 100×100 μm) was recorded (at 30 fps) and the subsequent video and images were analyzed for the size distribution of nanoparticles in sample.

Measurement of zeta potential

The zeta potential was measured by using a Zetasizer Nano ZS 90 (Malvern Instrument ltd, UK). Measurements were made by means of Dynamic Light Scattering (DLS) in the range of 0.1-1000 μm. 1000 μl of sample was transferred in the clear disposable zeta cell for the measurement of zeta potential. The zeta potential was calculated using Henry's equation.

Fourier transforms infra-red (FTIR) spectroscopy

Silver nanoparticles were further characterized by FTIR (Perkin-Elmer FTIR- 1600, USA) in the range 500–2000 cm^{-1} at a resolution of 4 cm^{-1} to understand the biomolecules responsible for the reduction of silver ions and stabilization of silver nanoparticles in the mixture. For sample preparation, 300 μl of concentrate colloidal silver nanoparticle solution was mixed with 10 mg potassium bromide (KBr) in clean crucible, until it becomes a fine powder. The sample was prepared and oven dried to remove the traces of moisture.

Transmission Electron Microscopic Analysis

To determine the size and shape of silver nanoparticles Transmission Electron Microscopic analysis (TEM) was carried out. The silver nanoparticles were characterized by TEM (Joel 1010, at 80kv), on conventional carbon coated copper grids (400 meshes,), it was cleaned using plasma treatment under oxygen for 45 sec. A 5 µl of sample was then placed on the grid and dried at room temperature for 1 hour. The samples were inspected by operating at 80 KV. Images of sample were taken to have a clear representation of its composition.

Removal of unreacted Ag^+

To remove the unreacted Ag^+ from nanoparticle sample, it was treated with NaCl solution. After addition of NaCl, Ag^+ reacts with Cl^- and form white precipitate of AgCl. The precipitate was then removed by centrifugation of mixture at 4,000 rpm for 15 minutes. After removing the pellet, the supernatant was centrifuged at 14,000 rpm for 30 min to concentrate the silver nanoparticles, and then dried at 50°C. Subsequently, their dry mass was estimated. To be used in further studies silver bionanoparticles were resuspended in deionized water, to obtain their desired concentration [23].

Analysis of antibacterial activity of silver nanoparticles

Antibacterial efficacy of silver nanoparticles was tested against: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* subsp. *spizizenii* ATCC 6633 and *Pseudomonas aeruginosa* ATCC 10145.

The studies comprised: (a) effect of biosynthesized silver nanoparticles on the bacterial growth, using double-layer agar plates, b) the effect of biosynthesized silver nanoparticles on survivability or lethality of bacteria

a) Effect of biosynthesized silver nanoparticles on the bacterial growth, using double-layer agar plates [48].

Molten and cooled to 45°C Trypticase Soy Agar (TSA) medium in culture tube was inoculated with the cells suspension 10^4 cfu ml^{-1} of the respective bacteria (obtaining a final concentration of bacterial cells 10^3 cfu ml^{-1}) and supplemented with silver nanoparticles up to a concentration 100 µg ml^{-1}. Subsequently, after mixing, medium was poured as a second layer onto an already prepared petri dish of TSA agar and set aside to solidify. Plates with the poured medium without nanoparticles were also kept as a control. Plates were incubated for 24 hrs at 37°C. Bacterial colonies grown on the plates were counted. An extent of antibacterial activity of silver nanoparticles was calculated by following formula:

$$AE\ (\%) = [(C - T)/C] * 100$$

where

AE: Antibacterial activity (expressed in percents),

C: Number of bacterial colonies on the control plate (cfu/ per sample),

T: Number of bacterial colonies on the experimental plate (with silver nanoparticles) (cfu/ per sample).

b) The effect of biosynthesized silver nanoparticles on survivability or lethality of bacteria

LIVE/DEAD® BacLight™ Bacterial Viability Kit, L7012 (Invitrogen) stain was used, which makes possible a discrimination between live bacterial cells (green fluorescence) and the dead ones (red fluorescence) under the fluorescence microscope. Respective bacterial cultures were grown in liquid TSB medium for 24 hours at 37°C, and after that it was centrifuged at 5000 rpm for 5 min at 5°C. The bacterial pellet was then rinsed with sterile physiological saline, suspended in physiological saline maintained to the optical density equal to 4 McFarland units. Thus obtained cell suspension was 10-fold diluted by addition of the appropriate volume of nanoparticles suspension or silver ions solution used for comparison to obtain the final concentration equal to 75 µg ml^{-1}. Silver nanoparticles were purified according to the procedure described by [23]. Bacterial suspensions without silver nanoparticles were used as controls. Samples were incubated for 30 min. at room temperature, and then rinsed with physiological saline and resuspended in sterile distilled water. After staining with LIVE/DEAD stain, the bacterial suspension was put onto glass microscopic slide and was observed under the epifluorescence microscope ZEISS Axiostar plus, with the light source mbq 52 ac (Carl Zeiss, Jena, Germany), using the oil immersion 100-fold magnifying objective. Live and dead bacterial cells on microphotographs were counted with computer aid, using image analysis software program MacBiophotonics Image J (version 1.41a) and its Cell Counter plug-in.

Results

Synthesis of silver nanoparticles using *Myxococcus virescens* was found to be a promising method because change in colour of cell filtrate was observed from yellowish or colourless to brown when treated with aqueous 1mM $AgNO_3$ (Figure 1 inset). Synthesized silver nanoparticles were then subjected to UV-Visible spectroscopy showing the absorbance peak at 420 nm (Figure 1), which is characteristic feature of silver nanoparticles.

Further, silver nanoparticles were characterized by Nanoparticle Tracking Analysis (NTA) using Nanosight LM 20 to analyse size and size distribution of particles on the basis of their brownian motion in

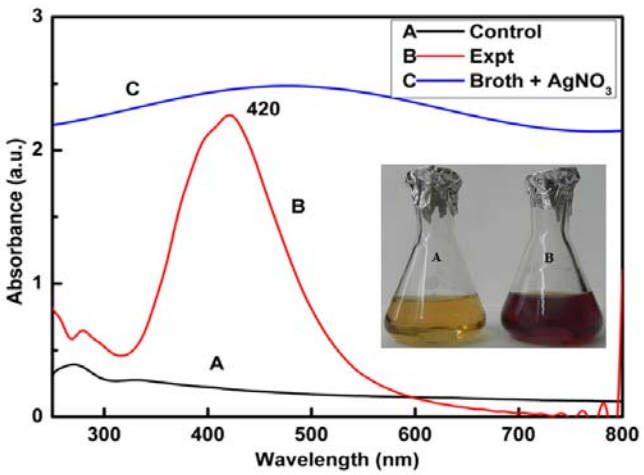

Figure 1: UV-Visible Spectra of (Control, **Spectra A**) cell free extract of *Myxococcus virescens*, silver Nanoparticles (Expt., **Spectra B**) synthesized by cell free extract of *Myxococcus virescens*, and broth + 1mM $AgNO_3$ (**Spectra C**). Inset figure: colour change of bacterial filtrate from pale yellow (control) (**A**) to dark brown (treated) after synthesis of Silver Nanoparticles (**B**).

suspension. The average size of silver nanoparticles synthesized by *M. virescens* was 81 nm and mode size 65 nm, means in suspension maximum nanoparticles are of 65 nm size (Figure 2). In addition, to determine the stability of synthesized silver nanoparticles, zeta potential was measured. Figure 3 reveals that the zeta potential of synthesized silver nanoparticles was -25.2 mV, which indicates that the synthesized nanoparticles were moderately stable. Further analysis was performed by FTIR spectroscopy to acquire the idea regarding the protein capping and interactions of proteins with silver, which might be responsible for synthesis and stabilization of silver nanoparticles. In FTIR spectrum (Figure 4) of control (Figure 4 spectra A) peaks were observed at 1074, 1415, 1548, 1649 and 2349 cm^{-1}. After the synthesis of silver nanoparticles these peaks were shifted to 1051, 1386, 1539, 1652 cm^{-1} respectively. Transmission Electron Microscopy (TEM) revealed (Figure 5) that the synthesized silver nanoparticles were polydispersed and spherical in shape with the size distribution 7- 50 nm.

Our study on, the effect of silver nanoparticles on the growth of bacteria with the use of double-layer plate method have shown that the concentration of nanoparticles used (100 µg ml^{-1}) exerted an antibacterial action, which was within the range 25.8-33.9%. The highest inhibitory effect was observed in the Gram-negative bacterium; *Pseudomonas aeruginosa* (Table 1).

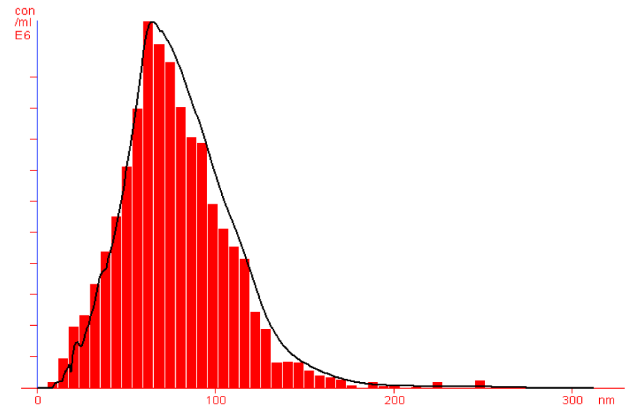

Figure 2: NTA (Nanosight-LM 20) nanoparticle size distribution histograms showing Polydisperse silver nanoparticles synthesized by cell free extract of *Myxococcus virescens*.

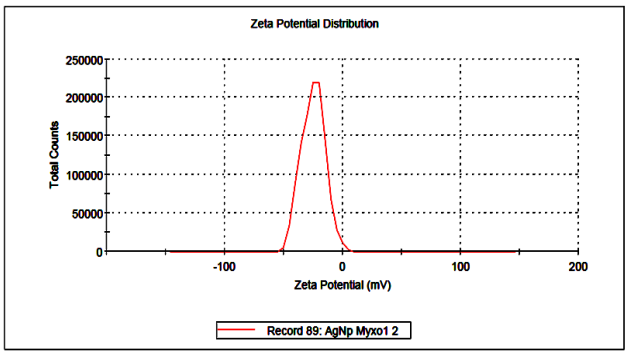

Figure 3: Surface zeta potential graph showing negative zeta potential value for silver nanoparticles synthesized by cell free extract of *Myxococcus virescens*.

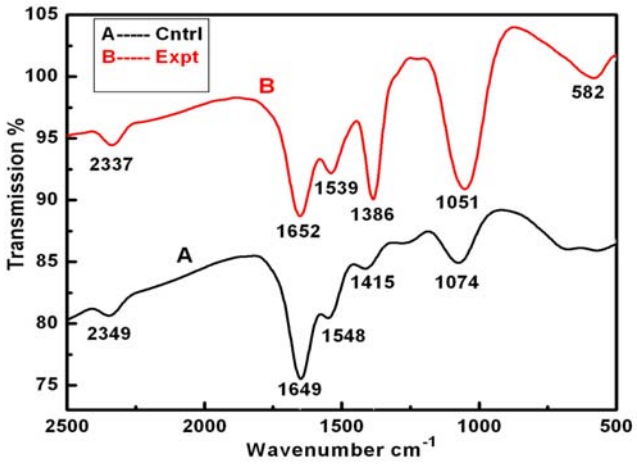

Figure 4: FTIR spectra of bacterial cell filtrate (control, **A**), and silver nanoparticles (experimental, **B**) synthesized by cell free extract of *Myxococcus virescens*.

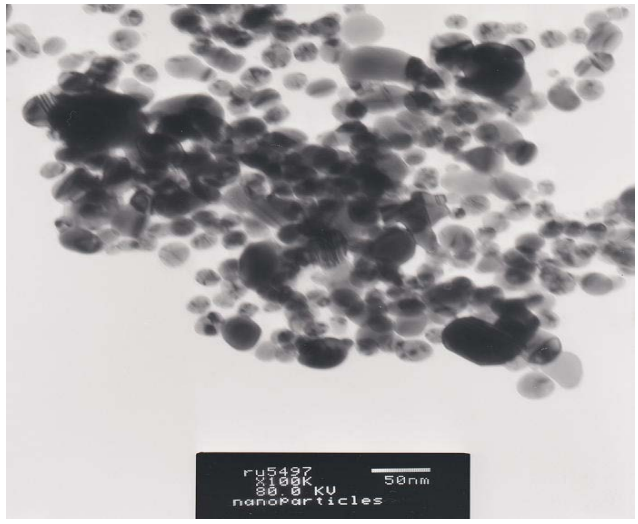

Figure 5: TEM micrograph showing spherical and polydispers silver nanoparticles synthesized by cell free extract of *Myxococcus virescens*.

Table 1: Effect of silver nanoparticles synthesized by cell free extract of *Myxococcus virescens* on bacterial growth on agar medium.

Bacterial species	Number of bacterial colonies on plate (cfu) x10^3 (control)	Number of bacterial colonies (cfu) x 10^3 grown on plate in the presence of silver nanoparticles (AgNPs)	AE [%] (percentage antibacterial activity)
		100 µg cm^{-3} of AgNPs	
B.subtilis subsp. *spizizenii*	252	187	25.80
S. aureus	510	382	25.10
P. aeruginosa	784	518	33.93

Results of our studies on the effect of silver nanoparticles or silver ions on the lethality of bacterial cells, estimated that lethality of all the strains studied was higher in the presence of silver nanoparticles (over 90%) as compared to silver nitrate as a control. The strongest action of silver nanoparticles was noted for *Staphylococcus aureus* (Figure 6). However, both in case of test dealing with the effect of

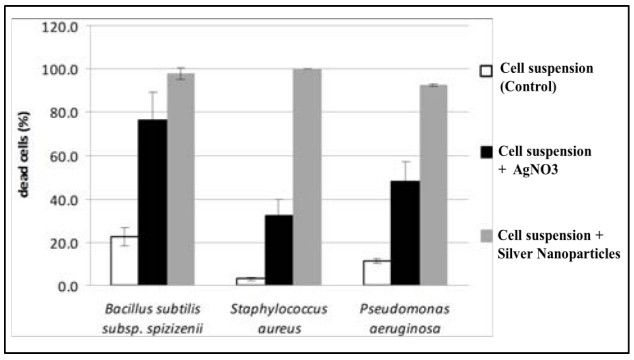

Figure 6: Effect of silver nanoparticles synthesized by cell free extract of *Myxococcus virescens* and silver ions on the lethality of pathogenic bacteria cells (BacLight method) (average values ± standard deviation).

silver nanoparticles on bacterial growth, and their effect bacterial survival, antimicrobial activity of nanoparticles studied essentially did not differ depending on the strain.

Discussion

The change in colour from pale yellow to dark brown was due to the reduction of silver ions (Ag^{+}) to silver nanoparticles (Ag^{0}) which is the evidence for synthesis of silver nanoparticles (Figure 1 inset). Silver nanoparticles exhibit strong absorbance at 420 nm in the visible range due to surface plasmon resonance [49]. The single and symmetric peak indicates the synthesis of spherical nanoparticles. synthesis of spherical nanoparticles. These findings support the earlier studies carried out by many researchers using different bacteria and fungi as biological agents for the synthesis of silver nanoparticles [15,2,26,50,16,18,51,52].

Negative zeta potential value of the particles might be due to adsorption of OH^{-} ions on it. Since adsorption of OH^{-} ions on the silver nanoparticles increase zeta value of the nanoparticles, there is an increase in stability of the nanoparticles due to electrostatic repulsion among the negative charges. An OH- ion helps in preventing the aggregate formation and maintains the smaller size of silver nanoparticles [53]. In FTIR analysis of silver nanoparticles peak observed at 1051cm^{-1} associated with stretch vibration of -C-O- bond. The peak at 1386 cm^{-1} is due to NO^{-}_{3} existed in the residual solution [54]. The band at 1539 and 1652 cm^{-1} arose due to N-O nitro group and Stretch vibration of -C=C- respectively. These results corroborate with findings of Haung *et al.* [54]. As shown in Figure 4 spectra B, the peak exhibits the binding of amide linkage with silver nanoparticles which was clearly indicated in the infrared region of the electromagnetic spectrum indicating the presence of protein as a capping agent for silver nanoparticles. Proteins have stronger ability to bind silver nanoparticles which increases the stability of synthesized nanoparticles [55].

Raheman *et al.* [50] observed greater antibacterial effect of silver nanoparticles in case of *Staphylococcus aureus* than in Gram negative bacteria *Salmonella typhi*., Sunkar and Nachiyar [18] also demonstrated bactericidal activity of silver nanoparticles to pathogenic strains of bacteria namely Gram negative (*Escherichia coli, Pseudomonas aeruginosa*) and Gram positive bacteria (*Staphylococcus aureus*). Effect silver nanoparticle was observed to be more profound in Gram negative bacteria than Gram positive. Authors suggested that it is attributed to the fact that the relative abundance of negative charges on Gram negative bacteria facilitated the interaction between the nanoparticles and the cell wall.

The bactericidal effect of silver nanoparticles has been well established; however, the mechanism is only partially understood. The silver nanoparticles show efficient antimicrobial property compared to other salts due to their extremely large surface area, which provides better contact with microorganisms. The nanoparticles get attached to the cell membrane and also penetrate inside the bacteria. The silver nanoparticles are considered to be a slow release source of silver ions, which interact with sulfur-containing proteins present in bacterial cell membrane as well as with the phosphorus containing compounds like DNA [56,57]. When silver nanoparticles enter in the bacterial cell it forms a low molecular weight region in the center of the bacteria due to which the bacteria conglomerates and protect the DNA from the silver ions. The nanoparticles preferably attack the respiratory chain, cell division finally leading to cell death. Moreover, the nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity [2].

Extracellular synthesis of silver nanoparticles by *Myxococcus virescens* is being reported for the first time. It has been confirmed that cell filtrate of *M. virescens* is capable of synthesizing the silver nanoparticles. *M. virescens* mediated synthesized silver nanoparticles are quite stable, and capped with proteins. This is a sustainable, eco-friendly and simple process for synthesis of desirable silver nanoparticles. Biosynthesized silver nanoparticles reveal antibacterial properties against some clinical bacteria showing lethal effect on their cells. These nanoparticles could make a starting point to obtain new antibacterial substances towards multiple antibiotic-resistant pathogenic bacteria.

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