



P-ISSN: 2349-8528  
 E-ISSN: 2321-4902  
 IJCS 2018; 6(4): 288-300  
 © 2018 IJCS  
 Received: 13-05-2018  
 Accepted: 16-06-2018

**Praveen Khatri**

Department of Biotechnology  
 Dr. Yashwant Singh Parmar  
 University of Horticulture and  
 Forestry (Nauni) Solan,  
 Himachal Pradesh, India

**Ankita Sharma**

Department of Biotechnology  
 Dr. Yashwant Singh Parmar  
 University of Horticulture and  
 Forestry (Nauni) Solan,  
 Himachal Pradesh, India

**Priyanka Kashyap**

Department of Biotechnology  
 Dr. Yashwant Singh Parmar  
 University of Horticulture and  
 Forestry (Nauni) Solan,  
 Himachal Pradesh, India

**Neha Sharma**

Department of Biotechnology  
 Dr. Yashwant Singh Parmar  
 University of Horticulture and  
 Forestry (Nauni) Solan,  
 Himachal Pradesh, India

**Poonam Shirkot**

Department of Biotechnology  
 Dr. Yashwant Singh Parmar  
 University of Horticulture and  
 Forestry (Nauni) Solan,  
 Himachal Pradesh, India

**Correspondence**

**Praveen Khatri**

Department of Biotechnology  
 Dr. Yashwant Singh Parmar  
 University of Horticulture and  
 Forestry (Nauni) Solan,  
 Himachal Pradesh, India

## Biosynthesis, characterization and antibacterial activity of bio silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1

**Praveen Khatri, Ankita Sharma, Priyanka Kashyap, Neha Sharma and Poonam Shirkot**

**Abstract**

Bio silver nanoparticles have been used as antimicrobial agents, bioremediation of waste material, effluent detoxification, decolorization of dyes, pesticides degradation and plant growth and development. In present investigation, *in vitro* synthesis of bio silver nanoparticles by *Bacillus algicola* strain UMP 1.1 was carried out by using various optimization parameters. Optimum condition parameters for maximum synthesis of bio silver nanoparticles was selected which included tryptone yeast broth containing 3g/l tryptone, 3g/l yeast extract, 30mg/l CuSO<sub>4</sub> and 2.0mM AgNO<sub>3</sub>. Tryptone Yeast broth (pH; 8.0) using optimum condition was used for *in vitro* synthesis of bio silver nanoparticles at 35°C for 24 hrs of incubation. Nanoparticles synthesis indicated by change in colour from pale yellow to brown red was confirmed by UV-Vis spectroscopy with maximum absorbance peak obtained at 420nm. Characterization of bio silver nanoparticles was carried out using FTIR, XRD, DLS and SEM. These were tested for antibacterial activity against three bacterial plant pathogens.

**Keywords:** Biosynthesis, bio silver nanoparticles synthesized, *Bacillus algicola*

**Introduction**

Nanotechnology is one of the most promising areas of scientific development for future decades. The rapid evolution of nano science and nanotechnology during the past two decades has demonstrated that nanotechnology holds the keys to many of the technological advancements of the 21<sup>st</sup> century in different sectors such as agriculture, pharmaceutical, electronic, energy, textile, coatings and paintings which relies on having novel materials with unique properties derived from their small size. Nanotechnology or nano science is defined as, the production or use of materials and structures so that at least one of their dimensions in the range of 1-100 nm [1]. The use of microorganisms to synthesize functional nanoparticles has been of great interest recently [8]. The ability of microorganisms to change oxidation state of metals and their microbial processes has opened up new opportunity to explore novel applications such as biosynthesis of metal nanomaterials. Nanoparticles synthesized either biologically or by any other means must be characterized in order to understand their intrinsic properties such as size, shape, dispersity, aqueous stability, net charge, adsorption to biomolecules, aggregation and flocculation in various media using Scanning electron microscopy (SEM), FTIR spectroscopy, X-Ray diffraction, Dynamic light scattering, UV-Vis spectroscopy. Silver nanoparticles have many applications among all the well-known activities of silver ions and silver-based compounds, silver nanoparticles proved to be the material of choice for killing microbes effectively [2]. Several mechanisms have been proposed to explain the inhibitory effect of silver nanoparticles on bacteria. It is assumed that the high affinity of silver towards sulfur and phosphorus is the key element of the antimicrobial effect [5]. Due to the abundance of sulfur-containing proteins on the bacterial cell membrane, silver nanoparticles can react with sulfur-containing amino acids inside or outside the cell membrane, leading to the inhibition of enzyme functions, which in turn affects bacterial cell viability [7]. It has also been suggested that silver ions released from silver nanoparticles can interact with phosphorus moieties in DNA, resulting in inactivation of DNA replication [5]. In the present study bio silver nanoparticles were synthesized by *Bacillus algicola* strain UMP 1.1 for the synthesis of bio silver nanoparticles process, synthesis medium and cultivation conditions play critical role because these affect formation, concentration and yield of a

particular end product. Therefore, it was found important to consider the optimization of medium and process conditions in order to maximize the profits from fermentation process and various physical factors which include incubation time, temperature, pH, concentration of components of nutrient broth and substrate concentration.

## Materials and methods

### Procurement, maintenance and characterization of bacterial culture

Bacterial culture *Bacillus algicola* strain UMP 1.1 was procured from laboratory of Department of Biotechnology, Dr. YS Parmar University of Horticulture and Forestry Nauni-Solan (H.P.) which was one of the 28 bacterial isolates isolated from silver mine located at Uchich village located in Manali, Himachal Pradesh. This bacterial isolate was selected for carrying out the present study and was maintained using nutrient agar medium with composition: Peptone (5.0g/l), Beef extract (3.0g/l), Sodium chloride (5.0g/l), Agar (20.0g/l), at pH: 7.0 followed by its colony, microscopic, biochemical and molecular characterization.

### Quantitative evaluation of *Bacillus algicola* strain UMP 1.1 for silver nanoparticles synthesizing ability

*Bacillus algicola* UMP 1.1 was assessed for its ability to synthesize silver nanoparticles. One percent inoculum (overnight culture) of *Bacillus algicola* strain UMP 1.1 was inoculated into a 50 ml tryptone yeast broth followed by incubation at 37 °C for 24 hrs. Supernatant of *Bacillus algicola* strain UMP 1.1 was collected by centrifugation at 8500 rpm for 10 mins at 4 °C (Cooling Centrifuge REMI CM-8 Plus) to investigate extracellular synthesis of silver nanoparticles. Ten ml of supernatant was mixed with 10 ml of 1.0 mM solution of AgNO<sub>3</sub> and incubated at 37 °C for 1 hr followed by exposure to sunlight for 10 mins and again incubated for 24 hrs. Formation of silver nanoparticles was indicated by colour change of the solution<sup>[4]</sup>. Formation of silver nanoparticles was further confirmed by the spectrophotometer (Spectronic 20, Milton Roy Company) at a wavelength of 420 nm.

### Optimization of culture conditions for growth and synthesis of bio silver nanoparticles by *Bacillus algicola* strain UMP 1.1

The culture conditions for *Bacillus algicola* strain UMP 1.1 are optimized to obtain higher yields of their important products. *Bacillus algicola* strain UMP 1.1 depicting maximum bio silver nanoparticles synthesizing activity was further investigated to study effect of different medium (Luria Broth, EMB Broth, Tryptone Yeast Broth, Nutrient Broth) and factors such as incubation time (0-120 hrs), temperature (20-40 °C), pH (5.0-10.0), tryptone concentration (0.0-5.0g/l), yeast extract concentration (0.0-5.0g/l), CuSO<sub>4</sub> concentration (0.0-50 mg/l), AgNO<sub>3</sub> concentrations (1.0-5.0mM), and inoculum size (1-5%) on bio silver nanoparticles synthesis as well as growth of *Bacillus algicola* strain UMP 1.1.

### *In vitro* Biosynthesis of Silver Nanoparticles

#### Preparation of selected bacterial culture

Optimum concentration of inoculum (overnight culture) was inoculated into TY broth and incubated at optimum temperature for optimum incubation period of time at a speed of 150 rpm (Orbital shaker REMI). The supernatant was collected by centrifuging the culture broth at 8500 rpm at 4 °C

for 10 minutes (Cooling Centrifuge REMI CM-8 Plus) for extracellular synthesis of silver nanoparticles.

### Silver nanoparticles synthesis by selected bacterial isolate

Ten ml of supernatant was mixed in 10.0 ml of optimum concentration of silver nitrate prepared with double distilled water and incubated at optimum temperature for optimum incubation period of time. Formation of silver nanoparticles was indicated by colour change of the solution, which was confirmed using spectrophotometer (Spectronic 20, Milton Roy Company). The silver nanoparticles suspension obtained was used for further experiments.

### Lyophilization of silver nanoparticles suspension

For the preparation of powdered form of bio silver nanoparticles, process of lyophilization was carried out. One litre of bio silver nanoparticles suspension was transferred to two petri plates which are placed in the lyophilizer chamber (BIOGENTEK (I) Pvt Ltd) for subsequent lyophilization involving freezing the bio silver nanoparticle solution at -30 °C for 48hrs and then reducing the surrounding pressure to allow the frozen water to sublime directly from liquid phase to gas phase. Thus after 48 hrs of lyophilization of the solution powdered/lyophilized bio silver nanoparticles was obtained from one litre of silver nanoparticles suspension.

### Characterization of silver nanoparticles

Silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1 were analyzed using various techniques such as UV-visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), X-Ray Diffraction (XRD), Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM).

### UV- visible spectroscopy

Optical characteristics of the biosynthesized silver nanoparticles were analyzed using UV-Vis spectrophotometer. For this, silver nanoparticles solution was subjected to absorption analysis between 300-600 nm range using UV-Vis spectrophotometer (Perkin Elmer Lambda 2, USA).

### Fourier Transform Infrared Spectroscopy

Microcup was washed with 100% absolute ethanol. 10 µl sample was filled in a 2 mm internal diameter microcup and loaded onto the FTIR set at 26 °C ± 1 °C. The sample was scanned in the range of 4,000 to 400 cm<sup>-1</sup> using a Fourier transform infrared spectrometer (Thermo Nicolet Model 6700, Waltham, MA, USA). The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample.

### X-Ray Diffraction

X-ray diffraction (Bruker, Karlsruhe, Germany) is one of the important technique for the structure characterization of the crystalline material. Prepared silver nanoparticles can be analyzed by this instrument using the lynx eye detector (silicon strip detector technology).

### Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) technique has been used to determine the size of particles by measuring the intensity of peaks which depends on the size distribution of particles. The detector was put into 90° position. Five drops of toluene were placed into the center hole of the XY-translation stage.

Sample was placed in glass test tube into the center hole of the XY-translation stage. The sample was filled into test tube in a way so that the liquid fills ~75% of the test tube and a spectrum was taken using software.

#### Scanning electron microscope

Sample was prepared for SEM analysis by separating the biomass from the liquor by centrifugation and washed twice in sterile distilled water. The sample was fixed for 1 hr in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.4), followed by 3 washes in 0.075 M phosphate buffer. After a second fixation step of 1 hr in 1% osmium tetroxide, cells were washed in distilled water. The cell pellet was subjected to dehydration with 30, 50, 70% ethanol, followed by three dehydration steps in 100% ethanol. Infiltration of the resin was carried out by placing the pellet in 30% Quetol in ethanol for 1 hr, followed by 1 hr in 60% Quetol. After centrifugation, the pellet was resuspended in 100% Quetol for 4 hrs before polymerisation at 65 °C for 24 hrs. Ultrathin sections were not stained prior to analysis, due to possible interference of the stain with the silver nanoparticles. SEM analysis was done on a FE SEM JSM 7800F prime. For SEM analysis of the cell-free extract, a drop of the sample was placed onto a carbon-coated copper grid. After about a minute, the extra solution was removed using blotting paper and the grid air-dried before analysis. Elemental analysis on single particles was carried out on an air-dried, carbon coated sample using an energy dispersive spectroscopy (EDS) attachment on a JSM 7800F prime scanning electron microscope using accelerating voltage of 20 keV and counting time of 100 secs.

#### Applications of silver nanoparticles

##### Antibacterial activity of silver nanoparticles preparations against pathogens

Silver nanoparticles possess appreciable antimicrobial activity against various bacterial plant pathogens. The following pathogenic bacteria were obtained from the Microbiology laboratory, Departments of Basic Sciences and Department of Plant Pathology Dr. Y.S. Parmar UHF, Nauni, Solan. Three bacterial pathogens procured were *Xanthomonas campestris*, *Agrobacterium tumefaciens* and *Xanthomonas axonopodis* causing black rot, crown gall and citrus canker respectively.

Silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1 were standardized for antibacterial activity against various plant pathogens. Nutrient agar was prepared and was poured into sterile plates followed by solidification. Using spread plate technique, bacterial culture (1.0ml) was transferred on to solidified agar plate and spread using sterilized L-shaped glass rod under aseptic conditions. Wells were made in the solidified agar plate using a sterile borer of size 250 mm. Different dosages of bio silver nanoparticle solution i.e. 25, 50, 75 and 100 µl were poured in each well with the help of sterile pipette followed by refrigeration of plates for one hour for diffusion of the suspension followed by incubation at 37 °C for 24 hrs. Zones of inhibition were measured in centimetres using measuring scale in each plate.

#### Result and Discussion

##### Procurement, maintenance and characterization of bacterial culture

Bacterial culture was procured and maintained using nutrient agar medium (Plate-1a). Morphological characterization showed that bacterial colonies were whitish in colour, medium in size, circular in shape with rough texture. Colonies of this bacterial isolate were found to possess undulate

margins and raised elevation and was found to produce translucent colonies and microscopic characterization depicted that the cells of this bacterial isolate was purple in color and thus gram positive in nature with rod shape and arrangement of cells was found to be in singles. The bacterial isolate was found to be a spore former (Plate-1b). The biochemical characterization of bacterial culture revealed that procured bacterial isolate was found positive for citrate utilization, malonate utilization activity, O-nitrophenyl-β-D-galactopyranoside, catalase, arginine, nitrate reductase and trehalose tests and negative for Voges-Proskauer, sucrose, mannitol, glucose and arabinose tests. As per Bergey's manual of Systematic Bacteriology (Claus, 1986) and on the basis of results obtained from morphological and biochemical characters, the bacterial culture was found to belong to the genera of *Bacillus*. Molecular characterization was carried out using 16S rRNA gene technology. Total genomic DNA of selected bacterial isolate UMP 1.1 was extracted and amplified using PCR technology. Universal primer B27F and U1492R for 16S rna gene was used and after 35 cycles of PCR amplification produced an amplicon of expected size of 1500 bp. To determine the nucleotide sequence of PCR product of 16S rRNA gene of bacterial isolate UMP 1.1, sequencing was carried out using same upstream and downstream primers which were used for amplification and as a result, the size of the 16S rRNA gene sequence of selected bio silver nanoparticles synthesizing bacterial isolate UMP 1.1 was found to be 1469 bp. BLASTn search of selected bio silver nanoparticles synthesizing bacterial sequence with the most similar 16S rRNA gene sequences of the Gen Bank database revealed that this bacteria showed 100% similarity with *Bacillus algicola* strain AB423f, 16S rRNA gene, complete sequence. Phylogenetic tree also verified bacteria *Bacillus algicola* as it clustered closely with *Bacillus algicola* strain AB423f, 16S ribosomal RNA complete sequence. Thus, based on molecular characterization, the isolate UMP 1.1 was eventually confirmed as *Bacillus algicola* strain.

##### Quantitative evaluation of *Bacillus algicola* strain UMP 1.1 for silver nanoparticles synthesis ability

*Bacillus algicola* strain UMP 1.1 was assessed for its ability to synthesize silver nanoparticles. Bacterial culture was inoculated into 50 ml tryptone yeast broth followed by incubation at 37 °C for 24 hrs. Supernatant was obtained by centrifugation at 8500 rpm for 10 mins at 4 °C to investigate extracellular synthesis of silver nanoparticles. One millimolar aqueous silver nitrate (AgNO<sub>3</sub>) was mixed with equal volume of supernatant and incubated at 37 °C for 24 hrs. Formation of silver nanoparticles was depicted by color change of the solution from pale yellow to brown red colour (Plate-2). Formation of silver nanoparticles was confirmed by measuring its optical density at a wavelength of 420 nm at time period ranging from 0-120 hrs and maximum OD value of 0.47 was obtained after 24 hrs depicting maximum silver nanoparticles synthesis at 24 hrs (Fig-1).

##### Optimization of culture conditions for maximum silver nanoparticles synthesis and growth of *Bacillus algicola* strain UMP 1.1

To achieve the objective of maximum nanoparticles synthesis, optimization of various culture conditions such as media, incubation time, temperature, pH, different concentrations of tryptone, yeast extract, copper sulphate, inoculum size and silver nitrate were optimized for silver nanoparticles synthesis

as well as for maximum growth of *Bacillus algicola* strain UMP 1.1.

### Medium for optimization

For selection of best growth medium, four different culture media viz., Luria bertani broth, Eosine Methylene Blue broth, Tryptone Yeast broth and Nutrient broth were investigated for growth and silver nanoparticles synthesis by selected bacterial isolate. Tryptone Yeast broth was found to be the best medium as silver nanoparticles synthesizing activity of 0.489 O.D. and growth of 1.25 O.D. was found to be maximum as compared to other three media. Silver nanoparticles synthesizing activity of 0.356, 0.011 and 0.003 O.D. was observed at 420 nm and growth of 1.19, 0.023 and 0.010 O.D. was observed at 540 nm for Nutrient broth, EMB broth and LB broth respectively. On the basis of these results Tryptone Yeast broth was selected for further study (Fig.2).

### Effect of incubation time

Effect of incubation time on growth of *Bacillus algicola* strain UMP 1.1 was monitored up to 120 hrs in TY broth and it has been observed that bacterial growth increased at a very fast rate depicting an OD of 1.114 at a wavelength of 540 nm during the first 24 hrs and then there was a gradual increase upto 48 hrs with an OD of 1.286 and after that the growth started declining upto 120 hrs (Fig.-3). Thus 48 hrs was selected as an optimum incubation time for maximum growth of *Bacillus algicola* strain UMP 1.1.

Effect of incubation time at different time intervals on silver nanoparticles synthesizing activity by selected *Bacillus algicola* strain UMP 1.1 was determined up to 120 hrs. It has been observed that silver nanoparticles synthesizing activity increased at a fast rate and maximum activity was observed at 24 hrs with an OD value of 0.498 after that it was found to decrease up to 120 hrs. Thus, maximum silver nanoparticles synthesis was achieved at an incubation time of 24 hrs and was selected for further experiments (Fig.-3).

### Effect of incubation temperature

A study on growth and silver nanoparticles synthesizing activity by *Bacillus algicola* strain UMP 1.1 was investigated at different temperatures ranging from 20-40 °C for 48 hrs. Bacterial growth was found to increase with increase in temperature up to 35 °C followed by decrease upto 40 °C. The bacterial strain showed a maximum growth OD value of 1.352 at an incubation temperature of 35 °C which was selected as optimum incubation temperature parameter for maximum growth of *Bacillus algicola* strain UMP 1.1 (Fig.-4).

Though silver nanoparticles synthesizing activity by *Bacillus algicola* strain UMP 1.1 also showed increased with increase in temperature however maximum activity of 0.440 at 420 nm wavelength of light was obtained at 35 °C. Thus the optimum incubation temperature condition selected for maximum silver nanoparticles synthesis was 35 °C (Fig.-4).

### Effect of pH

Effect of pH range of 5.0-10.0 of TY broth was examined and it was found that growth of bacteria increased sharply with increase in pH of the medium up to pH 7.0 followed by decrease in growth upto pH 10. Maximum growth OD of 1.415 at 540 nm wavelength was observed at pH 7.0, thus was selected as optimum pH for maximum growth of *Bacillus algicola* strain UMP 1.1 (Fig.-5).

Silver nanoparticles synthesizing activity increased with increase in pH up to pH 8.0 and then a sharp decrease in

activity was observed up to 10.0 pH. At pH 8.0 maximum silver nanoparticles synthesizing activity of 0.428 OD at 420 nm wavelength of light was observed and thus pH optimization studies indicated that pH 8.0 was found to be the optimum pH for maximum silver nanoparticles synthesis by *Bacillus algicola* strain UMP 1.1 (Fig.-5).

### Effect of Tryptone concentration

Effect of different concentrations of tryptone were investigated for growth and silver nanoparticles synthesizing activity of *Bacillus algicola* strain UMP 1.1 using TY broth. Growth of bacteria was observed at different tryptone concentration ranging from 0.0-5.0 g/l at pH 8.0, 35 °C for 48 hrs and it was found that growth of bacteria increased with increase in tryptone concentration in the medium up to 3.0 g/l. The bacteria showed a maximum growth OD value of 1.206 at a wavelength of 540 nm in TY broth containing 3.0 g/l tryptone and after that with increase in the concentration of tryptone bacterial growth was found to decrease (Fig.-6).

Silver nanoparticles synthesis activity by *Bacillus algicola* strain UMP 1.1 also was found to increase with increase in tryptone concentration up to 3.0 g/l. Maximum silver nanoparticles synthesis activity in TY broth was obtained at 3.0 g/l tryptone with OD of 0.456 followed by decrease in silver nanoparticles synthesizing activity with increasing concentration (Fig.-6). Thus 3.0 g/l tryptone concentration was found optimum for silver nanoparticles activity.

### Effect of yeast extract concentration

Concentration range from 0.0-5.0 g/l of yeast extract was studied for its effect on the growth as well as on silver nanoparticles synthesis activity of *Bacillus algicola* strain UMP 1.1. Growth of *Bacillus algicola* strain UMP 1.1 was found to be maximum at 3.0 g/l of yeast extract with OD value of 1.663 (Fig.-7). Thus 3.0 g/l was selected as an optimum yeast extract value for maximum growth of *Bacillus algicola* strain UMP 1.1.

In case of silver nanoparticles synthesis activity was found to increase with increase in yeast extract concentration up to 3.0 g/l followed by decreased up to 5.0 g/l. Maximum silver nanoparticles OD value of 0.395 at 420 nm wavelength of light was observed when 3.0 g/l of yeast extract was used (Fig.-7). Thus 3.0 g/l yeast extract was found maximum for silver nanoparticles synthesizing activity.

### Effect of CuSO<sub>4</sub> Concentration

Evaluation of growth of bacterial culture was also carried out at different concentrations of CuSO<sub>4</sub> using TY broth containing 3.0 g/l tryptone, 3.0 g/l yeast extract, pH 8.0 at 35 °C. Growth of *Bacillus algicola* strain UMP 1.1 for 48 hrs was observed at different CuSO<sub>4</sub> concentrations ranging from 0-50 mg/l and it was found that growth of the bacterial strain increased with increase in CuSO<sub>4</sub> concentration upto 30.0 mg/l showing a maximum growth OD of 1.240 at 540 nm in TY broth and after that at higher concentration of CuSO<sub>4</sub> the growth was found to decrease (Fig.-8).

Silver nanoparticles synthesis activity by *Bacillus algicola* strain UMP 1.1 also was found to increase with increase in CuSO<sub>4</sub> concentration up to 30 mg/l. Maximum silver nanoparticles synthesis activity in TY broth was obtained at 30.0 mg/l CuSO<sub>4</sub> concentration with OD of 0.319 followed by decrease in silver nanoparticles synthesizing activity with increasing concentration (Fig.-8). Thus 30 mg/l CuSO<sub>4</sub> concentration was found optimum for silver nanoparticles synthesizing activity.

### Effect of inoculum size

Effect of inoculum size ranging from 1.0-5.0% was examined on growth and silver nanoparticles synthesis of *Bacillus algicola* strain UMP 1.1. Growth of the bacteria was found to be increased with increase in inoculum size up to 4% and was found to decrease at 5%. Growth of *Bacillus algicola* strain UMP 1.1 was found maximum at 4% inoculum size showing maximum growth OD value of 1.214 at 540 nm. Thus 4% inoculum size was found optimum for the growth of the bacterial strain UMP 1.1 (Fig.-9).

Effect of inoculum size range of 1-5% on silver nanoparticles synthesis of *Bacillus algicola* strain UMP 1.1 was also observed and it has been revealed that an inoculum size of 2% showed maximum OD of 0.480 at 420 nm. However, an inoculum size below this level was inadequate for silver nanoparticles synthesis while the higher levels resulted in rapid depletion of nutrients in the media and reduced the silver nanoparticles yield. Also higher cell density would lead to the production of inhibitory metabolites that may be interfering with the enzyme production responsible for silver nanoparticles synthesis. Results of optimization studies indicated that 2% inoculum size was found to be optimum for maximum silver nanoparticles synthesizing activity (Fig.-9).

### Effect of different concentration of silver nitrate

Effect of different concentrations of silver nitrate was investigated on silver nanoparticles synthesis using TY broth by *Bacillus algicola* strain UMP 1.1. Maximum extracellular silver nanoparticles synthesis activity was observed in TY broth containing 3 mM silver nitrate produced maximum silver nanoparticles with OD value of 0.358 at 420 nm followed by decrease in silver nanoparticles activity up to 3.0 mM silver nitrate (Fig.-10).

### In vitro Biosynthesis of Silver Nanoparticles

Biosynthesis is a phenomenon where chemical compounds are produced from simple biological reagents. *In vitro* biosynthesis of metal nanoparticles is carried by decreasing the redox state of metal ions with the help of redox shuttlers which may be extracellular or intracellular which leads to conversion of metal ions into metal nanoparticles of defined size and shape.

### In vitro synthesis of silver nanoparticles by *Bacillus algicola* strain UMP 1.1

Extracellular biosynthesis of silver nanoparticles by *Bacillus algicola* strain UMP 1.1 was carried out using the standardized and selected optimized parameters in the previous experiment. Two percent inoculum size was used to inoculate tryptone broth containing 3.0 g/l tryptone, 3.0 g/l yeast extract, 30 mg/l CuSO<sub>4</sub>, pH 8.0 for 48 hrs of incubation. The culture supernatant was obtained by centrifugation at 8500 rpm, 4 °C for 10 mins which was used for synthesis of silver nanoparticles. Ten millilitre of supernatant was mixed in 10 ml of 3 mM solution of AgNO<sub>3</sub> (prepared with double distilled water) and inoculated at 35 °C for 24 hrs. Formation of silver nanoparticles was indicated by color change of the solution from pale yellow to brown red (Plate-2).

### Lyophilization of silver nanoparticles suspension

Powdered/lyophilized form of bio silver nanoparticles was obtained after subjecting one litre of bio silver nanoparticles suspension in a lyophilizer for lyophilization. After 48 hrs of lyophilization at -30 °C powdered/lyophilized bio silver nanoparticles were obtained. In present study 50 mg of silver

nanoparticles powder was obtained from one litre of silver nanoparticles suspension (Plate-3).

### Characterization of nanoparticles

Silver nanoparticles must be characterized in order to understand their intrinsic properties such as size, shape, aqueous stability, net charge, adsorption to biomolecules, aggregation and flocculation. This provides vital information in terms of application of these nanoparticles [6]. Nanoparticles obtained were analyzed using various techniques such as, Fourier transform infrared spectroscopy (FTIR), powder X-rays diffractions (XRD), scanning electron microscopy (SEM), UV-visible spectroscopy and dynamic light scattering (DLS).

### UV-visible spectroscopy

Optical characteristics of the biosynthesized silver nanoparticles were analyzed using UV-vis spectrophotometer. Addition of 3mM silver nitrate to the supernatant of centrifuged aqueous solution led to the development of a brownish red solution after 24 hrs of reaction, indicating the formation of silver nanoparticles as shown in the UV-vis absorption spectrum. Ultraviolet spectroscopy confirmed the reduction of Ag<sup>+</sup> to Ag<sup>0</sup>, silver nanoparticles that can be identified from the peaks obtained between 330-460 nm and maximum absorbance has been observed at 420 nm with value 2.4 (Fig.-11), which is the signature for the silver nanoparticles formation, apart from the color change (Plate-2).

### Fourier transform infrared spectroscopy (FTIR)

FTIR analysis of the reaction mixture has helped to understand the nature of the biomolecules involved in the formation of silver nanoparticles. FTIR spectrogram of the reaction mixture has showed the presence of five bands one at 3374.66 cm<sup>-1</sup> which can assign O-H stretching, whereas another very strong band at 1641.66 cm<sup>-1</sup> is due to C=C stretching in the aromatic ring, confirming the presence of the aromatic group, third intense peaks at 1382 cm<sup>-1</sup> correspond to C-N stretch vibrations, as well as to the amide I bands of proteins, bands at 1082.45 cm<sup>-1</sup> are due to ether linkages and the band observed at 828.34 cm<sup>-1</sup> assign to C-Cl stretching in the alkyl group involved in reduction of Ag<sup>2+</sup> to Ag<sup>0</sup> (Fig.-12).

### Powder X-ray diffraction

XRD study revealed crystalline nature of silver nanoparticles. The XRD pattern clearly showed that extracellular synthesis of silver nanoparticles formed by the reduction of the silver nitrate ions using *Bacillus algicola* strain UMP 1.1. Silver nanoparticles exhibited four prominent Bragg reflections at around 38.21, 44.26, 65.40 and 77.30. The fraction between the intensity of the (200), (220), and (311) diffraction pattern was found to be much lower, suggesting that the plane (111) is in predominant orientation (Fig.-13). The XRD facets of the silver nanoparticles match with standard silver which was published by JCPDS (file no 04-0783).

### Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) technique has been used to determine the hydrodynamic diameter of the particles by measuring the intensity of peaks which depends on the size distribution of particles in aqueous phase. DLS analysis revealed that average size of silver nanoparticles synthesized



by *Bacillus algicola* strain UMP 1.1 was 80.0 nm approximately.

### Scanning electron microscope

The silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1 were examined with scanning electron microscopy to confirm the morphology of synthesized silver nanoparticles. Scanning electron microscope revealed that silver nanoparticles were polydisperse and spherical, cuboidal and hexagonal in shape (Fig.-14).

### Antibacterial activity of silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1 against bacterial pathogens

Different dosages viz., 25, 50, 75 and 100  $\mu$ l of bio silver nanoparticles solution were tested against each bacterial pathogen and it was found that zone of inhibition in each pathogen i.e. *Xanthomonas campestris*, *Agrobacterium tumefaciens* and *Xanthomonas axonopodis* increased with increasing dosage of silver nanoparticles (Plate-4) and thus 100 $\mu$ l of silver nanoparticles dosage was selected for further experimentation for all the three plant pathogenic bacteria.

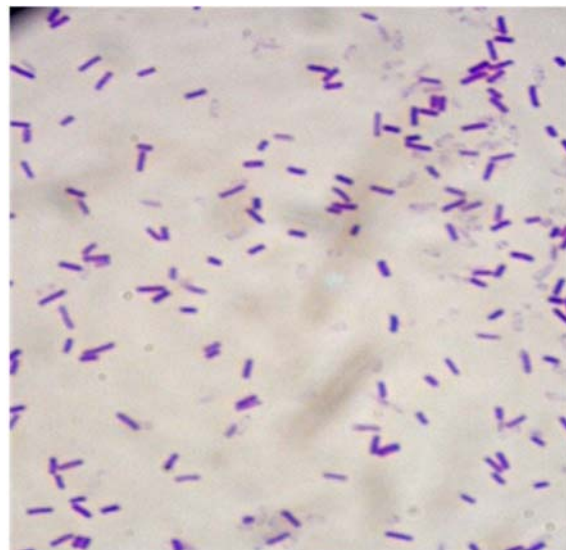
Silver nanoparticles (100 $\mu$ l) synthesized by *Bacillus algicola* strain UMP 1.1 were tested for their antibacterial activity against three bacterial plant pathogens viz., *Xanthomonas campestris*, *Agrobacterium tumefaciens* and *Xanthomonas axonopodis* causing black rot, crown gall and citrus canker respectively using agar well diffusion test and produced variable zones of inhibition against three bacterial plant pathogens after 24 hrs of incubation. In case of, *Xanthomonas campestris* and *Agrobacterium tumefaciens* silver nanoparticles suspension (100 $\mu$ l) showed 3.15 and 3.13 cms zones of inhibition respectively, whereas a maximum of 3.20 cm zone of clearance was observed using silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1 against *Xanthomonas axonopodis* (Plate-5 a-c).

### Conclusion

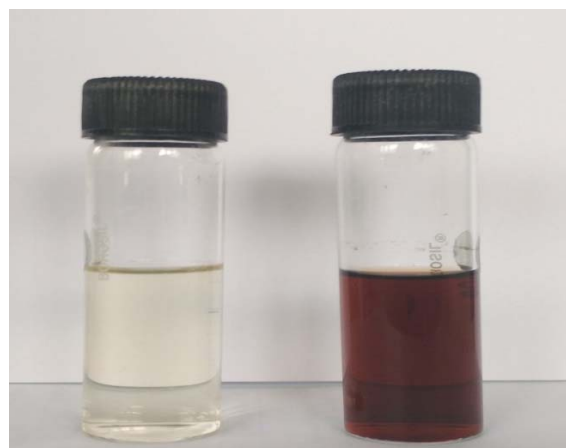
In present study maximum silver nanoparticles synthesis by *Bacillus algicola* strain UMP 1.1 was achieved at 35 °C, pH 8.0 and after 24 hrs of incubation with 3.0 mM silver nitrate, 3.0% tryptone, 3.0% yeast extract and 2.0% inoculum size and *in vitro* biosynthesis of silver nanoparticles was carried out using these selected optimum conditions. These silver nanoparticles of *Bacillus algicola* strain UMP 1.1 were characterized and were found to be 80nm in size with varying shapes of spherical, cuboidal and hexagonal and polydisperse and crystalline in nature. In the present study, the bio silver nanoparticles were found significant to inhibit various bacterial plant pathogens under *in vitro* conditions considerably.



**Plate 1a:** Colony morphology of procured silver nanoparticles synthesizing bacteria



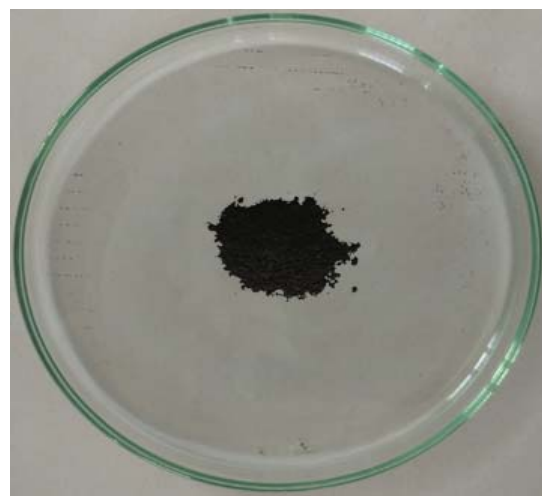
**Plate 1b:** Microscopic characteristics of Gram stained bacteria at 1000x as Gram positive rods



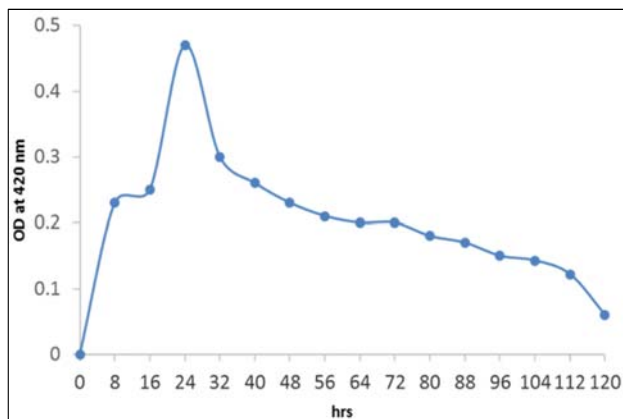
A

B

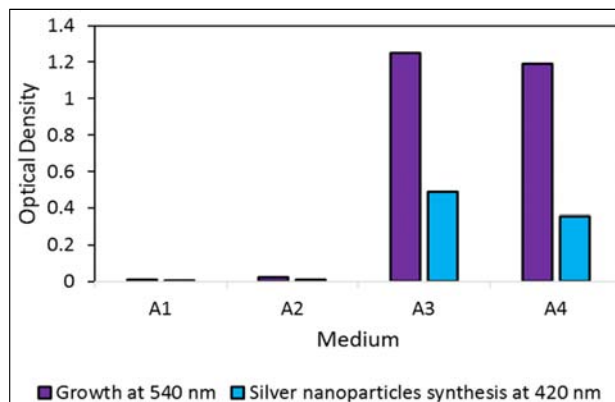
**Plate 2:** Colour change from pale yellow to brown red  
A) Control  
B) Silver nanoparticles suspension



**Plate 3:** Lyophilized biosilver nanoparticles powder

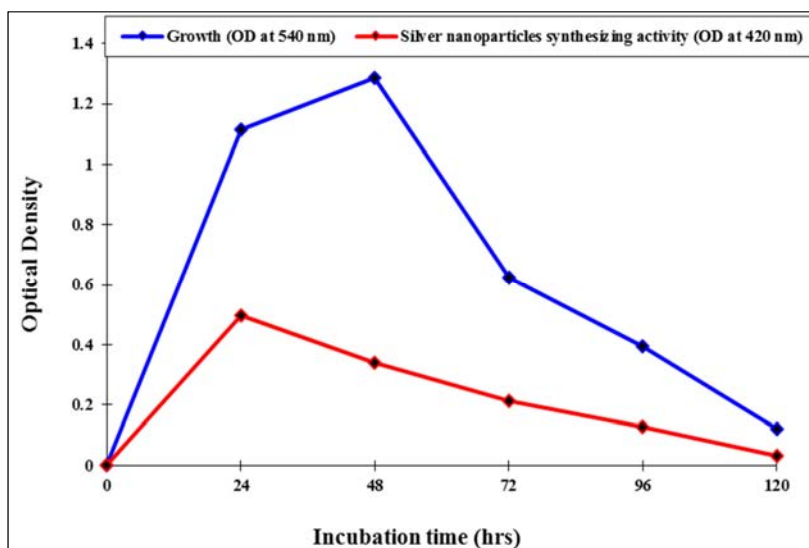


**Fig 1:** Quantitation evaluation of bacillus strain UMP 1.1 for Silver nanoparticles synthesis ability

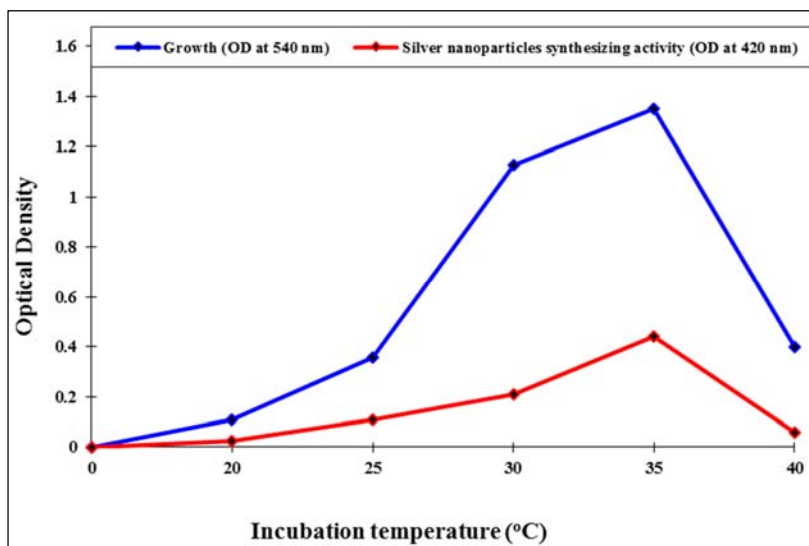


**Fig 2:** Effect of medium on growth and silver nanoparticles synthesis by *Bacillus algicola* strain UMP1.1

A1: Luria Bertani broth  
 A2: Eosine Methylene Blue broth  
 A3: Tryptone Yeast broth  
 A4: Nutrient broth



**Fig 3:** Effect of incubation time on growth and silver nanoparticles synthesizing activity of *Bacillus algicola* strain UMP 1.1



**Fig 4:** Effect of incubation temperature on growth and silver nanoparticles synthesizing activity of *Bacillus algicola* strain UMP 1.1

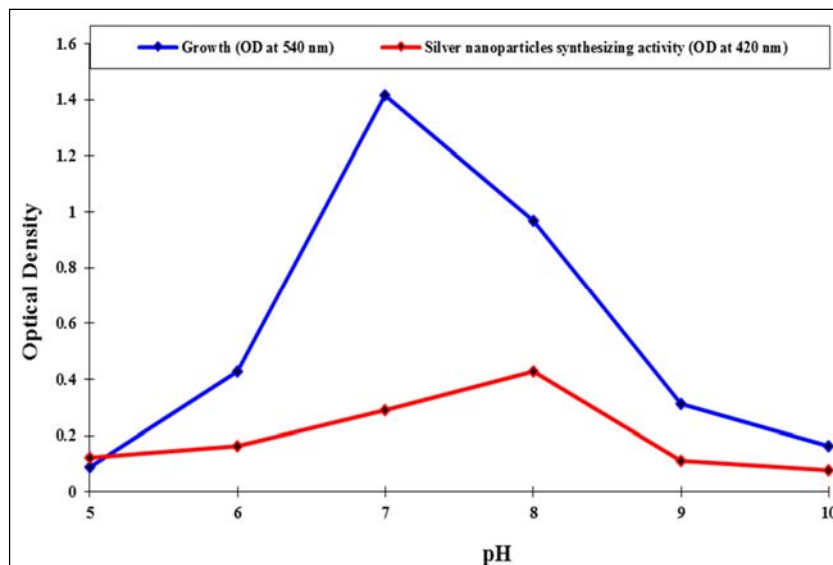


Fig 5: Effect of incubation pH on growth and silver nanoparticles synthesizing activity of *Bacillus algicola* strain UMP 1.1

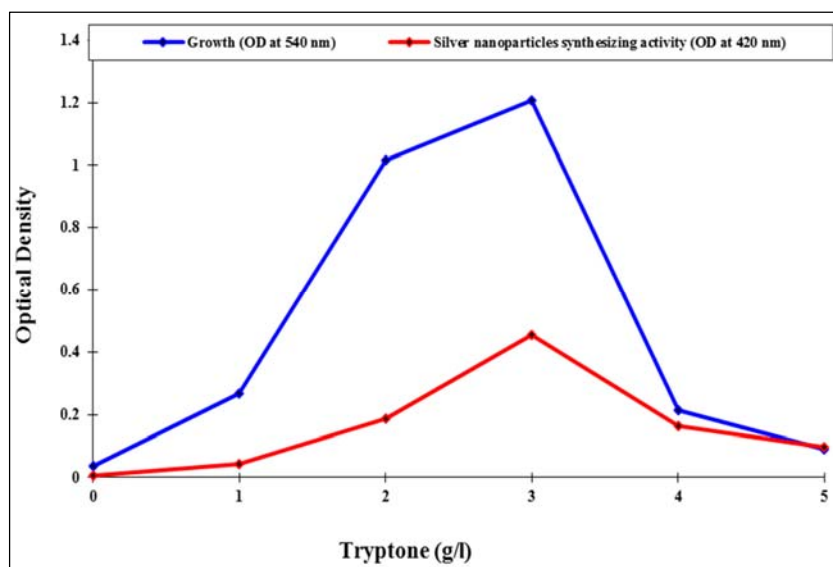


Fig 6: Effect of Tryptone concentration on growth and silver nanoparticles synthesizing activity of *Bacillus algicola* strain UMP 1.1

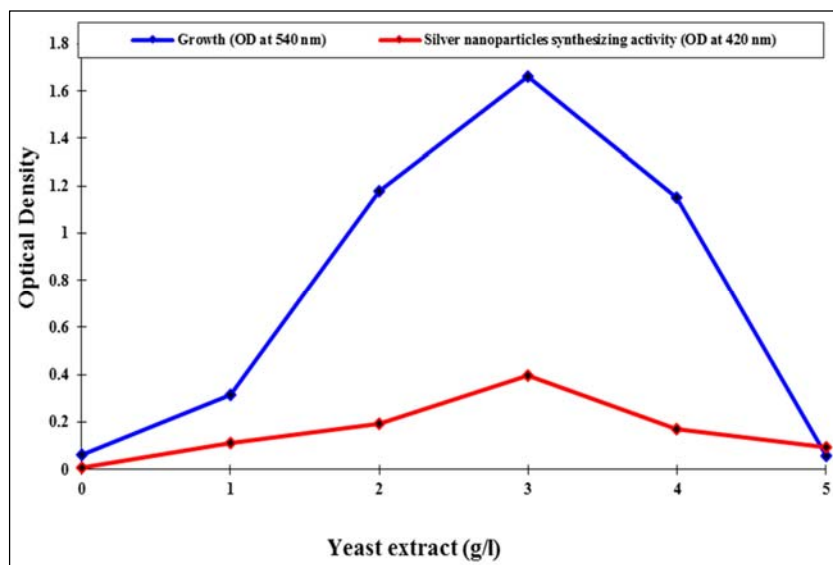


Fig 7: Effect of Yeast concentration on growth and silver nanoparticles synthesizing activity of *Bacillus algicola* strain UMP 1.1



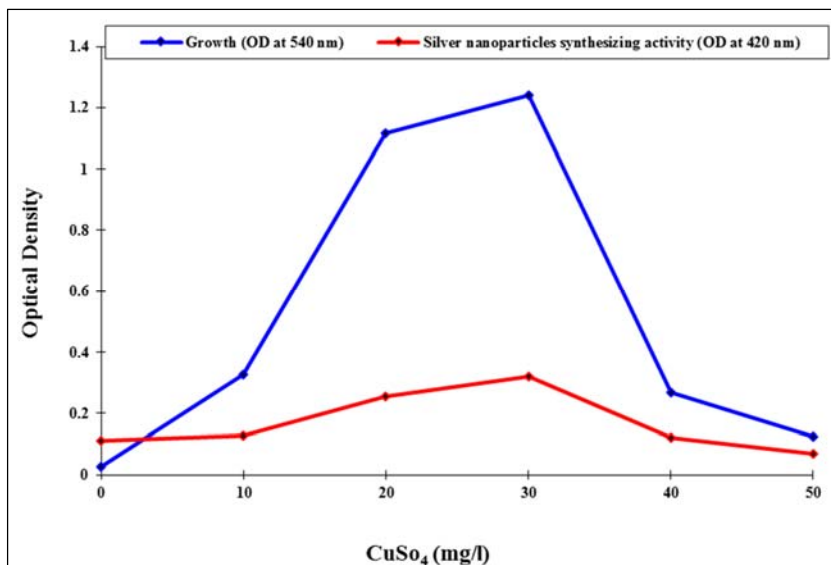


Fig 8: Effect of CuSO<sub>4</sub> concentration on growth and silver nanoparticles synthesizing activity of *Bacillus algicola* strain UMP 1.1

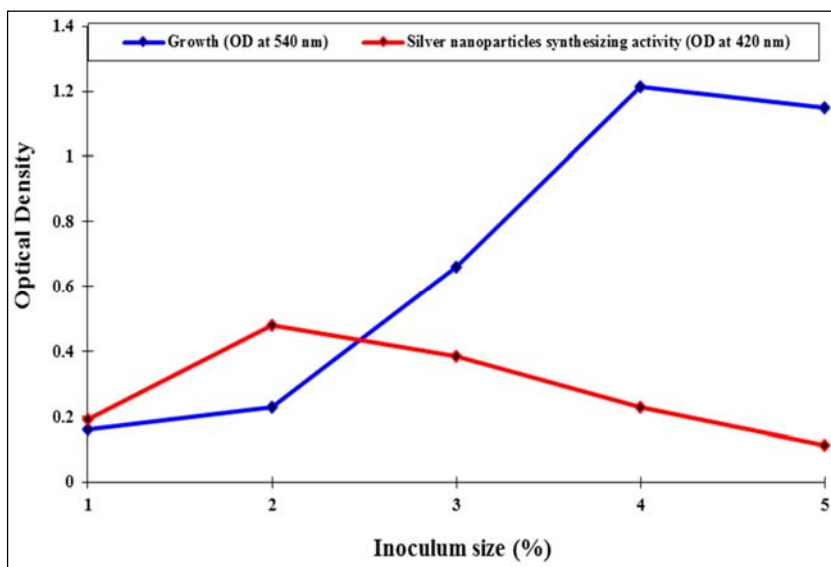


Fig 9: Effect of inoculum size concentration on growth and silver nanoparticles synthesizing activity of *Bacillus algicola* strain UMP 1.1

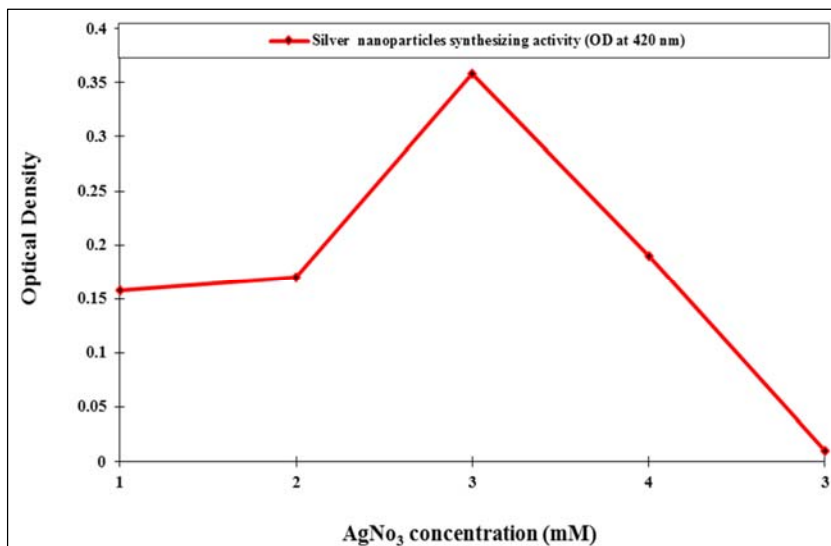
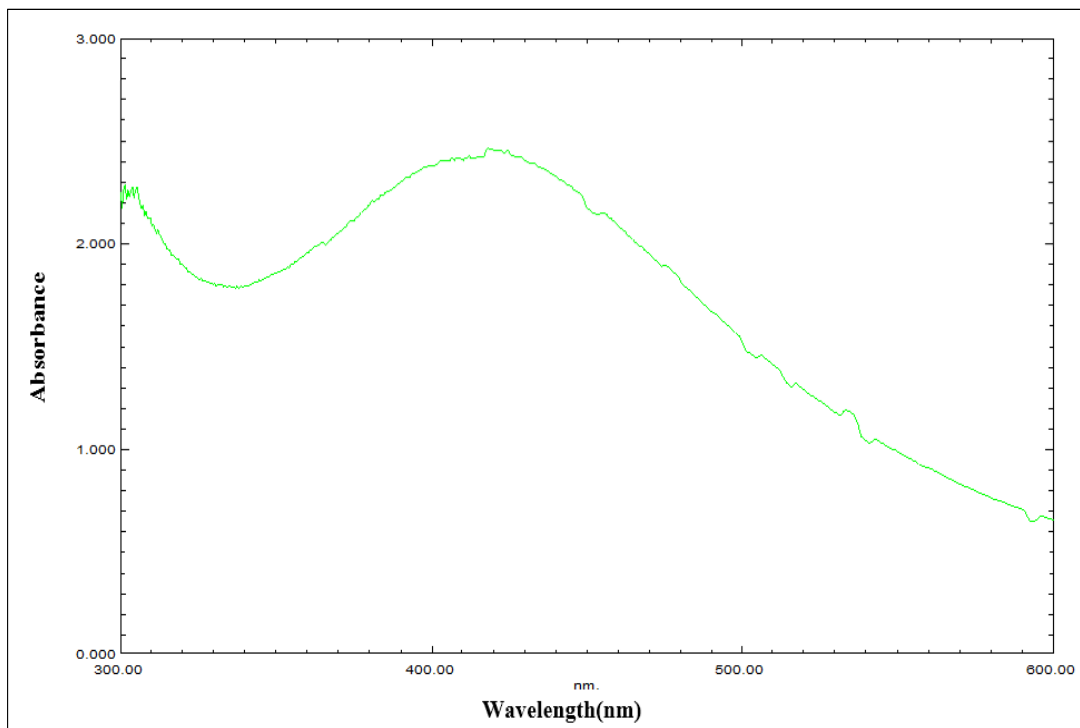
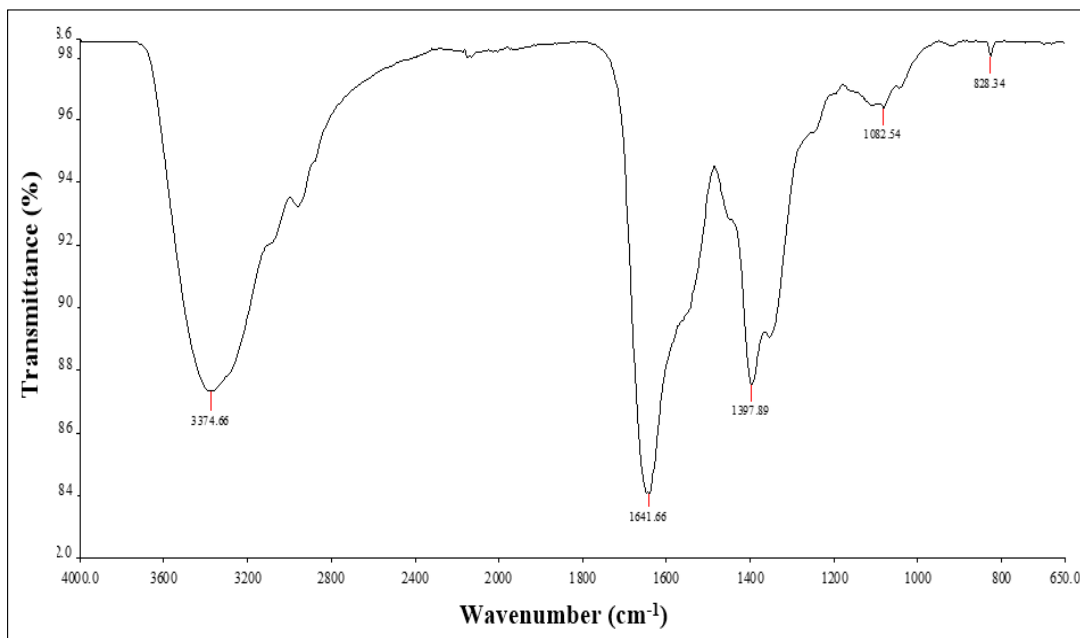


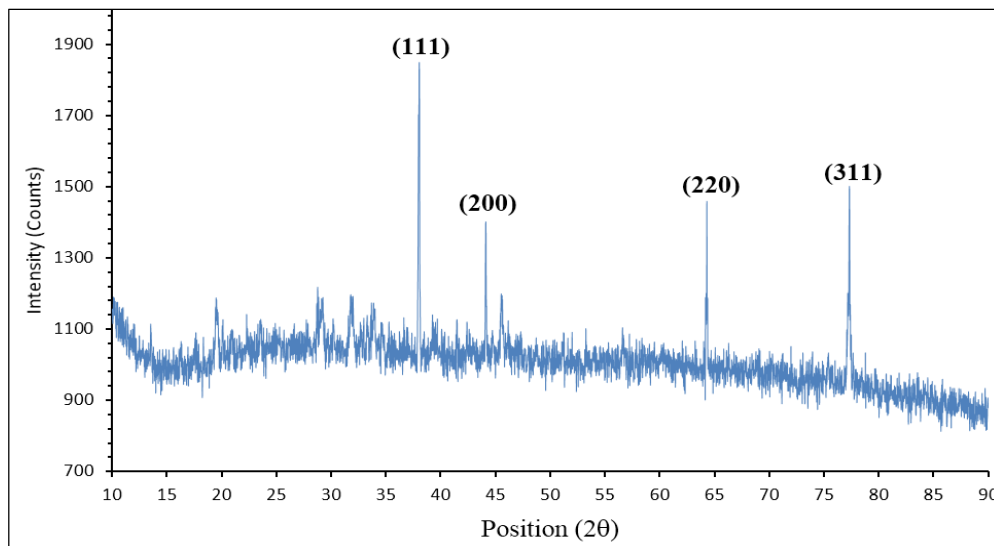
Fig 10: Effect of AgNO<sub>3</sub> concentration on silver nanoparticles synthesizing activity of *Bacillus algicola* strain UMP 1.1



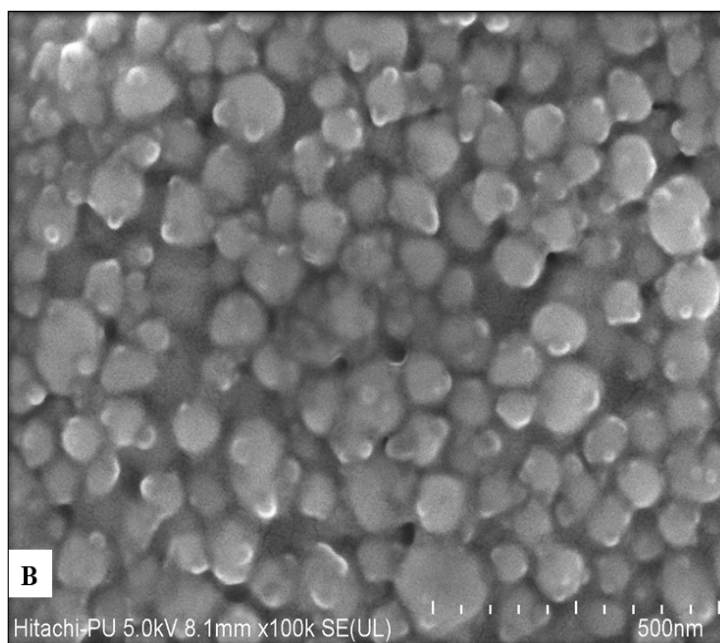
**Fig 11:** UV-Vis spectra of silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1



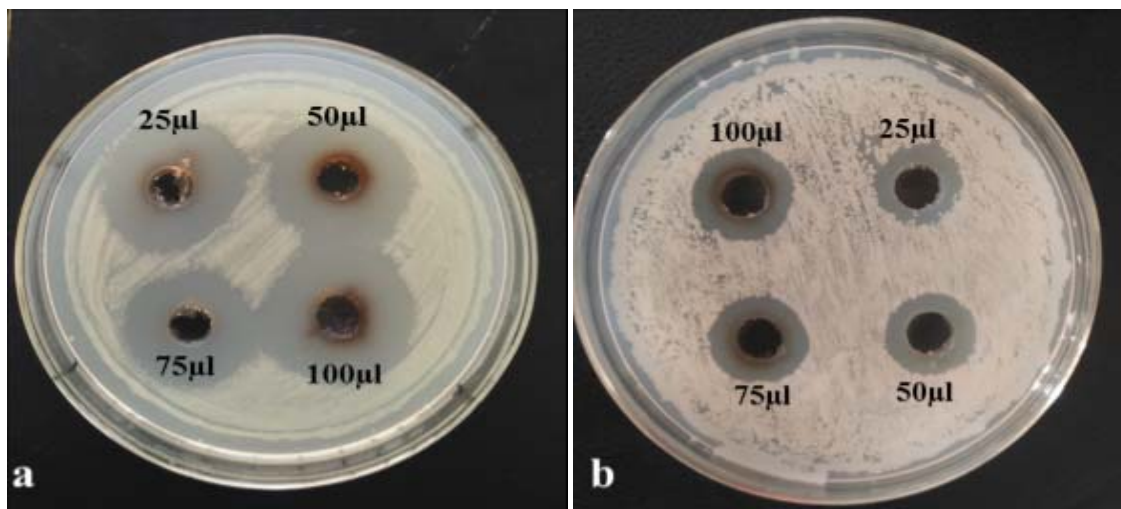
**Fig 12:** FTIR spectra of silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1

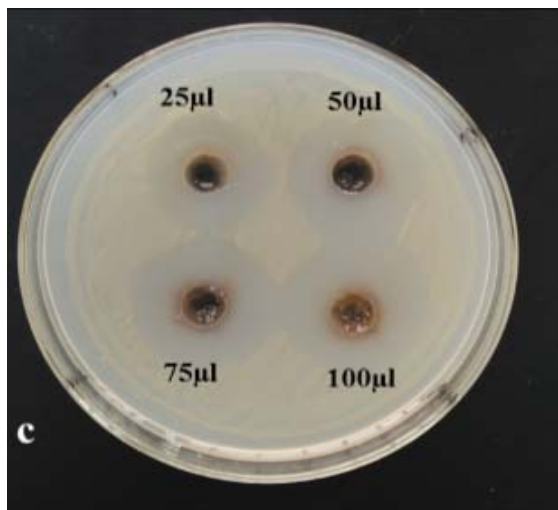


**Fig 13:** XRD spectra of silver nanoparticles synthesized by *Bacillus algalicola* strain UMP 1.1



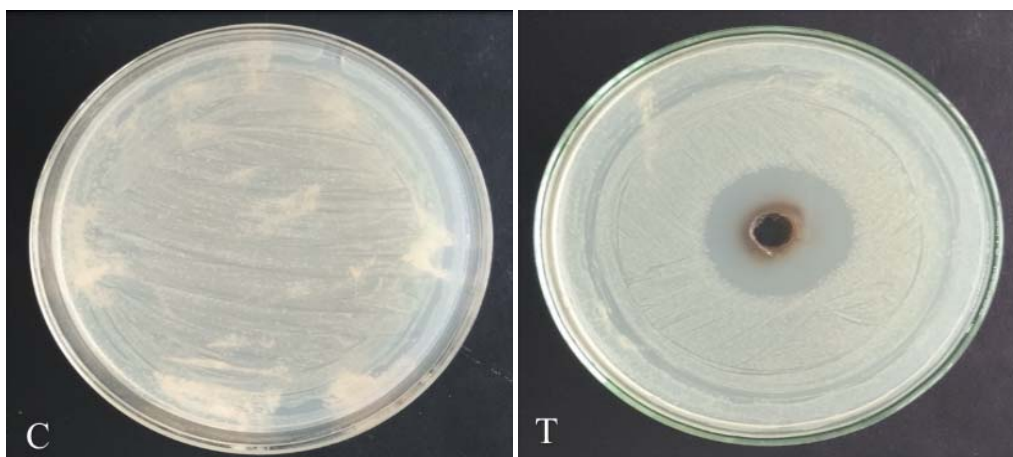
**Fig 14:** Scanning electron microscope image of silver nanoparticles synthesized by *Bacillus algalicola* strain UMP 1.1 at different scale





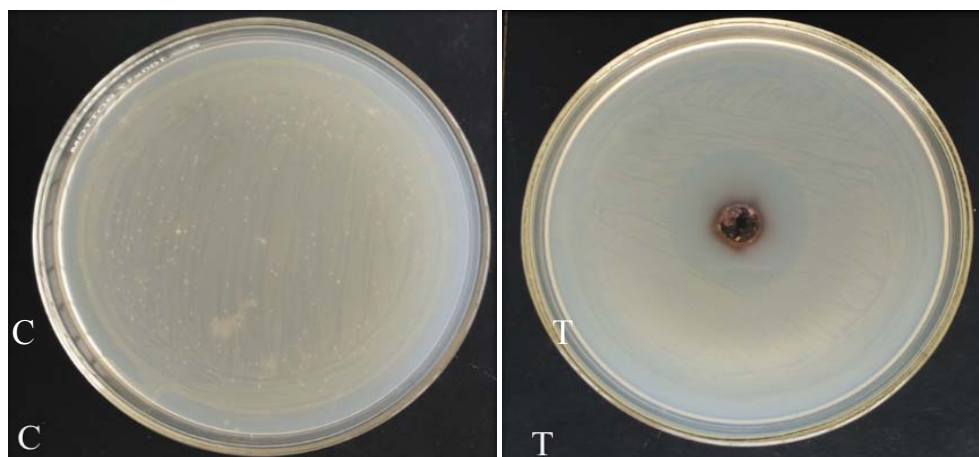
**Plate 4:** Effect of various dosages and silver nanoparticles suspension synthesized by *Bacillus algicola* strain UMP 1.1 against various plant pathogens using agar well diffusing method

- (a) *Xanthomonas axonopodis*
- (b) *Agrobacterium tumefaciens*
- (c) *Xanthomonas Campestris*



**Plate 5b:** Antibacterial activity of silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1 against various plant pathogens bacteria using agar well diffusing method

- (C) *Agrobacterium tumefaciens*
- (T) Zone of inhibition by 100ul bio silver nanoparticles suspension



**Plate 5c:** Antibacterial activity of silver nanoparticles synthesized by *Bacillus algicola* strain UMP 1.1 against various plant pathogens bacteria using agar well diffusing method

- (C) *Agrobacterium tumefaciens*
- (T) Zone of inhibition by 100ul bio silver nanoparticles suspension

### Acknowledgements

It is my proud privilege to express my deep sense of gratitude and indebtedness to my respected guide Dr. (Mrs.) Poonam Shirkot for her expert guidance, unfailing encouragement, scholarly suggestions and keen interest during the entire course of this study. I shall remain indebted to her for her affection, help and generosity bestowed upon me.

I emphatically owe my sincere thanks to the member of my coadvisors Dr. Rajnish Sharma and Dr. Anjali Chauhan for their valuable suggestions and encouragement.

My deep regards and gratitude to my father Mr. Shankar Lal Khatri and my mother Mrs. Santosh Khatri for their *constant inspiration, blessings and love* and special thanks to my sister Mrs. Nitu and my brother Mr. Sonu Khatri. My special thanks to my lab seniors Priyanka Kashyap, Himani Sharma, Sabina Rana and Shashikant Sharma.

I feel befitting here to express my endearment to Daulat Singh Jhala, Loon Karan Raika, Sachin Mittal, Puli Sridhar Reddy, Himanshu Pandey, Niharika Bakshi, Ruchi Gupta and Vinay Kumar Dhiman, Pooja for support.

I am sincerely thankful to office and lab staff of Department of Biotechnology of Dr. YSP UHF Nauli for their help & support.

### References

1. Albrecht MA, Evans CW, Raston CL. Green chemistry and the health implications of nanoparticles. *Green Chemistry*. 2006; 8:417-432.
2. Chopra I. The increasing use of silver based products as antimicrobial agents: a useful development or a cause for concern. *Journal of Antimicrobial Chemotherapy*. 2007; 6:587-590.
3. Claus D, Berkeley RCW. Genus *Bacillus* Cohn. 1872. In: Sneath PHA, Sharpe and Holt (eds): *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkins Co., Baltimore, MD, 1986, 1105-39.
4. Das VL, Thomas R, Varghese RT, Soniya EV, Jyothis M, Radhakrishnan EK. Extracellular synthesis of silver nanoparticles by *Bacillus* strain CS 11 isolated from industrialized area. *Biotechnology*. 2014; 4:121-126.
5. Lee HY, Park HK, Lee YM, Kim K, Park SB. A practical procedure for producing silver Nanocoated fabric and its antibacterial evaluation for biomedical applications. *Chemical Communications*. 2007; 28:2959-2961.
6. McNeil SE. Challenges for nanoparticle characterization. In: McNeil S.E *Characterization of Nanoparticles Intended for Drug Delivery*. London: Humana Press, 2011, 9-6.
7. Mijakovic I, Petranovic D, Bottini N, Deutscher J. Protein tyrosine phosphorylation in *Bacillus subtilis*. *Journal of Molecular Microbiology and Biotechnology*. 2005; 9:189-197.
8. Slawson RM, Van MI, Lee H, Trevor JT. Germanium and silver resistance, accumulation and toxicity in microorganisms. *Plasmid*. 1992; 27:73-79.