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In vitro biosynthesis and characterization of biosilver nanoparticles of *Pseudomonas putida* LUA 15.1 and their potential as antibacterial agents

Amna Devi, Ankita Sharma, Himani Sharma, Shashikant Sharma, Pankaj Raghuvanshi and Poonam Shirkot

Abstract
Biosilver nanoparticles are playing an important role in various field such as in agriculture, cosmetics, bioremediation and biomedical. In present investigation, *in vitro* synthesis of silver nanoparticles using *Pseudomonas putida* LUA 15.1 was carried out after optimization of various parameters. Optimum condition parameters for maximum synthesis of silver nanoparticles was selected which included TY broth containing 5g/l tryptone, 3g/l yeast extract, 1.5 mM $AgNO_3$, whereas $CuSO_4$ led to decline in silver nanoparticles synthesis. TY broth (pH; 8.0) using optimum condition was used for *in vitro* synthesis of 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 absorbance peak obtained between 330-460 nm. Characterization of biosilver nanoparticles was carried using SEM, DLS and FTIR. These biosilver nanoparticles found to be monodispersed, spherical in shape with size range of 10-25 nm and FTIR spectrum revealed presence of alkenes, phenylester and 1,2,4-trisubstituted biomolecules and these were tested for antibacterial activity against four bacterial plant using agar well diffusion test producing significant zones of inhibition against bacterial pathogens after 24 hrs of incubation.

Keywords: Biosilver nanoparticles, *Pseudomonas putida*, optimization and antibacterial activity

Introduction
Nanotechnology is amalgamation of information and communication technologies with biotechnology. Research and applied science has advanced to a place in which, instead of manipulating substances at the molecular level, we can control them even at the atomic level. Nanoparticles research has increased recently due to their properties such as size, high surface area to volume ratio, optical, electrical, magnetic, thermal, catalytic and thermal [1]. With the use of chemical and physical methods, the environmental contaminations are also increased. To overcome these disadvantages biological synthesis of nanoparticles is preferred due to low cost, ecofriendly and ease of nanoparticle synthesis method [2]. Different microorganisms such as bacteria, actinomycetes, and fungi have been investigated for synthesis of metal nanoparticles. Microorganisms synthesized silver nanoparticles when grab target ions from their environment and turn metal ions into the elemental form through enzymes generated by cell. Silver is a known antimicrobial agent and it has been observed that silver nanoparticles disrupt transport systems including ion efflux [3]. The dysfunction of ion efflux can cause rapid accumulation of silver ions, interrupting cellular processes at their lower concentrations such as metabolism and respiration by reacting with molecules. Further, silver nanoparticles have been found to produce reactive oxygen species (ROS) which are detrimental to cells leading to the death of cells [4]. Silver nanoparticles also used in clothing, sunscreen, cosmetics, and food industry due to its antimicrobial properties. In the present study silver nanoparticles were synthesized by an eco-friendly, cost effective method using *Pseudomonas putida* LUA 15.1. For the synthesis of silver nanoparticles process, synthesis medium and cultivation conditions play critical role because these affect formation, concentration and yield of a particular end product thus affecting overall outcome. 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, pH,

temperature, concentration of components of TY broth and substrate concentration. Biosynthesized silver nanoparticles must be characterized in order to understand their intrinsic properties via Scanning Electron Microscopy (SEM), UV-vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), Powder X-ray diffraction (XRD) and Dynamic Light Scattering (DLS). Silver nanoparticles were tested for their antibacterial activity against four bacterial plant pathogens *Ralstonia solanacearum*, *Xanthomonas axonopodis*, *Agrobacterium tumefaciens* and *Pectobacterium carotovorum* using agar well diffusion test and these nanoparticles showed variable zones of inhibition against bacterial pathogens.

Materials and methods

Procurement, maintenance and characterization of bacterial culture

Bacterial culture *Pseudomonas putida* LUA 15.1 was procured from laboratory of Department of Biotechnology, Dr. Y S Parmar University of Horticulture and Forestry Nauni-Solan (H.P.) which was one of the 409 bacterial isolates isolated from paddy fields and paper mills of Himachal Pradesh. The bacterial isolate was maintained using nutrient agar medium at pH 7.0 followed by its colony, microscopic, biochemical and molecular characterization.

Quantitative evaluation of *Pseudomonas putida* LUA 15.1 for silver nanoparticles synthesizing ability

Pseudomonas putida strain LUA 15.1 was assessed for its ability to synthesize silver nanoparticles. One percent (1%) inoculum (overnight culture) of *Pseudomonas putida* strain LUA 15.1 was inoculated into the 50 ml tryptone yeast broth followed by incubation at 37^{o}C (mesophilic) for 24 hrs**.** Supernatant of *Pseudomonas putida* strain LUA 15.1 was collected by centrifugation at 10000 rpm for 10 mins at 4^{o}C (Cooling Centrifuge REMI CM-8 Plus) to investigate extracellular synthesis of silver nanoparticles. Ten millilitre of supernatant was mixed with 10 ml of 1.0 mM solution of $AgNO_3$ and incubated at 37^{o}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 [5]. 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 silver nanoparticles by *Pseudomonas putida* strain LUA 15.1

The culture conditions for industrially useful microorganisms are generally optimized to obtain higher yields of their industrially important products. *Pseudomonas putida* strain LUA 15.1 depicting maximum silver nanoparticles synthesizing activity was further investigated to study effect of different factors such as incubation time (0-120 hrs), temperature (20-45 ^{0}C), pH (5.0-12), $AgNO_3$ concentrations (1.0-4.0 mM), tryptone concentration (0.0-10g/l), yeast extract concentration (0.0-5.0g/l), $CuSO_4$ concentration (0.0-70mg/l) and inoculum size (1-5%) on silver nanoparticles synthesis as well as growth of *Pseudomonas putida* strain LUA 15.1.

In vitro synthesis of silver nanoparticles by *pseudomonas putida* strain LUA 15.1

Preparation of bacterial culture

One percent concentration of inoculum (overnight culture) was inoculated into tryptone broth and incubated at optimum temperature for optimum incubation period of time. The supernatant was collected by centrifuging the culture broth at 10000 rpm, 4^{o}C for 10 mins to obtain extracellular synthesis of silver nanoparticles.

Silver nanoparticles synthesis by bacterial culture

Ten ml of supernatant was mixed in 10 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 from yellow to brown colour.

Characterization of silver nanoparticles

Silver nanoparticles synthesized by *Pseudomonas putida* strain LUA 15.1 culture were analyzed using various techniques such as UV- visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM)

UV- visible spectroscopy

The optical characteristics of the biosynthesized silver nanoparticles were analysed using UV–Vis spectrophotometer. For this, silver nanoparticles solution was subjected to absorption analysis at 200–700 nm range using UV–Vis spectrophotometer (Perkin Elmer Lambda 2, USA).

Fourier Transform Infrared Spectroscopy

Microcup was washed with 100% absolute ethanol. Teb microlitre 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^{-1} 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.

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 spectra 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% gluteraldehyde 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, the 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 were done on a JSM 6100. 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 Jeol 6100 LV scanning electron microscope using the following instrument conditions: accelerating voltage of 20 keV and counting time of 100 secs.

Antibacterial activity of silver nanoparticles preparations against bacterial pathogens.

Silver nanoparticles possess appreciable potential of antibacterial activity against various bacterial pathogens. The following pathogenic bacteria were obtained from the Departments of Basic Sciences (Microbiology-Section) and Plant Pathology, Dr. Y.S. Parmar UHF, Nauni, Solan. Four bacterial plant pathogens procured were *Ralstonia solanacearum, Xanthomonas axonopodis, Pectobacterium carotovorum* and *Agrobacterium tumefaciens.*

Lawn preparation

One ml inoculum of each pathogenic bacteria was swabbed on prepoured sterilized nutrient agar plates with the help of L shaped spreader. Swabbing was carried out in such a way that each test bacterial culture covers the whole surface of nutrient agar plate.

Assay of antibacterial activity of silver nanoparticles preparations

The inhibitory effect of silver nanoparticles preparation of *Pseudomonas putida* strain LUA 15.1 were studied using agar well diffusion test. Wells were cut on the bacterial lawn laid over the nutrient agar plates with the help of borer. An aliquot of 50 µl of silver nanoparticles were poured in each of well. The plates were then incubated at 37**°C** for 24 hrs and zones of inhibition were measured.

Results and discussion

Procurement, maintenance and characterization of bacterial culture

Bacterial culture was procured and maintained using nutrient agar medium (Plate-1). Morphological characterization showed bacteria was creamish in color, 0.7 mm in size, smooth texture, raised elevation, entire margin and microscopic characterization depicted Gram negative nature, rod shaped, arranged in single and clusters and nonsporulating. Biochemical characterization of bacterial culture revealed that bacterial strain was found to be positive for Oxidase, Citrate utilization test, Ornithine utilization test, Nitrate reductase, H_2S production, Glucose, Aldonitol, Arabinose and negative for Lysine utilization test, urease, phenylalanine deaminase, lactose and Sorbitol. On the basis of morphological and biochemical characterization this bacteria isolates was confirmed to belong to the genera *Pseudomonas* as per Bergey's Manual of Systemetic Bacteriology.

Molecular characterization was carried out using 16S *rrna* gene technology. Total genomic DNA of selected bacterial isolate LUA15.1 was extracted and amplified using PCR technology. Universal primer B27F and U1492R for 16S *rrna* 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 LUA15.1, 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 silver nanoparticles synthesizing bacterial isolate LUA15.1was found to be 1476 bp.

BLASTn search of selected silver nanoparticles synthesizing bacterial sequence with the most similar 16S *rrna* gene sequences of the Gen Bank database revealed that this bacteria showed 99% similarity with *Pseudomonas putida* strain KT2440, 16S ribosomal RNA, complete sequence. Phylogenetic tree also verified bacteria *Pseudomonas putida* as it clustered closely with *Pseudomonas putida* strain KT2440, 16S ribosomal RNA complete sequence. Thus, based on molecular characterization, the isolate LUA15.1 was eventually confirmed as *Pseudomonas putida*.

Quantitative evaluation of *Pseudomonas putida* strain LUA 15.1 for silver nanoparticles synthesis ability

Pseudomonas putida strain LUA15.1 was assessed for its ability to synthesize silver nanoparticles. One millimolar solution of silver nitrate ($AgNO_3$) when mixed with same volume of supernatant of *Pseudomonas putida* LUA 15.1 and incubated at 37ºC for 24 hrs. Formation of silver nanoparticles was confirmed by color change of the solution from pale yellow to brown red colour (Plate-2). Formation of silver nanoparticles was confirmed by spectrophotometer at a wavelength of 420 nm.

Optimization of culture conditions for maximum silver nanoparticles synthesis and growth of *Pseudomonas putida* strain LUA 15.1

Effect of incubation time

Effect of incubation time on silver nanoparticles synthesizing activity by *Pseudomonas putida* strain LUA15.1 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 0.595 after that it was found to decrease up to 120 hrs. Thus, maximum silver nanoparticles synthesis was achieved at an incubation time of after 24 hrs (Fig. 1a). In other reports, *Pseudomonas* sp. [6] has been found to produce maximum extracellular silver nanoparticles synthesis after 48 hrs of incubation, whereas [7] reported maximum silver nanoparticles synthesis by *Bacillus stearothermophilus* after 72 hrs.

Effect of incubation temperature

A study on silver nanoparticles synthesizing activity by *Pseudomonas putida* strain LUA15.1 was investigated at different temperatures ranging from 20-45ºC for 24 hrs. Silver nanoparticles synthesizing activity by *Pseudomonas putida* strain LUA15.1 showed increased with increase in temperature however maximum activity of 0.856 at 420 nm wavelength of light was obtained at 35 ºC (Fig.1b). In other hand maximum silver nanoparticles synthesis at 60^0C temperature by *Escherichia coli* [8] similarly [9] reported optimized temperature 5^0C for maximum silver nanoparticles synthesis by *Eshcherichia coli*.

Effect of pH

pH range of 5.0-12.0 of the TY broth was examined and it was found that silver nanoparticles synthesizing activity increased with increase in pH up to pH 8.0 followed by a sharp decrease in activity up to 12.0 pH. At pH 8.0 maximum silver nanoparticles synthesizing activity of 0.690 OD at 420 nm wavelength of light was observed (Fig.1c). Whereas [9]

recorded maximum silver nanoparticles synthesis at an optimum pH of 7.5 by *Eshcherichia coli* and *Bacillus stearothermophilus* synthesized maximum silver nanoparticles with pH 7.4 [10].

Effect of Tryptone concentration

Silver nanoparticles synthesis activity by *Pseudomonas putida* LUA15.1 was found to increase with increase in tryptone concentration up to 5.0 g/l. Maximum silver nanoparticles synthesis activity in TY broth was obtained at 5.0 g/l tryptone with an OD of 0.790 followed by decrease in silver nanoparticles synthesizing activity with increasing concentration (Fig. 1d). Thus 5.0 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 investigated and silver nanoparticles synthesis activity was found to increase with increase in yeast extract concentration up to 3.0 g/l followed by decrease. Maximum silver nanoaprticles OD value of 0.552 at 420 nm was observed when 3.0g/l of yeast extract was used (Fig. 1e). Similarily [7] reported 4g/l yeast extract gave maximum silver nanoparticles synthesis by *Bacillus stearothermophilus.*

Effect of $CuSO_4$ Concentration

Silver nanoparticles synthesis was also carried out at different concentrations of $CuSO_4$ using TY broth containing 5.0g/l tryptone, 3.0g/l yeast extract, pH 8.0 at 35°C. $CuSO_4$ was found to inhibit the silver nanoparticles synthesis as shown in the (Fig. 1f), maximum silver nanoparticles synthesis took place in control with no $CuSO_4$.

Effect of inoculum size

Size range of 1-5% on silver nanoparticles synthesis of *Pseudomonas putida* LUA15.1 was investigated and it has been revealed that an inoculum size of 3% showed maximum OD of 0.528 at 420 nm (Fig. 1g).

Effect of different concentration of silver nitrate

Different concentrations of silver nitrate were investigated for silver nanoparticles synthesis using TY broth and maximum extracellular silver nanoparticles synthesis activity was observed in TY broth containing 1.5 mM silver nitrate with OD value of 0.952 at 420 nm followed by decrease in silver nanoparticles activity up to 4.0 mM silver nitrate (Fig. 1h). In other hand, [11] reported maximum yield of silver nanoparticles was obtained with 1mM $AgNO_3$ whereas [6] reported *Pseudomonas putida* NCIM 2650 synthesized maximum silver nanoparticles with 1mM $AgNO_3$.

***In vitro* synthesis of silver nanoparticles by *Pseudomonas putida* LUA15.1**

Extracellular biosynthesis of silver nanoparticles by *Pseudomonas putida* LUA15.1 was carried out using the optimized parameters. Three percent inoculum size was used to inoculate tryptone broth containing 5.0 g/l tryptone, 3.0 g/l yeast extract, 30 mg/l $CuSO_4$, pH 8.0 for 24 hrs of incubation. Ten millilitre of culture supernatant was mixed in 10 ml of 1.5 mM solution of $AgNO_3$ and inoculated at 35^{0}C for 24hrs. Formation of silver nanoparticles was indicated by color change of the solution from pale yellow to brown red (Plate-2).

Characterization of nanoparticles

Silver nanoparticles must be characterized in order to understand their intrinsic properties such as size, monodispersivity, aqueous stability, net charge, adsorption to biomolecules, aggregation and flocculation. This provides vital information in terms of application of these nanoparticles [12]. Nanoparticles obtained were analyzed using various techniques such as UV- vis, FTIR, DLS and SEM.

UV- visible spectroscopy

Addition of 1.5 mM $AgNO_3$ solution to the culture supernatant 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^+ to Ag^0. Silver nanoparticles that can be identified from the peaks obtained between 330-460 nm (Fig. 2a), which is the signature for the silver nanoparticle formation, apart from the color change. In another studies [13] characterized silver nanoparticles using UV–visible spectroscopy and found that the nanoparticles exhibited maximum absorbance at 430 nm. Whereas [14] reported characterization of nanoparticles using UV- vis and maximium absorbance was noted at 420 nm and [5] characterized silver nanoparticles using UV-vis and maximum absorption peak was observed at 450 nm.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectrogram of the reaction mixture has shown presence of three bands one at 3000 cm^{-1} which can assign C-H stretching alkenes whereas another at 1700 cm^{-1} indicated C=O stretching of phenylester and third at 860 cm^{-1} which can be assigned to C-H bending 1,2,4-trisubstituted of biomolecules involved in reduction of Ag^{2+} to Ag^0 (Fig. 2b). [15] reported characterization of silver nanoparticles using FTIR and results displayed the presence of hydroxyl functional group which was found by peak transmission value at 3200-3500 cm-1. [6] repoted FTIR spectra of silver nanoparticle and observed absorption peaks located in the region of 4000 cm-1 to 400 cm-1.

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. It has been revealed that maximum silver nanoparticles size distribution lies in the range of 10-25nm (Fig. 2c). In other study [16] reported silver nanoparticles synthesized in size range of 15-60 nm.

Scanning electron microscope

Scanning electron microscope revealed that silver nanoparticles synthesized by *Pseudomonas putida* LUA 15.1 were monodispersed and spherical in shape (Fig. 2d). In another studies [12] characterized silver nanoparticles using SEM and SEM micrographs revealed spherical nanoparticles in the size range of 10–100 nm, similarily [17] characterized silver nanoparticles and SEM graph revealed relatively spherical shaped nanoparticles with the diameter in the range of 40-60 nm.

Antibacterial activity of silver nanoparticles synthesized by *Pseudomonas putida* LUA15.1 against bacterial plant pathogens

Silver nanoparticles showed variable zones of inhibition against four bacterial pathogens after 24 hrs of incubation. In

case of *Ralstonia solanacearum,* silver nanoparticles solution preparation showed 1.4 cm zone of inhibition whereas 3.5 cm zone of clearance was observed against *Xanthomonas axonopodis.* Similarly, in case of *Agrobacterium tumefaciens* and *Pectobacterium carotovorum* silver nanoparticles solution produced 1.6 and 1.2 cm zone of inhibition respectively (Plate-3a-d). Literature has been found regarding antibacterial activities of silver nanoparticles [18]. reported antibacterial activity of silver nanoparticles against various plant pathogenic bacteria like *Erwina cartovra*, *Dickya chransanthemi* and *Pectobacterium atrosepticum* with zone of inhibition 20.0mm, 10.0mm and 19.0mm respectively, whereas [19] reported antibacterial activity of silver nanoparticles against plant pathogens like *Xanthomonas* sp., *Agrobacterium* sp., *Erwinia amylovora* and *Pseudomonas compestris*. Similarily [20] reported antibacterial activity of silver nanoparticles against *Xanthomonas compestris* and *Ralstonia solanacearum* with 26.6mm and 24.2mm zone of inhibition respectively.

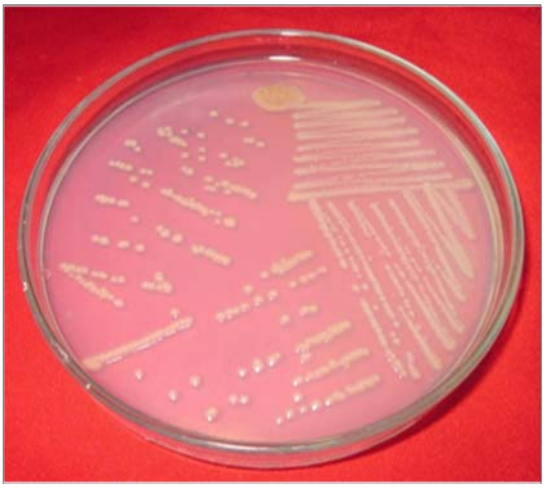
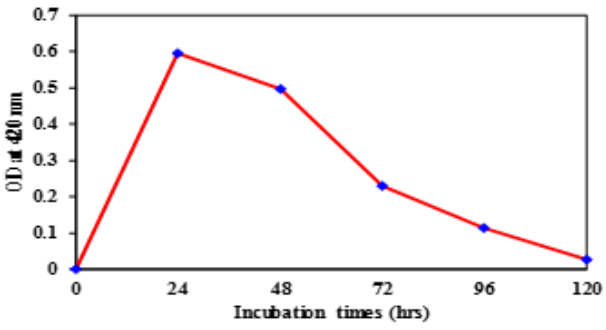


Plate 1: Procured culture of *Pseudomonas putida* LUA 15.1

Plate 2: Colour change from yellow to brown red
a) Control
b) Silver nanoparticles solution



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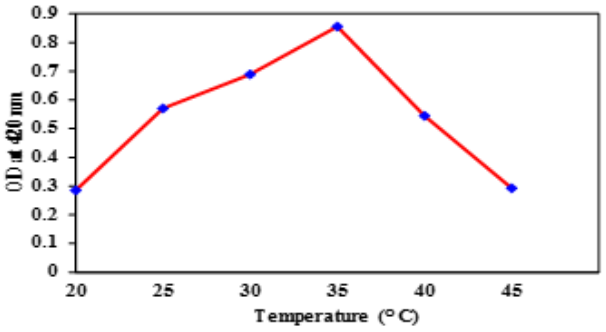

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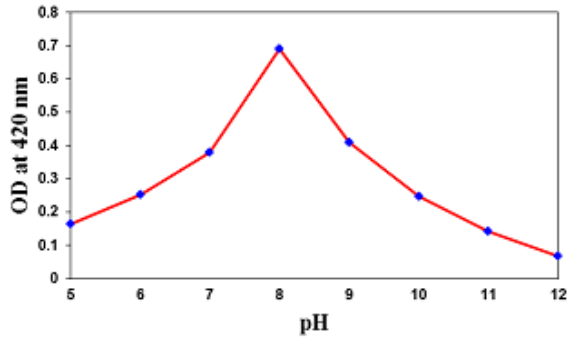

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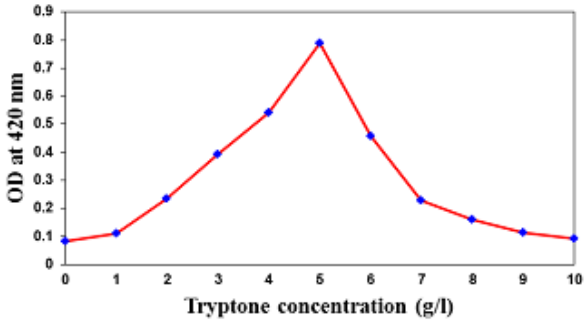

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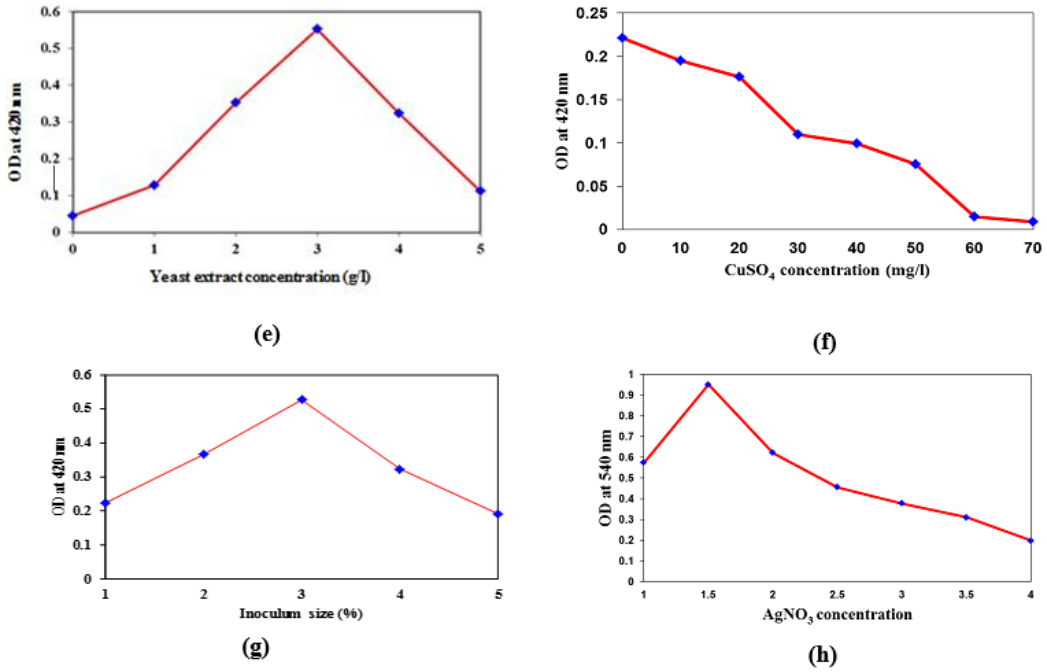
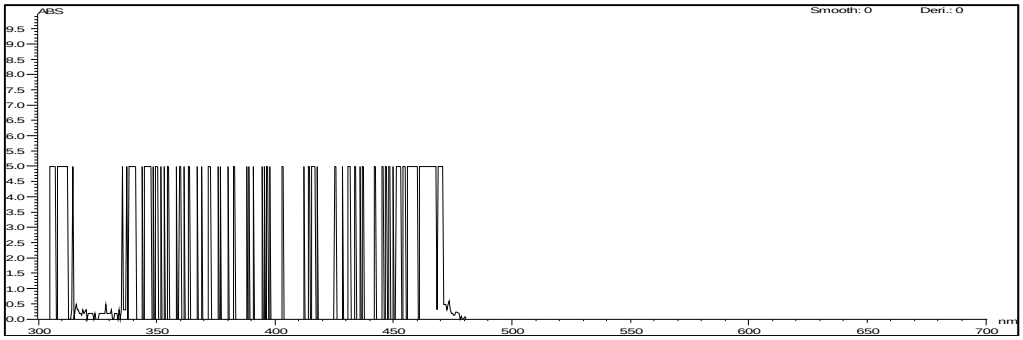


Fig 1: Effect of (a) Incubation time (b) Temperature (c) pH (d) Tryptone concentration (e) yeast extract concentration (f) $CuSO_4$ concentration (g) Inoculum size (h) $AgNO_3$ concentration on silver nanoparticles synthesis by *Pseudomonas putida* LUA 15.1.



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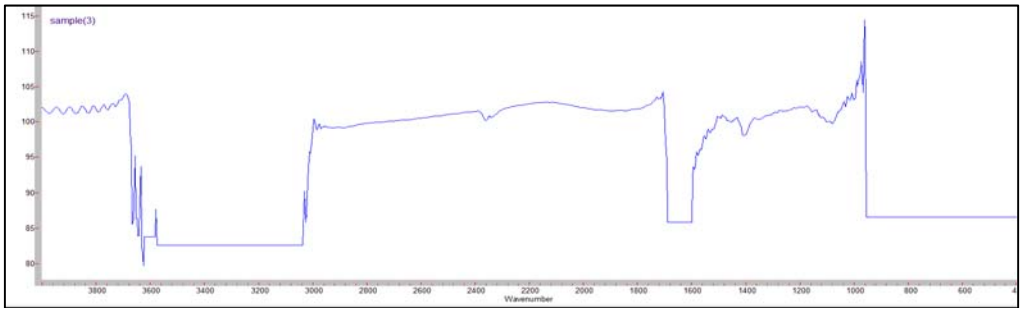

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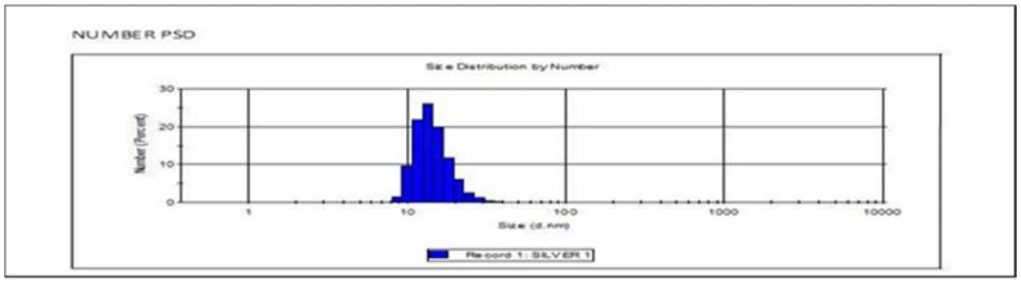

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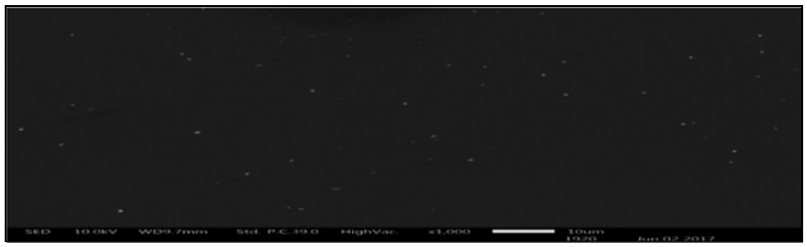

(d)

Fig 2: Characterization of silver nanoparticles synthesized by *Pseudomonas putida* LUA 15.1 (a) UV- vis spectroscopy (b) FTIR (c) DLS (d) SEM

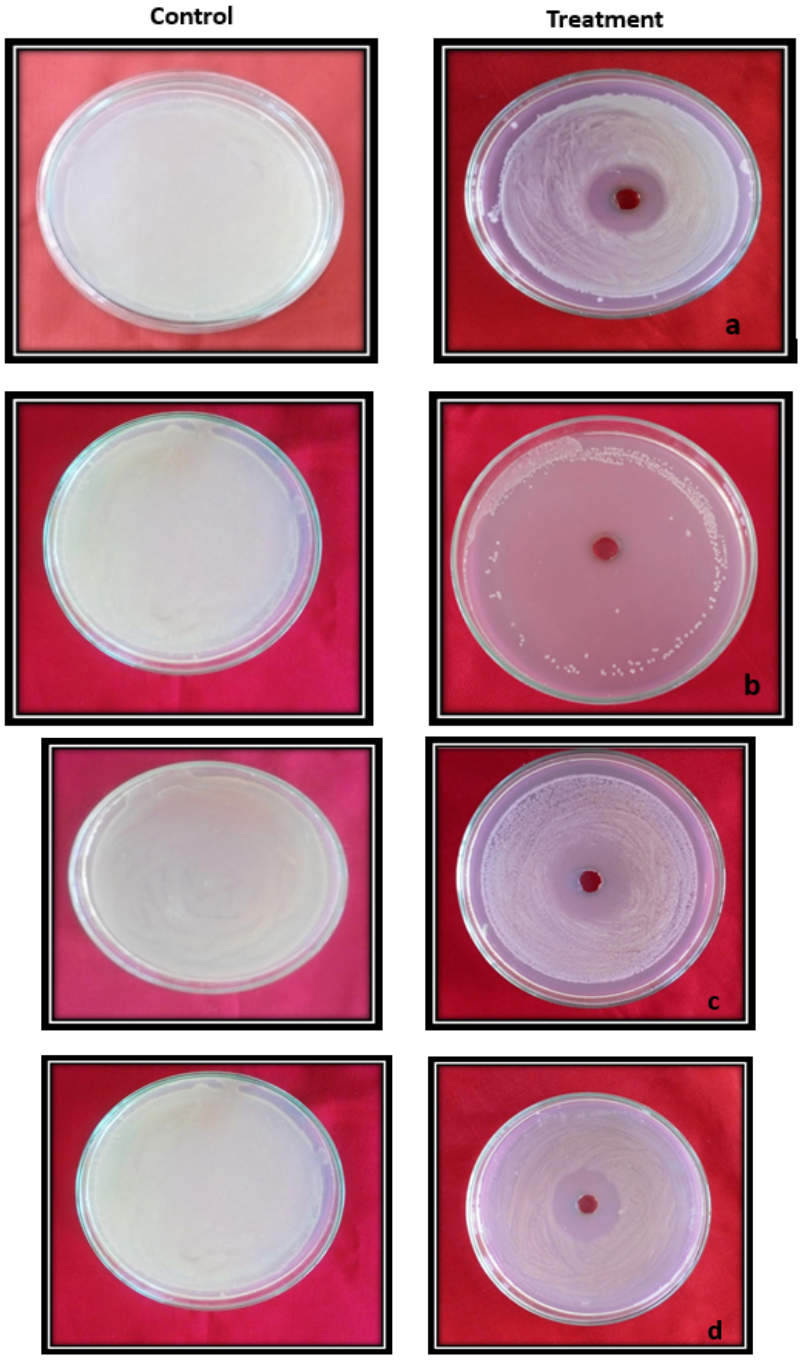

Plate 3: Antibacterial activity of silver nanoparticles synthesized by *Pseudomonas putida* LUA 15.1 against various bacteria using agar well diffusion method

(a) *Ralstonia solanacearum*
(b) *Xanthomonas axonopodis*
(c) *Agrobacterium tumefaciens*
(d) *Pectobacterium cartovorum*

Conclusion

In the present study spherical, monodispersed biosilver nanoparticles of 10-25 nm size synthesized by *Pseudomonas putida* LUA15.1 and maximum biosilver nanoparticles was achieved at 35 ^{0}C, pH 8.0 after 24 hrs of incubation with 1.5 mM silver nitrate, 5.0% tryptone, 3% yeast extract and 3% inoculum size. Biosilver nanoparticles has emerged as potential agent able to inhibit important plant pathogenic bacteria *viz., Ralstonia solanacearum, Xanthomonas axonopodis, Agrobacterium tumefaciens* and *Pectobacterium carotovorum* using agar well diffusion method. After 24 hrs of incubation silver nanoparticles showed maximum zone of inhibition against *Xanthomonas axonopodis* which was found to be 3.5 cm under *in vitro* condition, though further evaluation is required to be done under *in vivo* condition.

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