Original Research

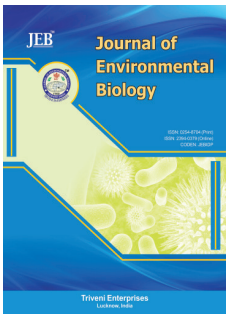
Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

DOI : http://doi.org/10.22438/jeb/39/4/MRN-489

JEB

p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP



Silver nanoparticles production by *Aspergillus niger* and their antibacterial efficacy against *Xanthomonas citri* and *Ralstonia solanacearum*

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Key words

Aspergillus niger
Bioassay
Pathogenic bacteria
Silver nanoparticles

Publication Info

Paper received : 18.10.2016
Revised received : 08.03.2017
Re-revised received : 26.09.2017
Accepted : 02.11.2017

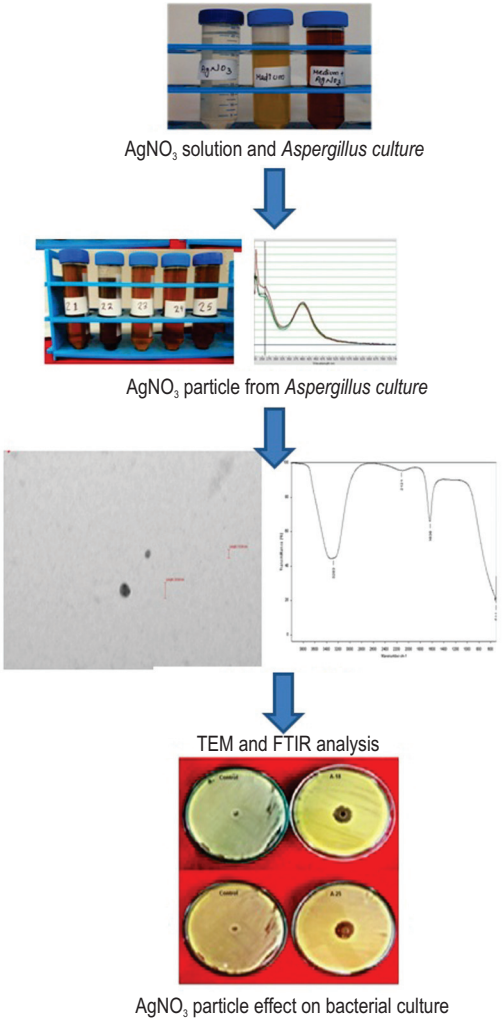
Abstract

Aim : Nanobiotechnology is being widely applied in multidisciplinary fields including biopesticide applications. Recently, there is an increase research in the fungal mediated production of silver and copper nanoparticles.

Methodology : In this investigation, the silver nanoparticles were synthesized from the isolates of industrial fungus, *Aspergillus niger* and their efficacy was examined using well diffusion assay against two phyto-pathogenic bacteria viz., *Xanthomonas citri* and *Ralstonia solanacearum*. The silver nanoparticles developed from *Aspergillus niger* cell filtrate were described through UV-Vis spectrophotometer, Transmission electron microscope (TEM) analysis and Fourier Transform Infrared Spectroscope (FTIR).

Results : The production of silver nanoparticles was confirmed by visualization of peak at 420 nm in UV-Vis spectrophotometer. FTIR studies showed the protein capping of these silver nanoparticles, while TEM microphotographs showed well dispersed silver nanoparticles which are isotropic and round having size between 3–25 nm. The 20-30 mm diameter inhibition zones were formed by biosynthesized silver nanoparticles when tested against *Xanthomonas citri* and *Ralstonia solanacearum*.

Interpretation : *Aspergillus niger* based silver nanoparticles were proved to be used against plant pathogenic bacteria due to their broad spectrum efficacy. Further studies are needed to investigate the efficacy of silver nanoparticles as antibacterial agent *in vivo*.



Introduction

Nanoparticles are the basic units for different nanotechnology applications. The sizes of nanometer dimensions with a range of 1-100 nm determine the properties (physical and chemical) of materials which shows different ways of future applications (Rai *et al*., 2009). To avoid application of hazardous chemical pesticides, there is a need for the production of environmentally safe nanoparticles. Many organisms produce inorganic materials, either intracellularly or extracellularly (Ahmad *et al*., 2003: Sastry *et al*., 2003). Even at high metal ion concentration, different microorganisms can live and grow because of their ability to fight metal stress.

The silver nanoparticles production from living organisms is ecofriendly, safe, cost effective and having effective antibacterial properties. Recently, many microorganisms especially fungi was proved as potential bio-agents for the nano-sizing of gold, silver and cadmium metals (Sastry *et al*., 2003). When the fungi, *Beauveria, Verticillium*, *Fusarium* and *Trichoderma* exposed to silver and gold ions, reduced the metal ion rapidly and formed respective metallic nanoparticles (Bhainsa and D'Souza, 2006: Balaji *et al*., 2009; Singh and Raja, 2011; Prameeladevi *et al*., 2013; Kamil *et al*., 2017). The structural analysis of nanoparticles was done with transmission electron microscope (Balaji *et al*., 2009; Shaligram *et al*., 2009).

Nanoparticles are synthesized when the microorganisms grab the metal ions from the environment and turn them into the element metal by the enzymes produced through cell activities. The extracellular biosynthesis of nanoparticles using fungi can also make the downstream processing much easier than bacteria (Li *et al*., 2011). Silver nanoparticles (AgNPs) are reported as a promising antimicrobial agents which acts on a high range of target sites both extra- and intra-cellularly and exhibited lysis of Gram positive and Gram negative bacteria even multi-resistant strains (Shrivastava *et al*., 2007; Zeng *et al*., 2007; Roe *et al*., 2008) with a size of 10–100 nm (Morones *et al*. 2005). The bactericidal activity was reported by Rai *et al*. (2012) against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* and also reported by Verma *et al*. (2013) against *Salmonella, Pseudomonas* and *Bacillus*. Silver nanoparticles synthesized from *Fusarium semitectum* have been found with strong antibacterial activity against *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Gitanjali and Ashok, 2014).

The extracellular production of silver nanoparticles using *Aspergillus fumigatus* and *Aspergillus flavus* was reported by D'Souza (2006). The process of silver nanoparticles synthesis is quick and the reaction starts immediately after metal ions mixed with filtrate (Vigneshwaran *et al*., 2007). The AgNPs produced using *Aspergillus terreus* can inhibit *Staphylococcus aureu*, *Pseudomonas aeruginosa* and *Escherichia coli* (Li *et al*., 2011). The present study was performed for the fungal mediation synthesis of silver nano particles from silver nitrate solution using *Aspergillus niger* and to find the antibacterial activity of these produced nanoparticles against *Xanthomonas citri* and *Ralstonia solanacearum*.

Materials and Methods

Preparation of culture filtrate of *Aspergillus niger* : All the microbial cultures for the work were procured from Indian Type Culture Collection, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi. All these isolates of the fungi mentioned above were cultured on Potato Dextrose Agar slants (for storage and further use) and Potato Dextrose Broth (for the extracellular synthesis of Ag NPs) and incubated at 25°C in BOD incubator for 10 days. The 10-day-old fungal cultures from PDB were transferred into 30 ml tube and then centrifuged (12000 rpm) for half an hour at room temperature. The clear liquid above the sediment was collected and used as a culture filtrate for Ag NPs biosynthesis.

***Aspergillus niger* mediated synthesis of silver nanoparticles (AgNPs) :** For extra cellular production of silver nanoparticles, 50 ml aqueous solution of $AgNO_3$ was mixed with 50 ml of culture filtrate of *A. niger* in a flask with 8.5 pH. The mixer was kept under dark condition at 27°C and 200 rpm for 5 days in shaker incubator.

Analysis of silver nanoparticles (AgNPs)

Spectroscopic studies : The UV-VIS spectrum was visualized at 24 hr interval up to 120 hrs and their absorbance was recorded at 420 nm using UV-Vis spectrometer.

Transmission electron microscopy studies : The fungal mediated silver nanoparticles were placed on carbon coated 40 X 40µl mesh sized TEM grids and analyzed through electron microscope (JEOL model 1200EX) which was operated at 120kV. The observations were noted through photomicrograph.

Fourier transforms infra-red spectroscopy : FT-IR spectra of AgNPs were obtained through Fourier transform infrared (FTIR) spectrophotometer in diffuse reflectance mode at four particles/cm resolution. The AgNPs were ground with the pellets of potassium bromide and examined under FTIR spectrophotometer at 4000–400 cm^{-1} region.

Extraction of Ag NPs : The mixture of cell debris and silver nanoparticles (AgNPs) was centrifuged for 20 min at 4500 rpm and 4°C and the supernatant was further centrifuged for 30 min at 14000 rpm and 4°C (Chan and Don, 2012). The pellet having NPs were suspended i milli Q water.

Anti bacterial activity of Ag NPs produced using *Aspergillus niger* : Both the bacterial cultures grown on nutrient agar were inoculated in nutrient broth for further use. AgNPs synthesized by the fungi were tested at 100 ppm for anti-bacterial activity by well diffusion assay against the phyto-pathogenic bacteria viz.,

Xanthomonas citri and *Ralstonia solanacearum*. In this test, the maximum concentration of bacterial culture was standardized as 300 µl and maximum SNP concentration against *Xanthomonas citri* and *Ralstonia solanacearum* were standardized as 30 µl. The pure cultures of *Xanthomonas citri* and *Ralstonia solanacearum* (300 µl) were inoculated uniformly onto the nutrient agar medium plates. The 6 mm hole was made at the center of the plate by using Cork borer on the bacterial-cultured plates and impregnated with 30 µl of AgNPs solutions and incubated at 37°C for 24 hrs. Water was used in place of AgNPs in the control plates to evaluate the anti bacterial activity of AgNPs.

Results and Discussion

Twenty five culture filtrates of *Aspergillus niger* isolates were mixed with $AgNO_3$ solution to select the potential isolates for

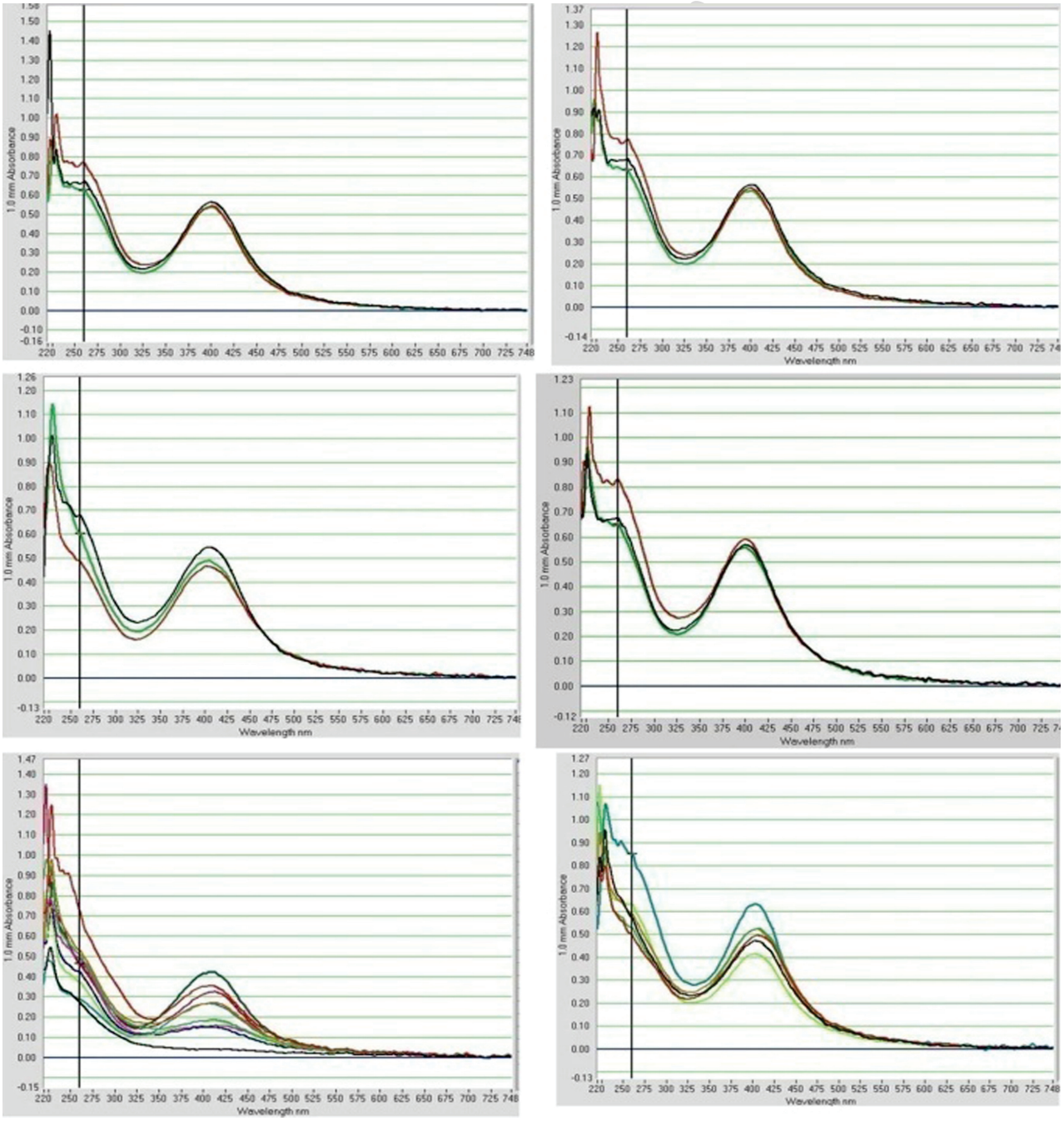


Fig. 1 : UV absorption peak of silver nanoparticles at 420 nm and 120 h

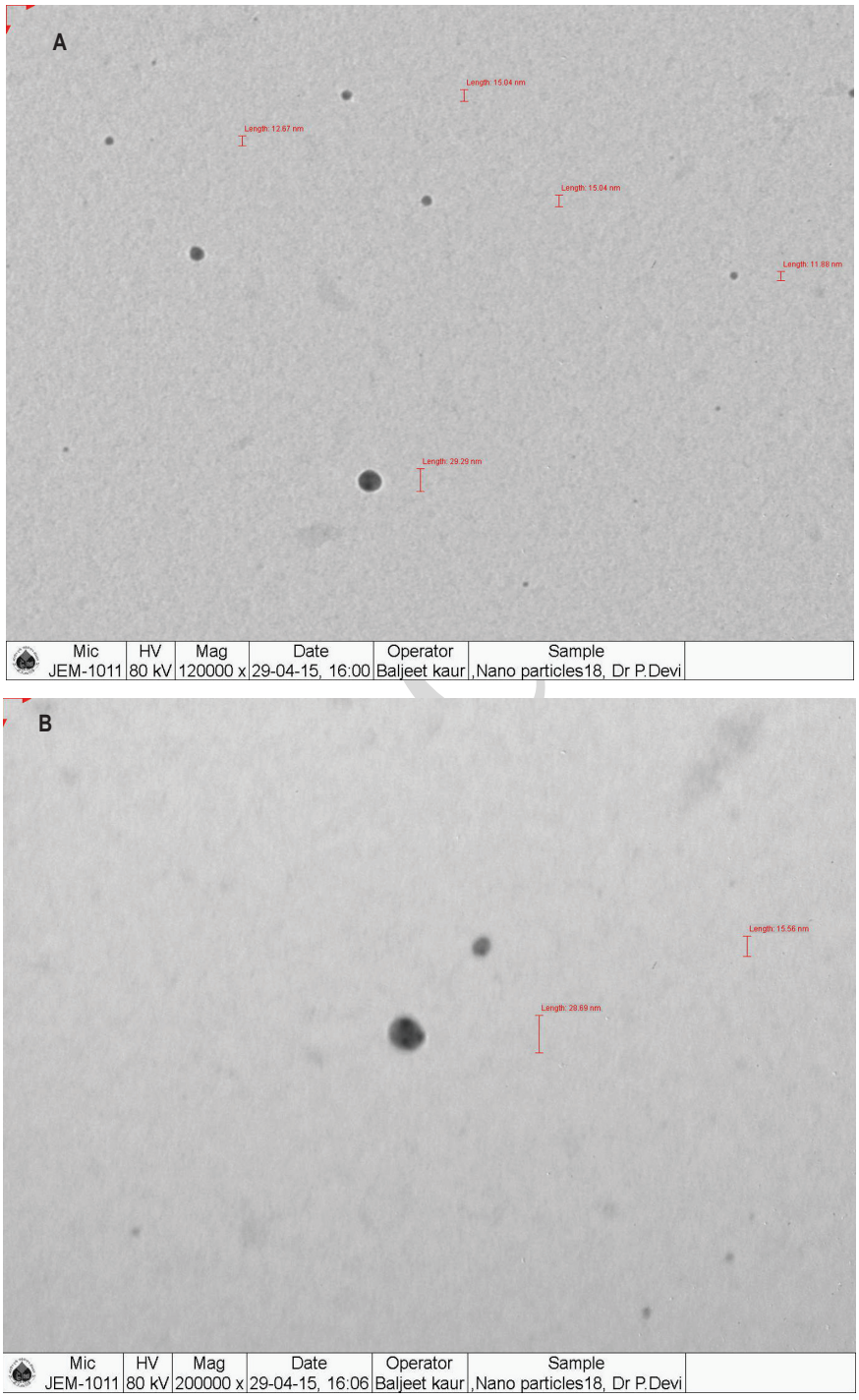


Fig. 2 : Transmission electron microscopy of silver nanoparticles biosynthesized by A-18 and A- 25 isolates of *Aspergillus niger*

the production of silver nanoparticles. The change in colour from transparent to brown was observed brown at 10 hrs due to deposition of silver nanoparticles, whereas the fungal supernatant (positive control) retained its original colour which denotes production of silver nanoparticles, and color intensity varied due to increasing quantity of nanoparticles. The silver nitrate treated fungal supernatant turned dark. The unreacted fungal biomass retained their original colour and reacted fungal biomass turned into dark brown colour. The UV-Vis reading of fungal biomass of *Aspergillus niger* treated with $AgNO_3$ is given in Table 1. The

Table 1: UV–vis absorption values of silver nanoparticles produced by *A. niger* at various time intervals

Culture	ITCC	Day 1		Day 2		Day 3		Day 4		Day 5	
No.	No.	380 nm	420 nm	380 nm	420 nm	380 nm	420 nm	380 nm	420 nm	380 nm	420 nm
A-1	6359	0.394	0.469	0.379	0.427	0.411	0.456	0.397	0.438	0.392	0.431
A-2	6370	0.094	0.097	0.072	0.051	0.066	0.038	0.042	0.039	0.037	0.043
A-3	6409	0.495	0.498	0.485	0.484	0.483	0.477	0.483	0.481	0.471	0.468
A-4	6410	0.240	0.363	0.245	0.392	0.236	0.374	0.305	0.336	0.331	0.342
A-5	6438	0.172	0.371	0.182	0.384	0.162	0.246	0.164	0.181	0.163	0.172
A-6	6503	0.180	0.375	0.188	0.375	0.184	0.390	0.262	0.237	0.227	0.236
A-7	6517	0.388	0.457	0.398	0.427	0.419	0.478	0.420	0.479	0.407	0.469
A-8	6543	0.418	0.483	0.178	0.216	0.243	0.297	0.453	0.487	0.450	0.473
A-9	6560	0.330	0.356	0.322	0.346	0.291	0.301	0.279	o.293	0.278	0.289
A-10	6561	0.445	0.512	0.454	0.505	0.447	0.494	0.449	0.499	0.438	0.486
A-11	6614	0.479	0.511	0.517	0.532	0.313	0.374	0.484	0.506	0.483	0.507
A-12	6642	0.467	0.525	0.458	0.507	0.437	0.479	0.489	0.537	0.480	0.526
A-13	6662	0.487	0.557	0.513	0.554	0.504	0.538	0.468	0.508	0.468	0.502
A-14	6677	0.535	0.539	0.419	0.471	0.418	0.461	0.443	0.477	0.425	0.468
A-15	6719	0.455	0.498	0.364	0.392	0.368	0.384	0.364	0.382	0.351	0.372
A-16	6738	0.197	0.270	0.398	0.434	0.431	0.434	0.374	0.409	0.400	0.436
A-17	6742	0.428	0.443	0.426	0.450	0.434	0.456	0.406	0.425	0.398	0.432
A-18	6775	0.492	0.540	0.518	0.542	0.489	0.514	0.486	0.515	0.477	0.503
A-19	6847	0.390	0.510	0.472	0.514	0.448	0.484	0.393	0.511	0.383	0.500
A-20	6860	0.280	0.304	0.452	0.485	0.448	0.488	0.438	0.477	0.466	0.507
A-21	7122	0.478	0.474	0.500	0.486	0.458	0.444	0.464	0.452	0.456	0.440
A-22	7056	0.474	0.456	0.486	0.466	0.469	0.447	0.468	0.448	0.472	0.450
A-23	7132	0.450	0.526	0.439	0.520	0.463	0.539	0.451	0.535	0.441	0.529
A-24	7136	0.298	0.311	0.361	0.357	0.351	0.354	0.342	0.344	0.352	0.350
A-25	5579	0.522	0.587	0.542	0.584	0.544	0.576	0.540	0.568	0.523	0.547

highest absorption band was found in the isolate A-25 followed by A-18. The spectrum clearly showed increase in the intensity of silver nitrate solution, indicating synthesis of increased silver nanoparticles with increasing time (Fig. 1). Some more details including morphology and size of silver nanoparticles synthesized by *Aspergillus niger* isolates (A-18 and A-25) were studied through Transmission electron microscopy (Fig. 2). The particles were isotropic and spherical and mono disperses and the size varied from 3–25 nm. The chemical functional groups were determined by IR spectroscopic analysis in the sample. Different functional groups absorb characteristic frequencies of infrared radiation. The nanoparticles of *Aspergillus niger* isolate (A-25) were analysed by FTIR spectrum which showed the presence of band at 1636, 2121 and 3283 cm^{-1}, the bands at 1636 cm^{-1} corresponds to primary amine NH band (Fig. 3).

Further, the bio-efficacy of silver nanoparticles was tested against plant pathogenic bacteria viz., *Xanthomonas citri* and *Ralstonia solanacearum* with well diffusion method. The antibacterial activity of silver nanoparticles produced using Aspergillus niger was found maximum in the isolates A-18 and A-25 with an inhibition zone ranging from 2.0 – 3.0 cm (Fig. 4).

Plants, bacteria and fungi showed the capability of nano-sizing of metal ions and were effectively used for controlling other organisms. The nano-sized silver particles (AgNPs) biosynthesized from *Nocardiopsis dassonvillei* bacteria inhibited 50% growth of *Fusarium solani, F. oxysporum*, *Alternaria* alternata and *Rhizoctonia solani* at 1.25, 3.23, 4.3 and 9.2 ng ml^{-1}, respectively (Abdel-Megeed *et al*., 2015). The silver nanoparticles from the root extracts of *Catharanthus roseus* showed potential larvicidal activity against larvae of *Aedes aegypti* and *Culex quinquefasciatus* (Rajagopal *et al*., 2015). Howeves, reports on the fungal mediated silver nanoparticle synthesis are meagre (Bhainsa and D'Souza, 2006, Kathiresan *et al*., 2009).

The excitation of surface Plasmon vibrations is a distinctive character which determines the presence of silver nanoparticles. The nanoparticles were produced immediately after silver ions were added to the culture filtrate. Reduction of silver ions was resulted as changed in the colour intensity of culture filtrate was directly proportional to the increasing number of nanoparticles formed. It has been reported that nano-sizing of silver ions were due to the action of reducing agents such as enzyme reductase and electron shuttle quinine

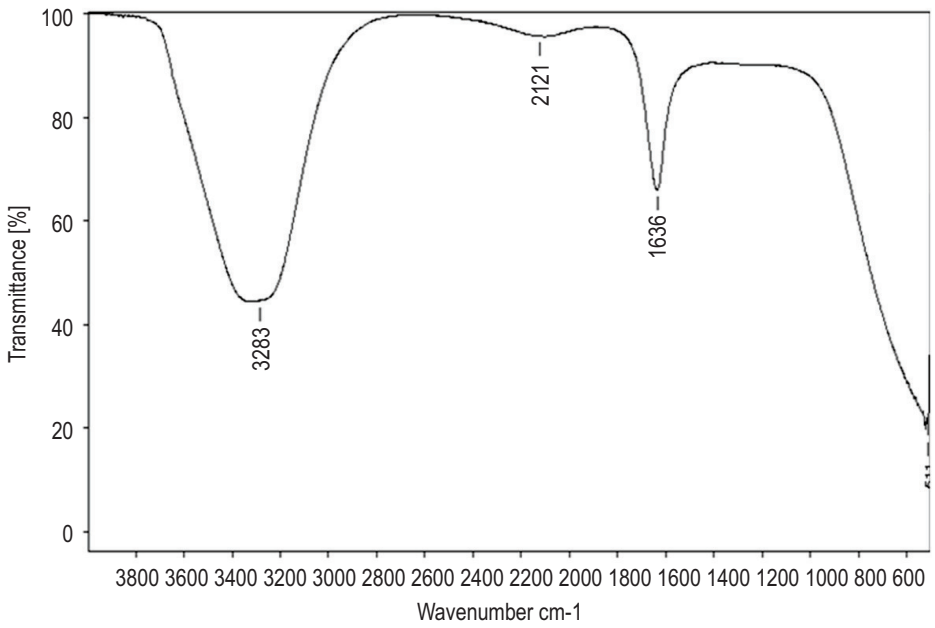


Fig. 3 : FTIR spectrum of silver nanoparticles produced by using *Aspergillus niger* isolates (A-25)

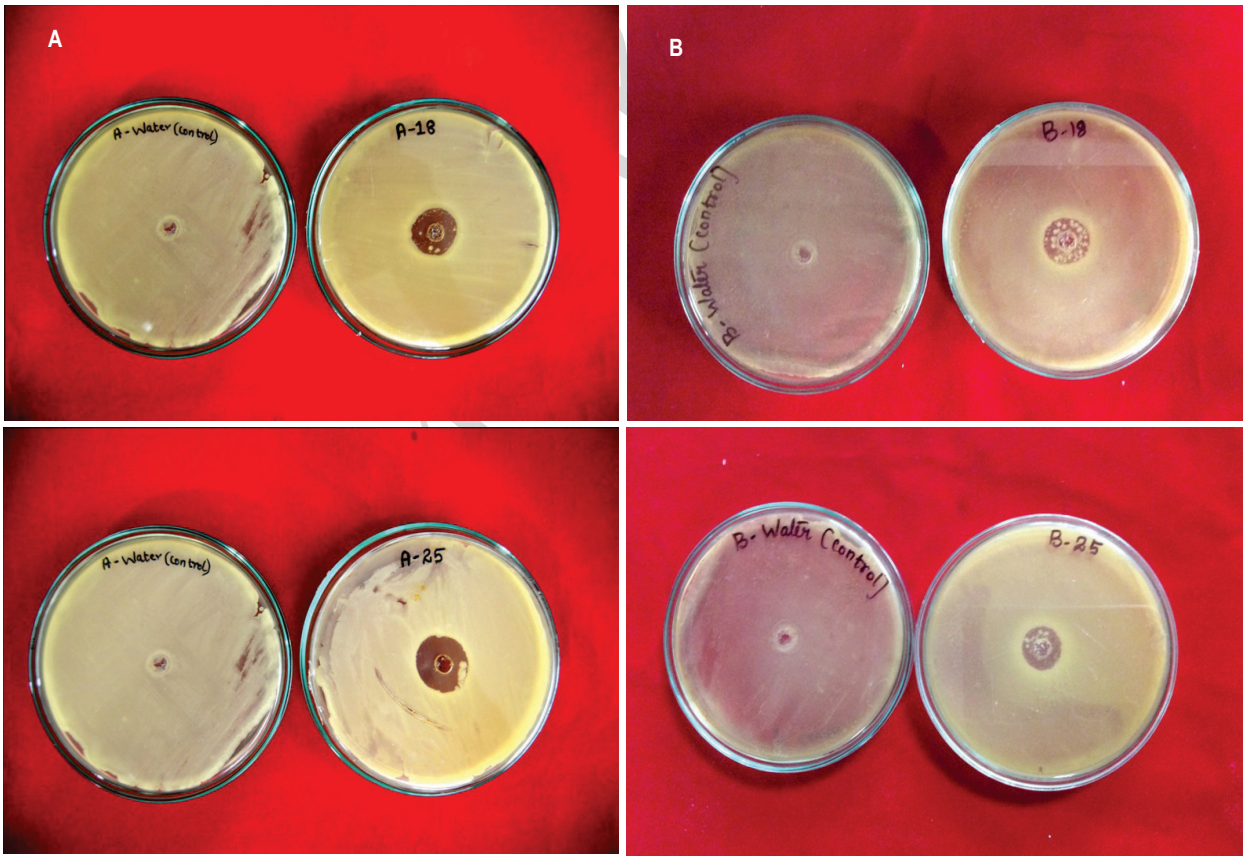


Fig. 4 : Zone of inhibition in the plates treated with silver nanoparticles of *A. niger* against (A): *Xanthomonas citri*; (B): *Ralstonia solanacearum*

(Prameeladevi *et al*., 2013). An extra-cellular biosynthesis of silver nanoparticles by *Aspergillus niger* was proved in this study through the change in the colour of the mixer of silver ion and culture filtrate solution from colourless to brown. In the current study, 120 hrs time was taken for complete transformation of metal ions to silver nanoaprticles, Roweves maximum production was observed after 24 hrs of incubation. Balaji *et al*. (2009) and Shaligram *et al*. (2009) studied size, shape, texture and arrangement of silver nanoparticles through Transmission electron microscope (TEM). The production of mono dispersed silver nanoparticles by using *Fusarium oxysporum* and their stability up to four months were reported (Ahmad *et al*., 2003). The mono-dispersed and spherical nano particles synthesized by *A. niger* were observed in the microphotographs of TEM in our studies. The size ranged from 3-25 nm. FTIR spectroscopic studies established that the carbonyl groups and peptides have strong capability to bind metal ion. The metallic nanoparticles were covered with proteins to avoid clumping in the medium. Recently, it has been reported that the fungal originated molecules maintains stability of silver nanoaparticles (Kamil *et al*., 2017).

The synthesis of SNP using *A. niger* is reported. The nano particles were characterised by UV-Vis, TEM and FTIR analyses. Maximum SNP production was observed in culture filtrate of Isolate A25. Crystalline nature of nanoparticles is evident for bright circular spots of 3–25 nm. It is proved from the study that the nanoparticles produced from *A. niger* can be used for the management of bacterial diseases caused by *Xanthomonas citri* and *Ralastonia solanacearium*.

Acknowledgments

The authors thank the Head, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi for providing the facilities. Authors are also thankful to the Indian Council of Agricultural Research for the financial support.

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