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Biosynthesis of Silver Nanoparticles and Their Biocontrol Potentials Against Aspergillus Niger and Fusarium Udum

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Abstract

Silver nanoparticles can be biosynthesized from bacteria, fungi, and plant extracts but due to their ability to synthesize nanoparticles in varying sizes and shapes at ease, bacterial has drawn interest. Bacterial based biosynthesis is effective, inexpensive, and simple thus, *Pseudomonas fluorescence* cell filtrates were used to synthesize silver nanoparticles in the present study. The chromatic shifts (yellow to brown) in the media after overnight incubation and the absorption of UV-Vis spectra at 420 nm confirmed the biosynthesis of AgNP's. Besides that, the SPR analysis of AgNP's showed a 400–500 nm band width, supporting the formation of silver nanoparticles and their small size with a uniform shape. AgNP's transmission electron microscopy (TEM) images confirmed their shape as quasi spherical, mean size as 30 nm and anisotropy. From the Zeta potential analysis (-42.7 mV at pH = 7 with a single peak), highly repulsive nature of nanoparticles was confirmed. On the other hand, bio-fabricated silver nanoparticles were tested for antifungal activity against *Fusarium udum* and *Aspergillus niger* under *in vitro* conditions. At 150 ppm concentration of AgNP's, *Fusarium udum* and *Aspergillus niger* were inhibited up to 100 and 80.50 %, respectively. In conclusion, synthesis of nanoparticle with aqueous *Pseudomonas fluorescence* extract is simple and environmentally benign.

Introduction

Nanotechnology was introduced by Physicist and Nobel laureate, Professor Richard Feynman in 1959. Synthesis and application of nanomaterials in different fields of investigation is called as Nanotechnology (Duran et al. 2005). Nanoparticles act as fundamental building blocks for nanotechnology. Self-assembly of atoms or molecules at nanoscale was first reported by Drexler, (1986) since then, rapid and extensive research has happened across the globe. Designing, synthesis and working with the molecular systems below 100 nm is popularly communicated as nanotechnology. Nanotechnology is a rapidly growing field with interdisciplinary applications especially in biology. Owing to their applicability, demand is increasing at an overwhelming rate concurrently manifested production is also recorded. Nanoparticles are synthesized from silver, gold, platinum, and palladium (noble metals) among these silver nanoparticles are most explored (Roy and Barik, 2010). Conventionally, nanomaterials are synthesized using either chemical or physical methods which include sol process, micelle, chemical precipitation, hydrothermal method, pyrolysis, and chemical vapour deposition. Often, these chemical and physical methods of preparing nanoparticles requires high-end toxic chemicals and consumes huge quantities of energy which are being reflected in their cost and restrict their avenues of applications, biology in particular, due to toxicity.

The future of nanotechnology relies on the development of efficient and eco - friendly nanomaterial synthesis protocols among a variety of over a range of biological compositions, proportions, shapes and elevated monodispersity. Biosynthesis of nanoparticles is an attractive opportunity to advance green nanotechnology, which has the opportunity to attain multiple applications, particularly in biology and agriculture. Bansal et al. (2011) stated that biosynthesis of nanoparticles is a kind of bottom-up method that combines the mechanism of reduction or oxidation. Silver, inhibits broad range of microorganisms

by altering the composition of cell membrane and function (Mc Donnell *et al.*, 1999), and interferes with the production of ATP by altering the expression of associated proteins (Yamanaka et al., 2005). Silver binds with the thiol groups of proteins and deactivate them (Davies and Etris, 1997).

Pulses exposed to several biotic and abiotic stresses because, in India they were poorly managed. Diseases like collar rot (*Aspergillus niger*) and wilt (*Fusarium udum*) reduces the yields of groundnut and pigeon pea by 50 (Ghewande et al. 2002) and 30–100 % respectively. The disease incidence of *Fusarium* and *Aspergillus* has been increasing year after year and most of the released cultivars became susceptible to these diseases, due to increased virulence in pathogen. However, the efficacy of commercial existed fungicides is low as most of organisms are resistant. Seed dressing with fungicides was found promising (Gangopadhyay et al., 1996) however, development of resistance was a big concern now a days and besides that damage of soil microflora need to be addressed for environmental stability. Therefore, there is a need to develop new fungicide formulations with enhanced efficiency against an array of disease-causing pathogens in agriculture using the emerging technologies like nanotechnology.

Several microorganisms have been utilized to synthesize AgNPs through either intracellular or extracellular (Klaus et al., 1999; Nair and Pradeep, 2002; Mukherjee et al., 2001; and Ahmad et al., 2003) mechanisms. For instance, Ag containing nanocrystals of different compositions were synthesized by *Pseudomonas stutzeri* AG259 bacterium (Klaus et al., 1999). In *Fusarium oxysporum* fungus, the reduction of Ag^{+} ions were attributed to an enzymatic process involving NADH - dependent reductase (Ahmad et al., 2003). *Pseudomonas fluorescens* produces extracellular enzymes and metabolites which are essential for the production of silver nanoparticles.

Material And Methods

Isolation of the pathogens

A total of twenty *Aspergillus niger* and *Fusarium udum* infected plant samples of groundnut and pigeon pea were collected. The collected samples were utilized for the isolation of respective pathogens by tissue segment method. In brief, the infected portions were cut into 1 cm bits with sterile surgical blades and the surfaces were sterilized by dipping in 0.1 percent (%) sodium hypochlorite (NaOCl) for 30 seconds followed by 3 washings in sterile distilled water. These cuttings were placed on the sterilized PDA plates and incubated at 28±2 ^{0}C in the BOD incubator for 5-7 days (Rangaswamy *et al.,* 1999). Pure culture of *Pseudomonas fluorescens* was obtained from the department of Agricultural Microbiology and Bioenergy, Hyderabad, India.

Bio-fabrication of silver nanoparticles from *Pseudomonasfluorescens*

A three-day old *Pseudomonasfluorescens* pure culture was inoculated into 100 ml of King's B Broth and incubated at 28±2 ^{0}C in an orbital shaker (120 rpm). The biomass was harvested after 3 Days of inoculation through sterilized Whatman No.1 filter paper. After the harvesting of biomass, silver nanoparticles were synthesized using the culture filtrate. Approximately, 2 g of culture filtrate of

Pseudomonasfluorescens was treated with the 50 ml of 1 mM $AgNO_3$ aqueous solution (odourless, boiling point 440 °C, molar mass 169.87 g/mol, density 4.35 g/cm^3) for the synthesis of silver nanoparticles. The whole mixture was incubated at room temperature for 72 h, change in media colour from colour less to brown colour confirms the formation of silver nanoparticles through reduction of silver ionic forms Ag^+ to Ag^0 (Adrian *et al.,* 2020).

Ultraviolet-Visible (UV-Vis) spectroscopy analysis of nanoparticles

The reduction of silver (Ag^+) ions in the reaction mixture was monitored using UV-Vis spectrophotometer at 24 h. The Localized Surface Plasmon Resonance (LSPR) of AgNP's in the reaction mixture was recorded using UV–Visible spectrophotometer (Shimadzu, UV2450) between the wavelengths 200 to 800 nm (Shreya *et al.,* 2015, Ojo *et al.,* 2017, Kumar *et al.,* 2017).

Transmission Electron Microscopy (TEM)

Dispersion, shape and size of bio-fabricated silver nanoparticles was analysed by using Transmission Electron Microscopy (JEOL (JEM-1010)), with an accelerating voltage of 80 kV (Adrian *et al.,* 2020). A drop of aqueous AgNP's was dried on the carbon-coated copper TEM grids and kept under vacuum in desiccators before loading them onto a specimen holder. The particle size distribution of silver nanoparticles was evaluated using ImageJ 1.45 software.

Silver nanoparticles characteristics

The aqueous suspension of silver nanoparticles filtered through a 0.22 µm syringe driven filter unit. Later, the filtrate was used for evaluating the size based on the principle of Dynamic Light Scattering (DLS) technique made in a Nanoparticle SZ-100 series compact scattering spectrometer (Mahmoodreza *et al.,* 2019). The surface zeta potentials of nano particles measured by using Laser zeta meter (Malvern zeta seizer 2000, Malvern). In brief, 5 ml of liquid samples (nanoparticles) were diluted with 50 ml of DDW (double distil water) using NaCl as suspending electrolyte solution (2×10^{-2} M NaCl) and the samples were shaken for 30 minutes. After shaking, zeta potential and pH was recorded and of the metallic particles was measured. A zeta potential values were used to determine the surface potential of the silver nanoparticles. In each case, an average of three separate measurements was reported and the stability of NPs were measured when the values of zeta potential ranged from higher than +30 mV to lower than -30 mV (Akman *et al.,* 2011).

Assessment of silver nanoparticles biocontrol potential against *Aspergillus niger* and *Fusarium udum*

To produce a final concentration of 10, 30, 50, 100 and 150 ppm of AgNP's and $AgNO_3$, 40 ml of modified PDA medium (Potatoes-400 g, Dextrose-40 g, Agar-40 g, distilled water-1000 ml) was combined with AgNP's (dried culture filtrate) and $AgNO_3$ solutions respectively. Approximately, 20 ml of this medium was poured in 90 mm diameter petri plate, control was maintained without AgNP's and $AgNO_3$ respectively. Mycelial discs (7 mm) of 24 h old pathogens were inoculated at the centre of the plates and incubated at

28±2 ^{o}C until full growth was observed in control. In each treatment, five replications were maintained. The percent inhibition of radial growth of the test pathogens *Aspergillus niger* and *Fusarium udum* were calculated by using the following formula.

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Percent reduction in growth of *A. niger* and *F. udum*

C = Radial growth (mm) in control

T = Radial growth (mm) in treatments

Results And Discussion

Isolation of pathogens

Aspergillus niger (collar rot) affected seeds with blackish testa and rotted internal tissue and also mature plants with wilting and rotting symptoms at just below the ground level were collected. The affected portion turned dark, shrunken and shredded, and later covered by black spores of the pathogen. The pathogen was identified as *Aspergillus niger* based on microscopic examination (Figure 1). The fungus displayed upright and tiny conidiophores, finishing with globose swelling, holding phialides radiating from the entire surface. The conidia were found to be single celled, light to dark brown in colour, globose shaped and produced basipetally (Figure 1a and b). Mycelium was hyaline branched and septate.

The wilt (*Fusarium udum*) pathogen isolated from the infected plant roots of pigeon pea had slender hyaline hyphae with abundant branching, typically with small aerial growth (Figure 2). Chlamydospores were globose and smooth walled. Macroconidia were straight to falcate, thin walled, septate but predominantly 3-septate. Microconidia fusiform to reniform, septate (Figure 2a and b), formation of micro and macroconidia was observed under compound microscope.

Silver nanoparticles biosynthesis and specifications

The majority of metal nanoparticles have optical properties that are proportional to the size and form. The bio-fabrication of silver nanoparticles (AgNPs) has been verified by means of a notable transition in media colour from yellow to brown after 24 hours of incubation (Figure 3), signalling the biotransformation of Ag^{+} to Ag^{0} by reductive enzymes (Ahmad *et al.,* 2003; Saifuddin *et al.,* 2009). However, we point out that in the present research, over colour production was observed, which may be attributed to the difference in the existence of the organism and the size and shape of metal particles. In

addition, the variation in relative behaviour in the reduction of silver nitrate ions to mental nanoparticles due to the existence of the proteins formed may be also be the explanation for the shift in colour. Surface plasmon vibration gives silver nanoparticles a peculiar brownish yellow colour. Light evokes the free electrons in silver nanoparticles as observed under UV-Vis spectroscopy, and transmits to a higher energy level, but the electron is unstable in an excited state and returns to the level of base energy and a photon is emitted simultaneously (Thangaraju *et al.,* 2012). Correspondingly, the spectrum of silver nanoparticles demonstrated highest surface plasmon resonance at 420 nm. The existane of silver nanoparticles in an aqueous solution is usually verified by sharp SRP's in the 350-600 nm range (Sastry *et al.,* 1998; Henglein, 1993). Figure 4 reveals that the SPR is in the 400-500 nm band, suggesting that bio-fabricated silver nanoparticles are smaller size and identical in form. As per Mie theory, only a single SPR band is expected in the absorption spectra of spherical nanoparticles whereas, the number of peaks increases as anisotropy increases (Raut Rajesh *et al.,* 2009). We also recorded a single peak in the present study that indicates the silver nanoparticles are spherical in nature. This finding was the primary confirmation of size, form and distribution, further verified by TEM analysis.

The size and form of individual bio-fabricated silver nanoparticles is demonstrated by TEM micrographs with good visibility of lattice space (Figure 5). With an average size of 30 nm, they are quasi spherical in form and anisotropic in nature. Moreover, without major agglomeration and morphological variation, they are all well scattered. These observations are in accordance with the finding of the small sized nanoparticles of distinct shapes found by Abdel-Raouf *et al.,* (2018). The particles are strongly monodispersed, in good alignment with the high zeta potential reported (- 42.7mV).

The particle size distribution of bio-fabricated silver nanoparticles has been evaluated with Dynamic Light Scattering (DLS). Based on the results, the mean size of bio-fabricated silver nanoparticles was approximately 66.0 nm (Figure 6) and the range of nanoparticles was 32 to 115 nm, based on the findings. As predicted, due to the variations in measurements by the devices, the DLS calculated value is marginally larger than TEM calculated value *i.e.,* TEM calculates the number based on size distribution of the physical dimension without capping agent, while the DLS measures the hydrodynamic diameter that is the particle diameter as well as the ions or molecules that are connected and travel along with them in silver nanoparticles in solution. Hence, DLS measurements are always greater than the TEM analysis (Huang *et al.,* 2007).

To obtain additional insights into the stability of the bio-fabricated silver nanoparticles, zeta potential analysis will typically be performed. The magnitude of zeta potential provides a hint a hint of the potential stability of the colloid, considering stable particles of more than +30 mV or more than -30 mV (Melendrez *et al.,* 2010). In this respect, the zeta potential value of bio-fabricated silver nanoparticles was -42.7 mV at pH=7 with a single peak (Figure 7), whereas, the relative high zeta potential suggests that particles are highly scattered due to heavy repulsion between synthesized nanoparticles. If every hydrosol has a strong negative or positive zeta potential, colloidal particles will appear to repel each other and the particles will not tend to agglomerate. It is thus shown that, because of its high negative charge, AGNP's are stable in nature.

Antifungal potentials of silver nanoparticles

Silver nanoparticles have recently received considerable interest in the field of phytopathogenic fungi control due to the increased resistance to fungicides and antibiotics. The capacity of silver nanoparticles to regulate phytopathogens must be investigated thoroughly. The bio-fabricated silver in the current research have demonstrated excellent *in vitro* inhibition of phytopathogenic fungi. The suspension of silver nanoparticles and $AgNO_3$ has been used to investigate the antifungal activity against *Aspergillus niger* and *Fusarium udum* on PDA. The effect of silver nanoparticles was compared with that of silver nitrate at concentrations of ($AgNO_3$) at 10, 30, 50, 100 and 150 ppm respectively. With increased concentration, the percent inhibition was improved and highest was recorded at 150 ppm. The percent inhibition of *Aspergillus niger* by silver nanoparticles is in the range of 45 to 80.55 % while, $AgNO_3$ supressed the pathogen 55.5 to 87.19 percent range. The percent inhibition of *Aspergillus niger* and *Fusarium udum* was registered as 87.19 and 74.8 percent, respectively, at 150 ppm of $AgNO_3$ concentration (Figure 8 and 9). Whereas the percent inhibition of *Aspergillus niger* and *Fusarium udum* was reported as 80.50 and 100 percent, respectively, at 150 ppm of AgNP's concentration. As compared with silver nanoparticles, *Aspergillus niger* was greatly inhibited by $AgNO_3$. The antifungal activity of silver nanoparticles varies with the type of fungus and the size of silver nanoparticles that are closely associated with the pit formation in fungal cell wall (Shafaghat, 2015). However, against *Fusarium udum*, silver nanoparticles demonstrated superior antifungal efficacy. Interestingly, the same concentration (150 ppm) of silver nitrate and bio-fabricated silver nanoparticles displayed superior antifungal activity against various species, including *Aspergillus niger*, and *Fusarium udum*. In general, important inhibitory action against *Aspergillus niger* and *Fusarium udum* was induced by the silver nanoparticles was studied. By selectively invading the cell membranes, silver nanoparticles interrupt the membrane potential of fungus (Kim *et al.,* 2009a), and conidial germination (Lamsal *et al.,* 2011).

Conclusions

Silver nanoparticles biosynthesis was accomplished using culture filtrates of *P. fluorescence* and aqueous solution of 1 mM $AgNO_3$. Later, using UV– Vis spectroscopy, they were characterized and validated by SPR and TEM study. It was observed that the nanoparticles produced by the isolate had an average size of 30 nm. The study of transmission electron microscopy (TEM) indicated a strong dispersion among the biosynthesized silver nanoparticles. An *in vitro* analysis of silver nanoparticles revealed a clear antifungal effect against *Fusarium udum* and *Aspergillus niger*, and the diameter of inhibition zone differed depending on the fungal species. The findings recommend that silver nanoparticles from *P. fluorescence* culture filtrate can be used as biopesticide agents.

Statistical analysis

Experiments were conducted in Completely Randomized Design (CRD). Data was analysed following the statistical methods outlined by Gomez and Gomez (1984).

Declarations

Conflict of interest

The authors don't have any conflict of interest with any of the organizations or persons.

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