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Extracellular biosynthesis of silver nanoparticles using *Streptomyces griseoplanus* SAI-25 and its antifungal activity against *Macrophomina phaseolina*, the charcoal rot pathogen of sorghum

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ABSTRACT

Streptomyces griseoplanus SAI-25 isolated from rice rhizospheric soils with previously demonstrated insecticidal activity is currently characterized for silver nanoparticle synthesis using its extracellular extract. The synthesized particles showed the characteristic absorption spectra of silver nanoparticles at 413–417 nm. Spectral analysis by FTIR confirmed the presence of alcohols, amines, phenols and protein in the cell-free extracellular extract of SAI-25. These functional groups could have served dual roles in silver nanoparticle synthesis like reducing and stabilizing agents. Microscopic and spectroscopic analysis such as SEM, TEM, EDAX and XRD has provided the size, shape and composition of the synthesized nanoparticles. DLS and Zeta potential further confirms the size and characteristic negative charges of AgNPs respectively. The observed antifungal activity against charcoal rot pathogen *Macrophomina phaseolina* shows a base for the development of *Streptomyces* mediated nanoparticles in controlling this polyphagus pathogen and key role of biopesticides in improving agricultural economy.

1. Introduction

The metal nanoparticles are unique in their optical, chemical, electronic and photoelectrochemical properties and hence attractive from the past several decades (Cao, 2004). Such nanoparticles/materials involve the metals such as gold (Au), silver (Ag), copper (Cu), palladium (Pd), platinum (Pt) and rhodium (Rh). Among them, the noble metal Ag, have strong surface plasmon resonance oscillations and the nanosystems developed with Ag have higher sensitivity; hence it has wide range of applications in sensors, micro-electronics, biomolecular detection and diagnosis, catalysis, and filters (Rai et al., 2009; Abbasi et al., 2014; Gupta et al., 2017). In addition, they are highly considered in biomedical fields for their biocidal activity against a range of microbes as it has lower cytotoxicity on eukaryotic cells (Durán et al., 2016; Ouay and Stellacci, 2015).

The Ag nanoparticles (AgNPs) can be synthesized by various physical and chemical routes, but in recent years, emphasize is given for the green synthesis of AgNPs which includes the use of plant extracts, and microbial sources like bacteria, fungi, algae and yeast. This is to avoid the issues related to yield, use of toxic chemicals and hazardous by-products in contrary to their physical and chemically synthesized counterparts (Abdelghany et al., 2017). Among the biological entities, microbes are of great importance. In general, microbes have defense system involving the reduction of toxic metal ions (Modak and Fox, 1973) into inert metal particles which aids for their presence in versatile microenvironment and this phenomenon is employed for nanomaterial synthesis. It is comparative to chemical synthesis, as it also follows the bottom-up approach for the development of NPs and reduces the use of a series of reducing and stabilizing agents. Researchers across the country have developed many protocols for microbial mediated nanoparticle synthesis using biomass, supernatant, and derived components of various microorganisms. Among them extracellular synthesis has become a key method, as it eliminates the downstream process like sonication and centrifugation which is required for the recovery of nanoparticles obtained from intracellular methodologies (Singh et al., 2016).

The initial studies related to microbe mediated NPs synthesis begin with Ag and bacteria *Pseudomonas stutzeri* AG259 isolated from silver mine by observing a single crystalline Ag-based particle of well-defined compositions and shapes (Klaus et al., 1999). This was followed by the exploration of many other bacterial genera such as *Bacillus*, *Escherichia*, *Enterobacter*, *Klebsiella* and *Lactobacillus* (Gomaa, 2016; Kalimuthu et al., 2008; Saifuddin et al., 2009; Shahverdi et al., 2007; Shivaji et al., 2011). Fungi, another major microbial domain also evidenced for their ability in NPs synthesis (Amerasan et al., 2016; Kathiresan et al., 2009; Kowshik et al., 2003). Their characteristic feature on large quantities of

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enzymes secretions serves as key factor and has been demonstrated in *Aspergillus*, *Fusarium*, *Penicillium* and *Metarhizium*. NPs synthesis by yeast like *Saccharomyces cerevisiae* (Korbekandi et al., 2016) and algae like *Caulerpa racemosa* (Edison et al., 2016) is also available.

The microbial domain actinobacteria, known to secrete higher amounts of enzymes, proteins, small molecules and secondary metabolites with reducing properties; and thereby it significantly enhances the biosynthesis of metal nanoparticles (Tsibakhashvili et al., 2011). Representative reports for AgNPs synthesis are available on *Streptomyces hygroscopicus* from Pacific shore region (Sadhasivam et al., 2010), *Streptomyces glaucus* 71 MD from a soy rhizosphere (Tsibakhashvili et al., 2011), *Streptomyces* sp. BDUKAS10 from mangrove sediments (Sivalingam et al., 2012), *Nocardiopsis* sp. MBRC-1 from marine sediments of South Korea (Manivasagan et al., 2013), *Streptomyces* sp. LK3 from marine sediments (Karthik et al., 2014), *Streptomyces* sp. 09 PBT 005 from sugarcane rhizosphere soil (Saravanakumar et al., 2014) and *Streptomyces atrovirens* from marine sediments (Subbaiya et al., 2017). *Streptomyces* are still being explored from broad range of environments from farm soil (Baygar and Ugur, 2017; Gupta et al., 2017) to amazon rainforest (Silva-Vinhote et al., 2017) and acid forest (Składanowski et al., 2017), for silver nanoparticles synthesis and also explored for broad range of applications including antipathogenicity, antioxidants and sensors.

Even though the actinobacteria have stability and significant biocidal activities, the extent of its exploration mainly focused on marine environments and also comparatively lesser than other microbial domains, (Manivasagan et al., 2016).

Macrophomina phaseolina, a polyphagous fungal pathogen causing charcoal rot, dry root rot, damping off, leaf blight, stem blight and wilt in various economically important crops (Kumari et al., 2012). Since, the pathogen is seed and soil borne, use of biofertilizers offers alternative management to chemicals inputs and reduces the associated negative impacts *M. phaseolina* causes significant yield losses up to 64% in India under conditions favoring the incidence of the disease in post-rainy sorghum occupying more than 5 million ha in Maharashtra, Karnataka and Andhra Pradesh (Das et al., 2008). To the best of our knowledge, there are no reports for actinobacteria mediated NPs for the control of *M. phaseolina*. In view of above, the present study has been designed to synthesize and characterize actinobacteria mediated AgNPs synthesis through *Streptomyces griseoplanus* SAI-25, a rhizospheric isolate of rice field. It was previously characterized and proved for its insecticidal activity against lepidopteran insects such as *Helicoverpa armigera*, *Spodoptera litura* and *Chilo partellus* (Vijayabharathi et al., 2014). An insecticidal metabolite belongs to diketopiperazine class called cyclo(Trp-Phe) has also been identified (Sathya et al., 2016) from *S. griseoplanus* SAI-25. Therefore, the aim of the present study is to synthesize and characterize AgNPs from SAI-25, and to evaluate its antifungal activity against the charcoal rot pathogen.

2. Materials and methods

2.1. Microbial synthesis of AgNPs

The strain *S. griseoplanus* SAI-25 was isolated from the rhizospheric soil of rice field at, Karnataka, India. The partial 16S rRNA gene sequence was deposited in GenBank (Accession No: KF770901). The particles were synthesized according to Sadhasivam et al. (2010) with some modifications. The strain of SAI-25 was freshly cultured on ISP-2 (International *Streptomycetes* Project) broth with Yeast extract (4.0 g/L), Malt extract (10 g/L) and Dextrose (4 g/L) (Yang et al., 2012) and incubated at 28 °C, 200 rpm for 96 h. At the end of the incubation period, the culture was centrifuged at 10,000 rpm for 15 min and the supernatant was collected. The supernatant was filtered using 0.2 μm filter and the cell free extract obtained was used for the synthesis of AgNPs. Nanopure water was used for the experiments to avoid interferences. Cell-free extract (10%) of SAI-25 was added to 1 mM $AgNO_3$ (Sigma, USA) solution and incubated at 28 °C, 200 rpm for 96 h under dark conditions.

2.2. Characterization of AgNPs

2.2.1. UV–visible spectra analysis

Aliquots of the reaction solution were removed at regular intervals and the formation of AgNPs was monitored by UV–visible spectroscopy (UV-1800, Shimadzu, Kyoto, Japan) by recording the absorption spectra at 300–800 nm. Monitoring was done for 96 h at the regular intervals of 12 h. 1 mM $AgNO_3$ and 10% of SAI-25 cell-free extract were used as controls. The synthesized NPs were collected by centrifugation, washed with nano-pure water, freeze dried and used for further studies.

2.2.2. Fourier transform infrared (FTIR) spectra analysis

The samples including $AgNO_3$, AgNPs and freeze-dried cell-free culture extract of SAI-25 were maintained in a vacuum desiccator over KOH pellets for 48 h and mixed with KBr pellets. The spectrum was recorded on FT/IR-420 (Jasco, USA) over a range of 4000–400 cm^{-1}.

2.2.3. X-ray diffraction analysis

XRD analysis was done for AgNPs using in a continuous scanning 2θ mode and the data were collected with a Bruker AXS D8 ADVANCE x-ray diffractometer using Cu Kα (λ = 1.54 Å) radiation. The peaks obtained were assigned and compared with the database published by the Joint Committee on Power Diffraction Standards (JCPDS).

2.2.4. Electron microscopic analysis

The shape and elemental composition of freeze-dried AgNPs powder was analyzed through high resolution scanning electron microscope (HR-SEM, FEI Quanta FEG 200, FEI Company, Eindhoven, The Netherlands) coupled with energy dispersive analysis of X-ray (EDAX). Surface morphology of the AgNPs was analyzed by high resolution transmission electron microscope (HR-TEM, JEOL 3010, JOEL, Tokyo, Japan) operated at an accelerating voltage of 300 keV. A drop of the reaction mixture was casted on carbon-coated copper grids and allowed to dry prior under ambient conditions for measurement.

2.2.5. Dynamic light scattering and zeta potential

Hydrodynamic size distribution and surface charge of synthesized AgNPs are measured using dynamic light scattering (DLS) and zeta potential measurements. To perform DLS, AgNPs solution is further diluted and allowed for sonication 10–20 min to disperse the particles. Size distribution measurements and zeta values are obtained using the Malvern Nano-ZS 90 analyzer. A volume of 3 ml of colloidal AgNP solution is placed into a specific cuvette. The refractive index, absorption coefficient, viscosity of the solvent and temperature (20 °C) is provided by the software. For each sample, the autocorrelation function is the average of five runs of 10 s each.

2.3. Antifungal activity of AgNPs

The biosynthesized AgNPs were examined against *M. phaseolina* by agar well diffusion method as per Medda et al. (2015) with slight modifications. *M. phaseolina* was acquired from the Directorate of Sorghum Research, Hyderabad, India. Potato dextrose agar (PDA) plates were prepared and fungus was sub-cultured freshly. Each PDA plate was punctured to create 5 wells of 8.0 mm size using gel puncture. Increasing concentrations of AgNPs solution (250, 500 and 1000 μg/ml) loaded on to the wells. 1 mM $AgNO_3$ and sterile distilled water were used as controls. Each well was loaded with 80 μl of the respective samples. A disc of 6 mm fungal inoculum from the 7 day old *M. phaseolina* culture is placed on the centre of the plate and incubated at room temperature for 24–72 h h and the zone of inhibition was measured.

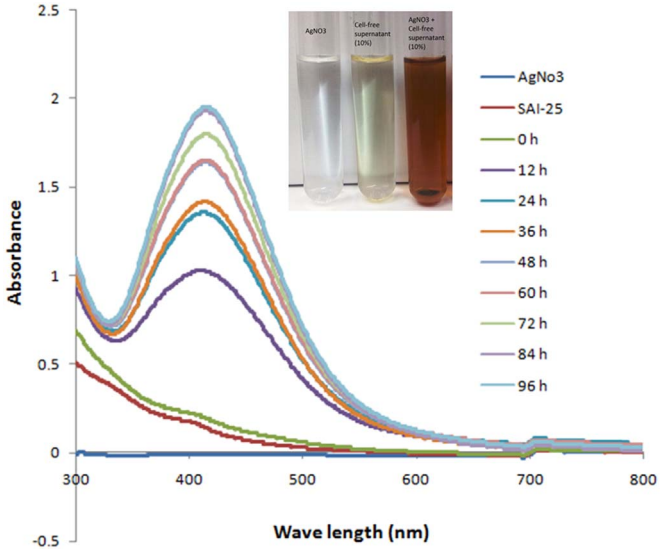

Fig. 1. UV–visible spectra of silver nitrate (1 mM), extracellular extract of *S. griseoplanus* SAI-25 and their reaction in forming AgNPs at different time intervals (h).

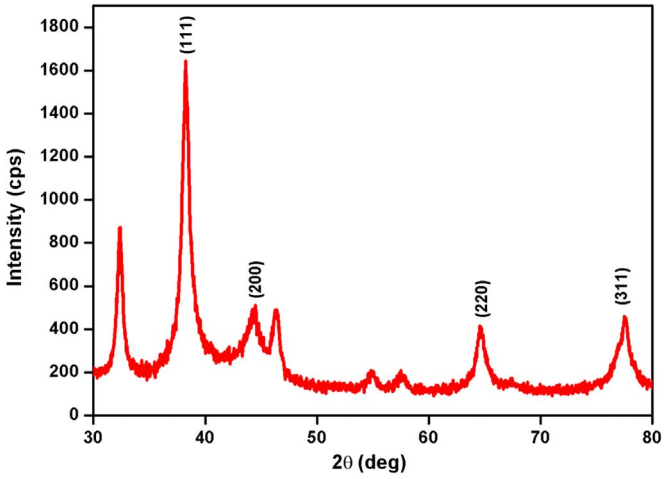

Fig. 3. XRD diffraction pattern of synthesized AgNPs.

3. Results and discussion

3.1. Characterization of AgNPs by UV–visible spectroscopy

The formation of AgNPs using *Streptomyces* sp. SAI-25 cell-free filtrate was monitored at 12 h regular interval for a period of 96 h by both visual examination and UV–visible spectrophotometry. Color change of the reaction mixture from colorless to yellowish brown indicates the formation of AgNPs (Fig. 1, inset). Absence of color change in controls such as $AgNO_3$ and cell-free filtrate solution was also noticed. Absorption spectrum shown in Fig. 1 further supports the visual examination in which none of the nanoparticles are formed at 0 h for any of the reaction mixtures tested including silver nitrate, cell-free culture filtrate and silver nitrate treated with cell-free culture filtrate; later a characteristic absorption spectra of AgNPs observed as a strong and broad band between 413 and 417 nm at 12 h on silver nitrated treated

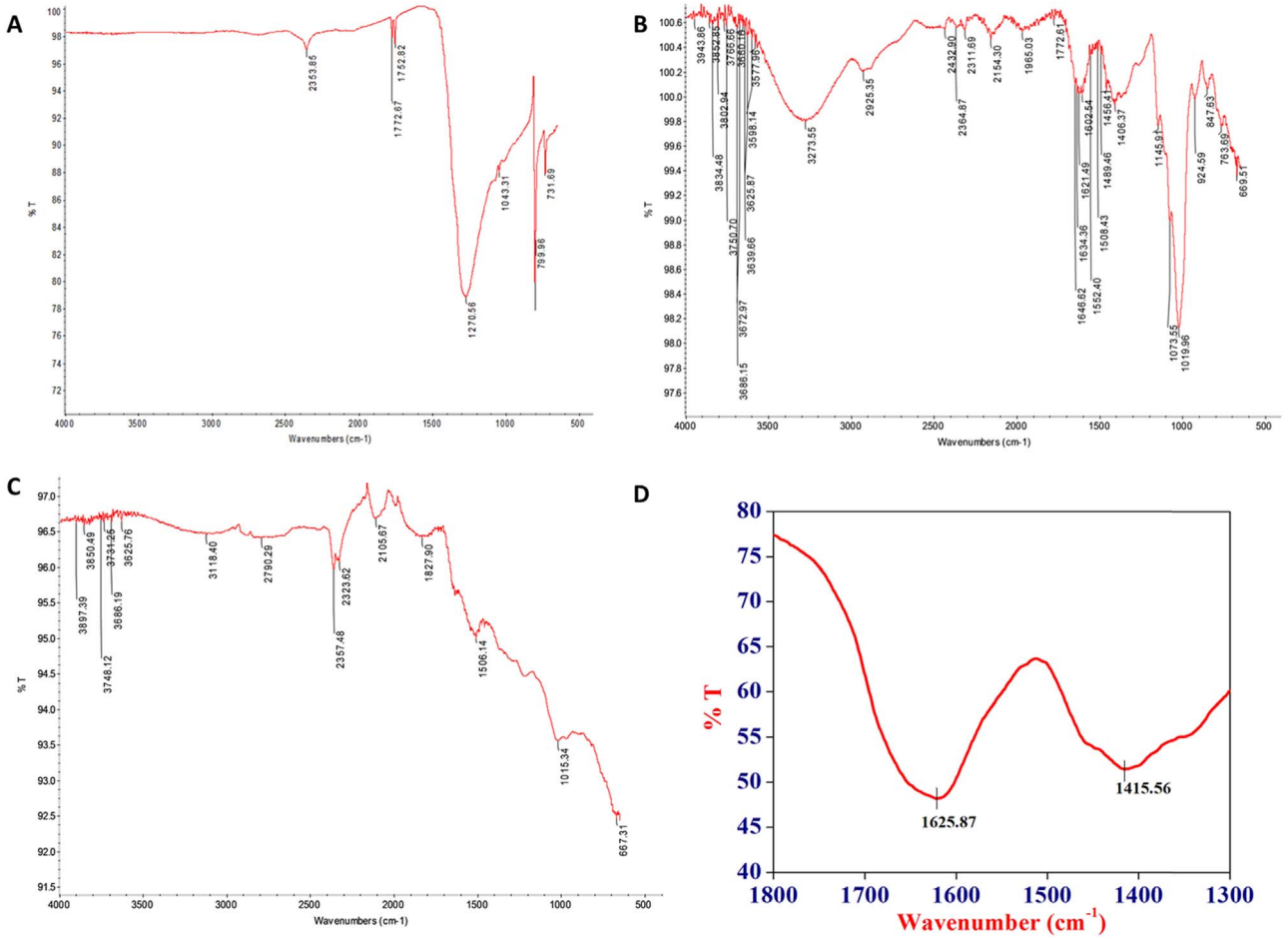

Fig. 2. FTIR spectra of (A) silver nitrate, (B) Cell-free supernatant of *S. griseoplanus* SAI-25, (C) synthesized AgNPs and (D) protein FTIR of AgNPs.

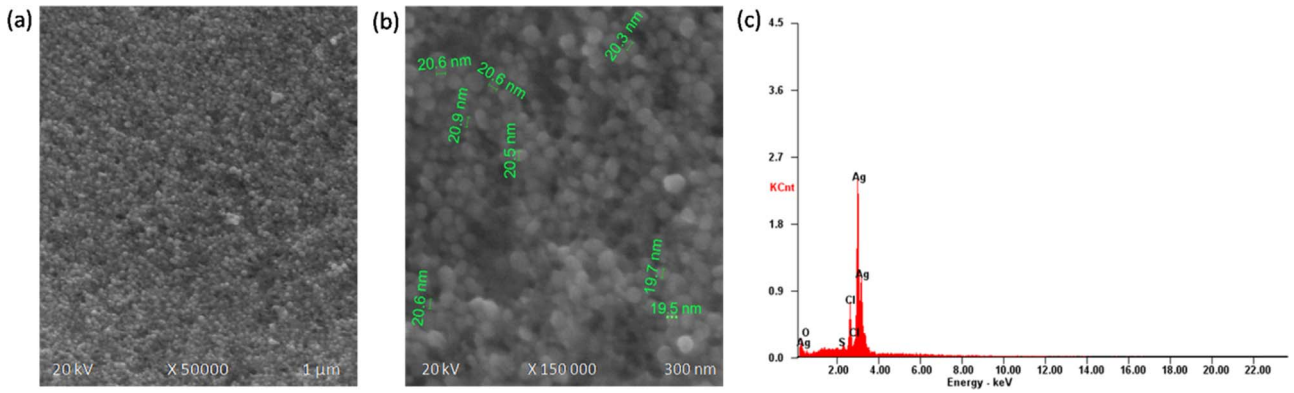

Fig. 4. HR-SEM and EDAX spectrum of synthesized AgNPs.

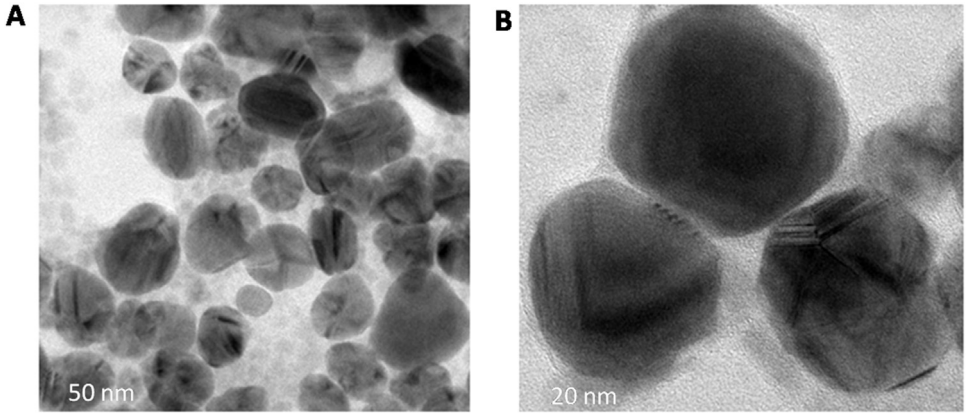

Fig. 5. HR-TEM of synthesized AgNPs.

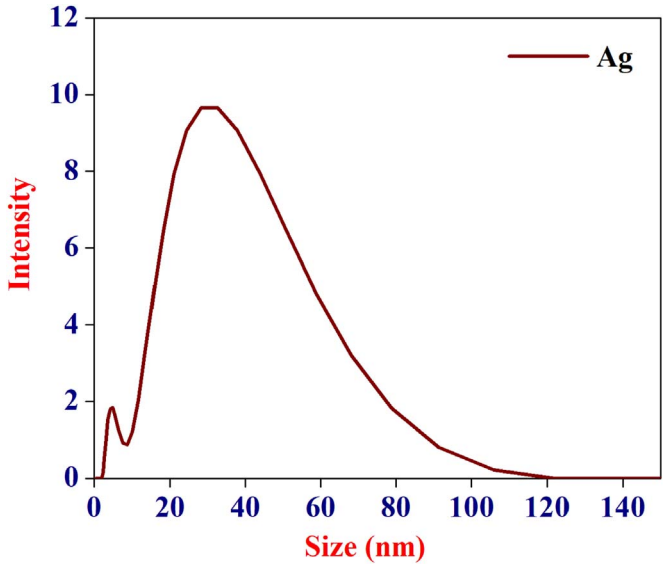

Fig. 6. DLS of synthesized AgNPs.

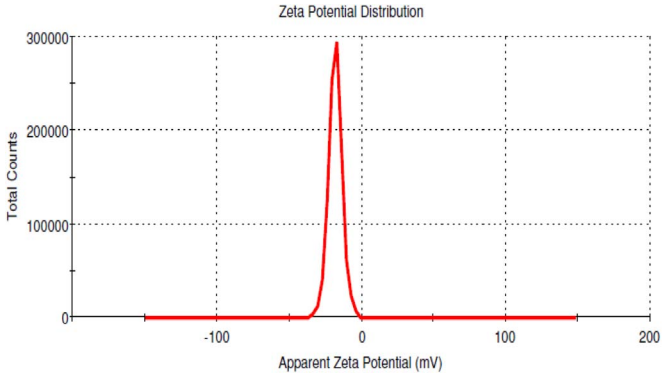

Fig. 7. Zeta potential of synthesized AgNPs.

with cell-free culture filtrate. This may have been due to the excitation of surface plasmon resonance and the reduction of $AgNO_3$ by the secondary metabolites present in the cell-free filtrate. In addition, a dramatic increase on intensity of the brown color noticed till 96 h indicates the lack of aggregation. This might be by the dual role of microbial secondary metabolites as reducing and capping agents. It is observed that, time required for the completion of nanoparticle synthesis using both bacteria (Klaus et al., 1999) and fungi (Mukherjee et al., 2002) ranges between 24 and 120 h, whereas actinomycetes can achieve in 24 h of incubation period (Sadhasivam et al., 2010) and this correlates with our study.

3.2. Characterization of AgNPs by FTIR

FTIR analysis of the silver nitrate, freeze-dried cell-free filtrate and AgNPs were shown in Fig. 2A–C. Cell free filtrate of SAI-25 showed characteristic amine stretching and bending vibrations at wavelengths 3273, 2925 and 1646, 1552 cm^{-1} correspondingly. Peaks at 763 represent presence of aromatic C-H bend vibrations and aromatic C=C bending vibrations can be seen at different peaks between 1700 and 1500. Carboxylic stretch of O-H can be observed at wave length of 2925. Peaks at 1019 and 1073 confirm the presence of C-N and C-O groups and a broad peak between 3550 and 3200 shows the presence of alcohol/phenol O-H stretch vibrations. This data of pure filtrate put together gives a confirmation of presence of amines, carboxylic acids

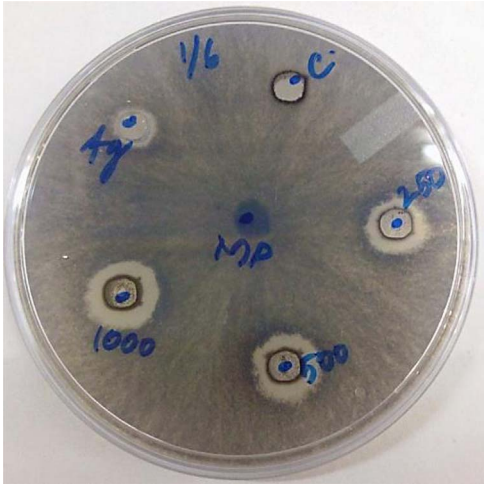

Fig. 8. Antifungal activity of AgNPs on *M. phaseolina*.

and aromatic phenols/alcohols which cumulatively show the presence of proteins/ sugars in filtrate. From the FTIR data of nanoparticle stabilized with filtrate we can assume that the nanoparticles were possibly stabilized by the interaction of amine and alcohol/phenol groups. Proteins present in the filtrate played a major role in stabilizing silver nanoparticle which was evident from condensed peak in the amine region and the characteristic bands found in the infrared spectra of proteins and polypeptides include the amide I (1600–1700 cm^{-1}) and amide II (1400–1500 cm^{-1}) (Fig. 2D). These arise from the amide bonds that link the amino acids. This can be supported by Shaligram et al. (2009) and Vigneshwaran et al. (2007).

3.3. Characterization of AgNPs by XRD

The characteristic XRD pattern of the synthesized AgNPs is shown in Fig. 3. The presence of peaks at 2θ values 38.2°, 44.53°, 64.59° and 77.6°, corresponds to (111), (200), (220) and (311) planes of silver, respectively. There were no additional peaks for impurities have been seen. Hence, the crystalline structure of silver nanoparticles is confirmed by this XRD spectrum. Indexing of the obtained XRD peaks to a face-centered cubic structure of silver from the available literature (JCPDS, File No. 4-0783) further supports the silver nanoparticles.

3.4. Characterization of AgNPs by microscopy

Topology and size of the synthesized AgNPs was analyzed by SEM and shown in Fig. 4. From the images, it is concluded that the SAI-25 mediated AgNPs are relatively spherical with diameter of 19.5–20.9 nm. EDAX spectrum of AgNPs at 3 keV with strong signal for Ag (Fig. 4c) confirmed that Ag is the major constituent. Absence of N indicates the reduction of $AgNO_3$ to AgNPs. Some minor peaks were observed for Cl, O and S. The peaks on O and S might be due to X-ray emissions of proteins/sugars/other microbial metabolites in the culture filtrate. It is a known phenomenon that, free amino groups or cysteine residues of proteins can bind to nanoparticles (Stroscio and Dutta, 2002). Similar observations were observed for other microbial mediated AgNPs (Sivalingam et al., 2012). The observed features on topology and size of the AgNPs by SEM were reflected by HR-TEM analysis as shown in Fig. 5.

3.5. Characterization of AgNPs by DLS and zeta potential

The particle size distribution measurement for AgNPs is displayed in Fig. 6 which shows the average particle size of 30 nm. The comparison of TEM and DLS measurements showed that the average particle sizes calculated from TEM were smaller than that obtained by DLS studies. This deviation in particle size measurements is ascribed to the fact that the particle size obtained by DLS measurements usually represents the hydrodynamic diameter of a sphere (i.e., diameter of particle with hydration shell), having the same volume as the particle and the added solvent or stabilizer moving with the particle (Vujačić et al., 2013). AgNPs exhibited a zeta potential value of −18.0 mV (Fig. 7). The zeta potential value suggested that Ag NPs surface is negatively charged.

3.6. Antifungal activity of AgNPs

In recent years, efforts have been given to safer pest management techniques for food and ornamental crops that pose less impact on humans, animal and environments. NPs synthesized through green approaches are one such category. In this study, *M. phaseolina* was inhibited by the synthesized AgNPs with the zone of inhibition of 13 mm at 1000 μg/ml (Fig. 8). The NPs may perturb the lipid bilayer and disturbs the membrane permeability and electrical potential which leads to leakage of ions and other molecules resulting in cell death (Kim et al., 2009). Similarly, microalgae *Chlorella vulgaris* based Ag chloride NPs showed inhibitory activity against bacterial pathogens such as *Staphylococcus aureus* and *Klebsiella pneumonia* (da Silva Ferreira et al., 2017). It is observed that *Streptomyces* mediated AgNPs can able to inhibit the phytopathogens such as *Aspergillus niger, Aspergillus flavus* and *Aspergillus fumigatus* (Thenmozhi et al., 2013). Recent studies by Mitra et al. (2017) documented AgNPs mediated inhibition of aflatoxin synthesis in *Aspergillus parasiticus* which further substantiates the role of AgNPs in agriculture.

4. Conclusions

In the present study, the AgNPs were synthesized by extracellular extract of *Streptomyces griseoplanus* SAI-25. Spectroscopic and microscopic characterization studies using FT-IR, XRD, SEM and TEM show its functional groups and the morphological properties such as size and shape. Small molecules of extracellular extract served as reducing and capping agents in synthesizing AgNPs. The antifungal activity observed on *M. phaseolina* serves as a preliminary efficacy data and can be further tested and explored as alternative pest management tools. Further characterization on pesticidal activities helps in exploring its utility in sustainable agriculture.

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Conflict of interest

The authors declare that, there is no conflict of interest.

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