Fungus (*Alternaria sp.*) Mediated Silver Nanoparticles Synthesis, Characterization and Application as Phyto-Pathogens Growth Inhibitor

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Research

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Abstract

Background: Biogenic nanoparticles have proved to be effective biocontrol agents for certain plant diseases. It possesses the potential for extensive use for sustainable agriculture. Many attempts have been made to synthesize nano-based antifungal compounds for the management of soil borne pathogenic fungi for crops.

Results: In our work, silver nanoparticles (AgNPs) was constructed with phytopathogenic fungi (*Alternaria* sp.) which was isolated from banana cultivated soil. *Alternaria* sp. was able to grow rapidly and produce highly bioactive compounds as safe antifungal agent against plant pathogenic fungi (*Fusarium* spp. and *Alternaria* sp.). The size of synthesized silver nanoparticles ranged between 5-10 nm. Analytic tools, such as UV-visible spectroscopy, Fourier transformed infra-red (FTIR) spectroscopy, scanning transmission electron microscopy (STEM), EDS and elemental mapping were used to visualize the formation of AgNPs. The UV-visible spectra showed the peak at 435 nm. The maximum inhibition zone was observed at 100 µl concentration of AgNPs for Fusarium oxysporum (21 ± 2 mm) following Alternaria sp. (20± 2 mm), suggested that the efficacy of the biosynthesized NPs against the phytopathogenic fungi.

Conclusions: The resulting AgNPs showed distinct antifungal activity against selected pathogenic plant fungi. The work indicates that green reduction and biogenic synthesis of nanoparticles with benign fungi is an effective, low cost, sustainable and environmentally friendly approach for prevention of soil borne plant diseases.

Introduction

Biological control agents should be prepared with less risk to human and livestock (Sastry, Ahmad et al. 2003). Currently the use of biological control agents at commercial level has some limitations such as adaptability to biotic and abiotic factors, deterioration of the desired activity between *in vitro* and *in vivo* (Askary, 2015). It is time to develop more effective and non-persistent biopesticides with the aid of nanotechnology using biogenic silver, zinc, copper, gold and iron (Oluwaseun and Sarin, 2017).

Fungitoxic metabolites (antibiotics and other bioactive metabolites) are seen to contribute to biocontrol applications in agriculture (Deacon and Berry, 1993). Fungi are able to produce a considerable amount of extracellular enzymes such as chitinases, glucanases and proteases, glycosyl hydrolases, xylanases, cellulases and mannanases under suitable conditions (Elgorban et al., 2016). Metallic nanoparticles from biological source have gained much attention mainly due to their alignment with the principles and concepts of green chemistry (de Andrade et al., 2017).

Many attempts have been made to synthesize nano-based antifungal compounds from zinc oxide and silver nitrate used for effective management of plant pathogenic fungi including *Fusarium oxysporum Penicillium digitatum, Alternariacitri, Alternaria alternate* and *Aspergillus niger* (Patra et al., 2012; Abdelmalek and Salaheldin, 2016). Kanhed et al. (2014) and Bramhanwade et al. (2016) also synthesized copper nanoparticles that exhibited strong antifungal activitys against tested plant pathogenic fungi

responsible for crop diseases. Al-Zubaidi et al. (2016) demonstrated the effectiveness and broad spectrum of antimicrobial activity of AgNPs against plant phytopathogenic fungi.

Biogenic nanoparticles have proved to be effective biocontrol agents for certain plant diseases. It possesses the potential for extensive use in agriculture as biocontrol agents for sustainable agriculture (Oluwaseun and Sarin, 2017). This study aimed to synthetize environmentally-friendly biogenic AgNPs by using metabolites from a fast growing fungitoxic fungus *Alternaria* sp. for the effective control of some phytopathogenic fungi. *Alternaria* sp.'s extracts were used for the first time in this study and were found to be highly effective in the reduction of silver ions into silver nanoparticles. Synthesis of metal NPs based on fungal extract is found to be the most effective and environmentally friendly way of preparing biocontrol agents for phyto-pathogen suppression.

Results And Discussion

Synthesis of Nanoparticles and UV-Vis spectral analysis

Alternaria sp. was isolated and grown on plates for extract preparation (Fig. 1A). After mixing the *Alternaria* sp. extracts with the $AgNO_3$ solution, it was noted visually that the colour of the mixture was changed from transparent to dark brownish (Fig. 1B). After 20 min of reaction, both alterations in colour and the absorbance were recorded at regular intervals, as the colour change was the first evidence of success in nanoparticle formation. Generally, the colour change occurred due to reducing agents released from fungal extracts into solution (Birla et al., 2013). This was also supported by UV–visible spectra, when exposed to light of specific wavelength, the NPs displayed a unique surface phenomenon called surface plasmon resonance (SPR); due to this, a specific peak formation occurred for each kind of NPs by UV–Vis spectroscopy (Zada et al., 2018). The synthesis of AgNPs using (*Alternaria* sp.) extract was monitored by using UV–Vis spectroscopy; the light absorption pattern of the fungal biomass was observed in the range of 350–500 nm. Figure 1.

The silver ions reduction peak of the SPR occurred at 435 nm (Fig. 2). The same peak area for AgNPs at 435 nm was also reported by other researchers, one of them used incubation of Ag ions with fungal biomass of *Trichoderma koningii* (Tripathi, Gupta et al. 2013). Spectral analysis showed that the UV–Vis SPR peak at 435 nm typically observed for AgNPs that without altering the peak position increased as a function of time. It further confirmed the conversion of silver ions into silver nanoparticles. This observation suggested the *Alternaria* sp. mediated biogenic formation of mono-dispersed AgNPs. It is obvious that SPR peaks of metal nanoparticles depend on particles size, shapes and the reaction medium. The SPR peaks normally show a red shift with increase in the particles size. In this study, the SPR peak retained its spectral position, confirming a uniform size particles distribution. Fungal extracts are rich in bioactive molecules and functional groups like –OH, amide and carboxylic groups which improved the quantitative production of AgNPs and their stable dispersion. Therefore, fungal extracts have the power to be used for biogenic synthesis of nanoparticles. Moreover, the stability was evaluated

after 30 days of synthesis and there was not change in absorption peak value indicating that the nanoparticles are highly stable, after 1 month under ambient conditions (28˚C). Figure 2.

Characterisation of fungal based synthesis of AgNPs

1 Zeta potential analysis

AgNPs displayed a particle size range of 5 – 10 nm. The Zeta potential analysis of the biosynthesized AgNPs was found as a single sharp peak between – 60 and 0 mV while having a maximum intensity at -31.9 mV (Fig. 3). It indicates that the negatively charged moieties are present on the surface of the AgNPs that expanded in the medium. The repulsion among the particles might be due to the negative values that proved that the particles are very stable. Low zeta potential values of particles suggest no flocculation and no tendency to assemble together due to repulsion forces among particles (Carlson et al., 2008; Roda et al., 2017). Figure 3.

2 Scanning transmission electron microscopic (STEM) analysis

Morphology and size distribution of the biosynthesized AgNPs was revealed via STEM analysis. The as prepared AgNPs exhibited a spherical morphology (Fig. 4A and 4B). The biogenic AgNPs displayed a particle size range of 5 – 10 nm (Fig. 4C). It is also evident that the biomolecules in the extracts of *Alternaria* sp. promoted synthesis of AgNPs. The SEM images of AgNPs reported by other researchers, from different extracts showed spherical particles, aggregated spherical particles, irregularly shaped particles and cubic particles (Birla et al., 2013; Zada et al., 2018). The moderate particles size observed were 5, 12, 25, 35 and 50 nm, for AgNPs synthesized by different biological extracts using water as a solvent. Similar results were also found by other researchers recently (Birla et al., 2013; Lee et al., 2014; Anandalakshmi et al., 2016; Kasithevar et al., 2017; Zada et al., 2018). Figure 4.

3 Energy dispersive x-ray (EDX) analysis

EDX of the NPs was performed to investigate the elemental composition of the biosynthesized AgNPs (Fig. 4D). The EDX spectra revealed the presence of silver peaks around 3 and 3.1 keV, which were appeared due to the discharge of different electrons from L and K shells of silver, respectively. Therefore, the EDX pattern clearly indicates that the AgNPs are crystalline in nature. The lower energy peak (3 keV) was responsible for the outer shell electrons (L) and higher energy peak (3.1 keV) was responsible for inner shell electrons (K). The observation was in agreement with the previous report by Muthupandi Kasithevar et al. (2017). The carbon peak present in the spectra was mainly due to the carbon adhesive tape used and the rest of the peaks might be due to inorganic impurities in the biomolecules from the *Alternaria* sp. extract. The EDX data of AgNPs showed that the weight percentage of Ag was 92%. This is also in agreement with the results reported earlier (Lee et al., 2014)

4 FTIR spectral analysis

FTIR was employed to quantify and determine the functional biomolecules in the *Alternaria* sp. extracts which were important for the reduction of silver ions into relative silver nanoparticles. The *Alternaria* sp. extracts have a variety of biomolecules, which might be involved in the synthesis of these NPs (Fig. 5). The occurrence of many prominent peaks in the IR region of electromagnetic spectrum is due to different functional groups. The active and extended band was observed at 3421 cm^{-1} that confirmed the presence of polyphenolic –OH group. The second band was observed relative to alkyl group of C–H stretching vibrations at the absorption band 2870 cm^{-1}, the third narrow band occurred at 2815 cm^{-1} is ascribed to C–H of alkane group. The fourth peak at 1638 cm^{-1} indicating the presence of amide I group, the fifth short band appeared at 1450 cm^{-1} assigned to carboxylic acid while the sixth peak around 1156 cm^{-1} is representing stretching of aromatic ring, the seventh band at 1027 cm^{-1} corresponds to C–N stretching vibrations of aliphatic amines of protein. The last wide and short band at 470 cm^{-1} represented alkyl halides. This investigation also demonstrated that protein and amino acids are the main components and have the capacity to bind metals. It is evident that polysaccharides, sulfonated compounds and amide linkages are strongly involved in the reduction of silver ions into nanoparticles. The biomolecules present in fungal extract have dual functions of reducing silver ions and stabilizing the NPs (Rajeshkumar et al., 2013; Anandalakshmi et al., 2016). Figure 5.

Antifungal potential of the AgNPs

Well diffusion method was used to assess the antifungal potential of the biosynthesized AgNPs against four phyto-pathogenic strains of fungi (*Fusarium oxysporum*, *F. maniliforme*, *F. tricinctum* and *Alternaria* sp.). Four Petri-dishes were prepared with PDA medium having wells of 8 mm diameter at equal distance. After inoculation, the wells were loaded with different concentrations (25, 50 and 100 µl) from stock solution of AgNPs (1 mg ml^{-1}). The plates were incubated at 28 ± 1°C and after 5 d the inhibition zones were measured (Fig. 6). The minimum inhibitory concentration (MIC) of AgNPs was 25 µl while the growth rates of all strains of fungi were quite low at 50 and 100 µl. The diameter of inhibition zones were measured as a score, indicating that the diameter of inhibition zones increased with increasing the concentration of AgNPs. The average diameter of inhibition zones were 15 ± 2 mm, 17 ± 2 mm and 21 ± 2 mm at 25, 50 and 100 µl, respectively for *Fusarium oxysporum* (Fig. 6A). Inhibition zones for *Fusarium moniliforme* were measured as 7 ± 2 mm, 10 ± 2 mm and 17 ± 2 mm at 25, 50 and 100 µl concentrations of AgNPs, respectively (Fig. 6B). Inhibition zones for *Fusarium tricinctum* were 9 ± 2 mm, 11 ± 2 mm and 20 ± 2 mm at 25, 50 and 100 µl concentrations of AgNPs, respectively (Fig. 6C). For *Alternaria* sp. the average diameter of inhibition zones were 16 ± 2 mm, 18 ± 2 mm and 20 ± 2 mm at 25, 50 and 100 µl, respectively (Fig. 6D). The maximum inhibition zone was observed at 100 µl concentration of AgNPs for *Fusarium oxysporum* (21 ± 2 mm) following *Alternaria* sp. (20 ± 2 mm), suggested the efficacy of the biosynthesized NPs against the phyto-pathogenic fungal strains. Figure 6.

Conclusion

The green reduction and biogenic synthesis of nanoparticles is an effective, low cost, sustainable and environmentally friendly approach. The Phytopathogenic *Alternaria sp.*'s extracts were used for the first time in this study and were found to be highly effective in the reduction of silver ions into silver nanoparticles. Compared with the earlier reported various biological extracts, the *Alternaria* sp. was found to be more effective in silver reduction, as the NPs obtained as a result of treatment with this extract has lowest zeta potential (-31.9 mV). Furthermore, the Fungi mediated AgNPs were utilized as an efficient biocontrol agents for suppression of various fungal phyto-pathogens. The maximum inhibition zones (21 ± 2 mm) and (20 ± 2 mm) were observed at 100 µl concentration of AgNPs for *Fusarium oxysporum* and *Alternaria* sp., respectively. This suggests the efficacy of the biosynthesized NPs against the fungal phyto-pathogens. It is concluded that fungal extract based synthesis of metal NPs is the most efficient and environmentally friendly way to prepare biocontrol agents for phyto-pathogen suppression.

Materials And Methods

Reagents and instruments

Silver salts (analytical grade) and culture media were purchased from Aladdin, China and the solvent used throughout the experiments was double distilled water (Millipore 18.2 MX cm). Instruments used in the study were High speed refrigerated centrifuge (JW-3021HR), UV/Vis spectrophotometer (Shimadzu, UV-3600), Scanning transmission electron microscope (STEM) and energy dispersive X-ray (JEOL JEM-ARM200F 200-kV), zeta potential (HORIBA Zetasizer SZ100), FTIR (Nicolet 6700 FT-IR spectrometer).

Fungal isolation, characterization and preparation of metabolites

Alternaria sp. was isolated from severely wilt affected banana plants rhizospheric soil in the way below: Soil samples were collected from Guilin, China. Serial dilution method was used to isolate fungal pathogens and cultured on PDA (potato dextrose agar) plates which were incubated at 26–28 °C for 5 days. Purified cultures were visually identified using their cultural and microscopic morphology. Presumptive identifications were confirmed with ITS rDNA sequence analysis. The entire ITS region of the fungal isolates was amplified with the primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The resulting amplicons were sequenced by 3730 XL DNA analyser (Applied Biosystems, USA). Sequence identification was performed and deposited to NCBI, BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Nucleotides). Its accession number is MN096578.

Alternaria sp. (seven days old) was grown in 200 ml of PDB media at 26–28 °C for 5 d. Biomass were recovered by centrifugation at 7000 rpm for 10 min. Supernatant was decanted and the biomass pellet was subsequently washed with sterilized deionized water to remove all the remaining components of growth medium. After washing, the biomass was re-suspended in 100 ml dH_2O, incubated at 28 °C for 3

d, and then filtered through 0.22 µm membrane filter to get small molecular weight metabolites. The resulting filtrates were used for silver nanoparticles synthesis (Birla et al., 2013).

Extracellular biosynthesis of silver nanoparticles

For the preparation of AgNPs, 10 ml of the *Alternaria* sp.'s metabolic filtrate was treated with 50 ml of 1 mM silver nitrate ($AgNO_3$) solution and kept on stirring (500 rpm) at room temperature (25 ± 1 °C) (Birla et al., 2013). The development of reddish colour in the reaction mixture designates the synthesis of AgNPs. Optical property such as localized surface plasmon resonance (LSPR) for the AgNPs was identified by UV–visible spectrometry (Shimadzu UV3600, Japan). The reaction was terminated after SPR bands saturation. The acquired product in suspension was recovered by centrifugation at 12,000 rpm for 15 min. The collected AgNPs were repeatedly washed and freeze-dried. The fungi mediated green fabrication of silver nanoparticles and examination of its antifungal activity against phytopathogens were monitored (Rajeshkumar et al., 2014).

Characterization of silver nanoparticles

1 UV-Visible spectroscopy analysis.

The biogenic AgNPs synthesis was confirmed using UV-vis absorption spectra. The absorption spectra of the samples were measured using a spectrophotometer (Shimadzu UV-3600, Japan) in the wavelength range of 190–800 nm, DI water was used as blank.

2 Scanning transmission electron microscope (STEM) and energy dispersive X-ray (EDX) analysis

The AgNPs were analysed using a JEOL JEM-ARM200F 200-kV STEM. The instrument was equipped with a light element 100 mm^2 SDD EDS detector. Mineralogical information and two dimensional elemental maps were obtained for Ag, C and K by electron diffraction of selected areas using a spatial resolution of 50 nm.

3 Zeta potential

In order to determine the surface electric charge of AgNPs, zeta potential measurement was carried out by using zetasizer (Zetasizer SZ-100, HORIBA).

4 Fourier transformed infrared (FTIR)

The freeze dried powder of AgNPs was subjected to FTIR analysis. FTIR spectral bands in the prepared materials (AgNPs) were determined using FT-IR spectrometer (Nicolet 6700, Thermo Scientific), 400–4000 cm^{-1} in transmittance mode. Samples for FTIR analysis were prepared using the KBr pellet technique, which involves mixing thoroughly the AgNPs with KBr before forming a pellet at high pressure.

Phytopathogens collection

The plant-pathogenic fungi used in this study were *Fusarium oxysporum*, *Fusarium moniliforme*, and *Fusarium tricinctum*. These were provided by Dr. Jing Lv, State Key Laboratory of Heavy Oil Processing, University of Petroleum, Beijing, China. All the fungi were grown on potato dextrose agar (PDA) according to manufacturer's instruction. *Fusarium oxysporum* appeared as an abundant cottony white colony with white aerial mycelium. *Fusarium moniliforme* had a black colony on the back side with a white aerial and marginal mycelium. *Fusarium tricinctum* colony had abundant cottony white mycelium. *Alternaria* sp. was isolated from wilt-affected banana rhizospheric soil.

Screening of antifungal potential of silver nanoparticles

Well diffusion method was used to assess the antifungal potential of the biosynthesized AgNPs against four pathogenic strains (*Fusarium oxysporum*, *Fusarium moniliforme*, *Fusarium tricinctum*, and *Alternaria* sp.). Petri-dishes were prepared with PDA agar medium having wells of 8 mm diameter at equal distance. After inoculation, the wells were loaded with different concentrations (25, 50 and 100 µl) from stock solution of AgNPs (1 mg ml^{-1}). The plates were incubated at 28 ± 1^{o}C and after 5 d the inhibition zones were examined (Tripathi et al., 2013).

Declarations

Acknowledgement

Not applicable

Authors' contributions

TTW designed, performed the research. TTW and SK wrote the manuscript. PCF supervised the research work and manuscript writing. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

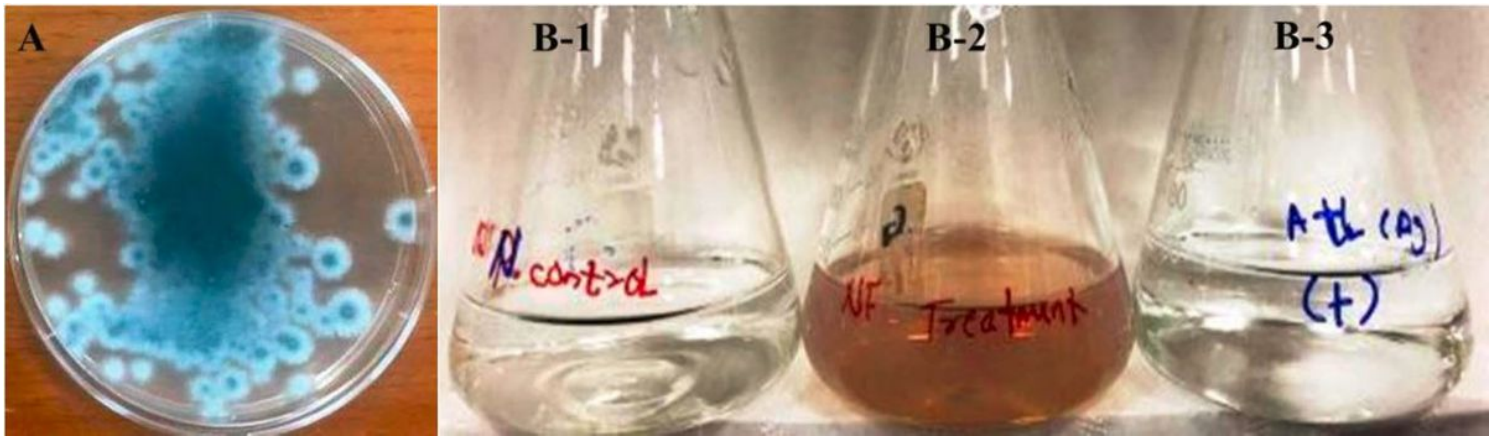

Figure 1

Macroscopic morphology of Alternaria sp. (A), Color changes after AgNPs synthesis (B-1: fungal metabolites only, B-2: fungal metabolites+ 1 mM AgNO3 1:5v/v, B-3: ddH2O+ 1 mM AgNO3 1:5v/v).

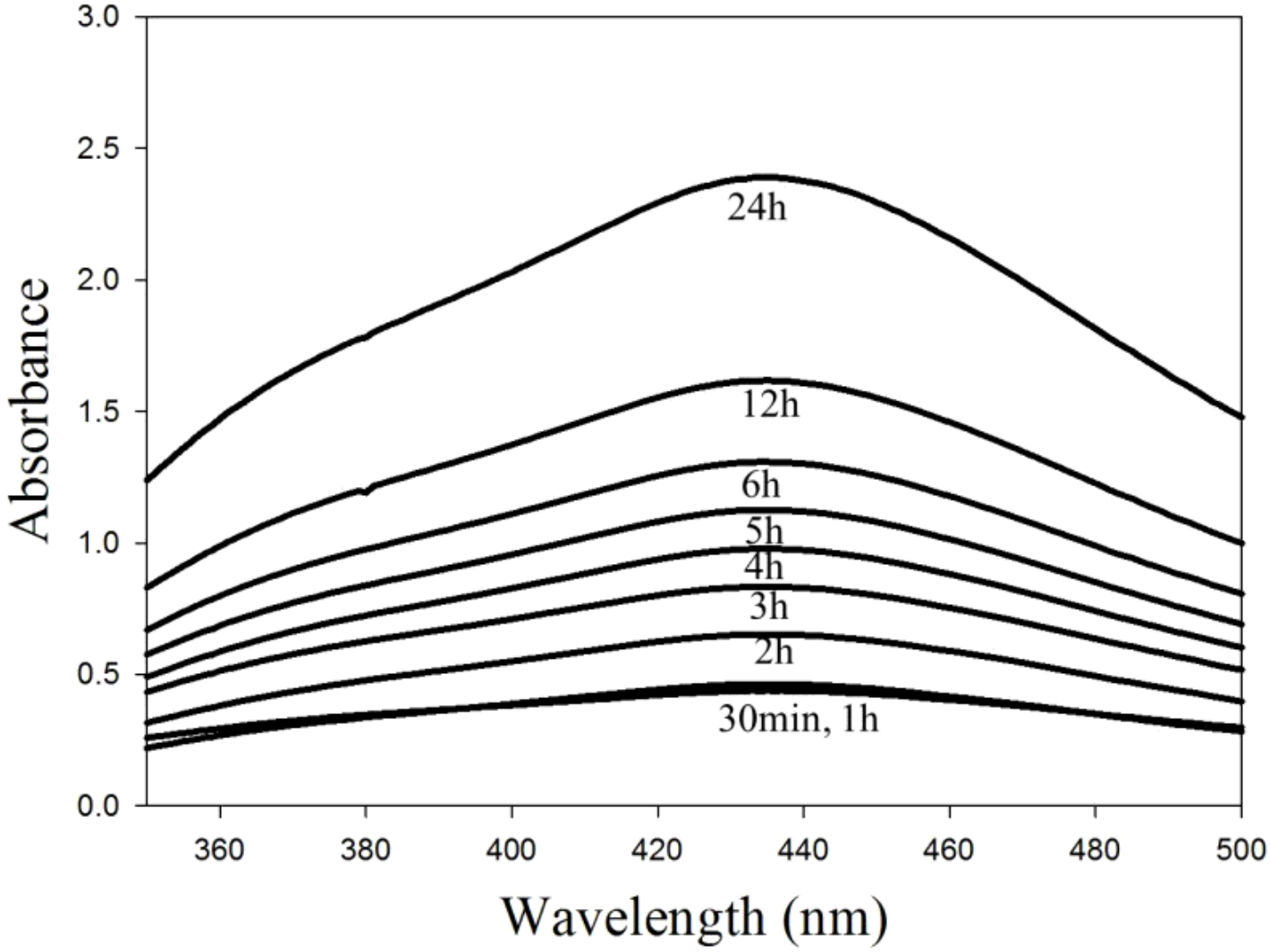

Figure 2

SPR banding patterns of AgNPs (435nm) recorded with UV-Vis Spectrometry at regular time intervals of 30 min up to 24 hr.

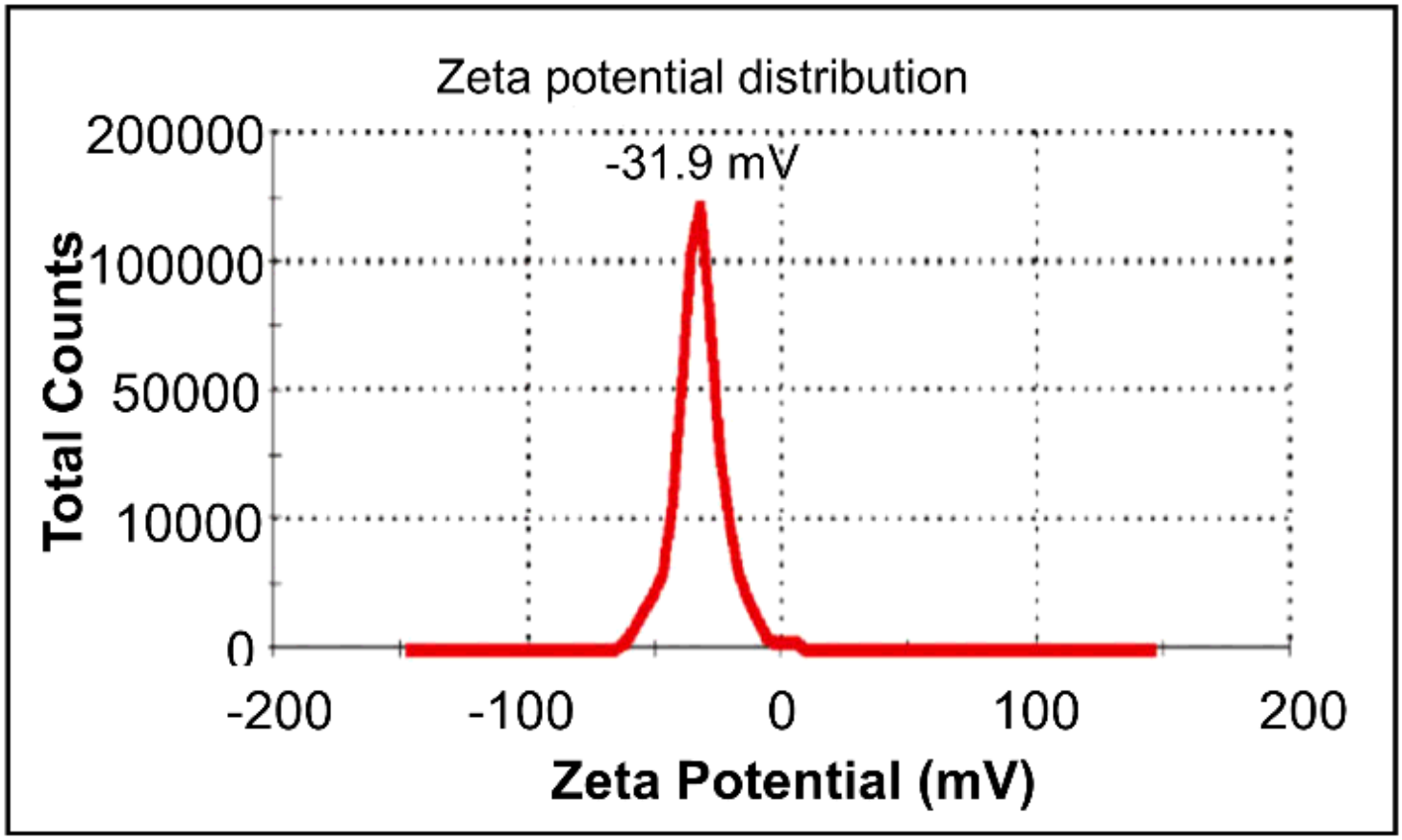

Figure 3

Zeta potential of biosynthesized silver nanoparticles illustrating the surface charge value.

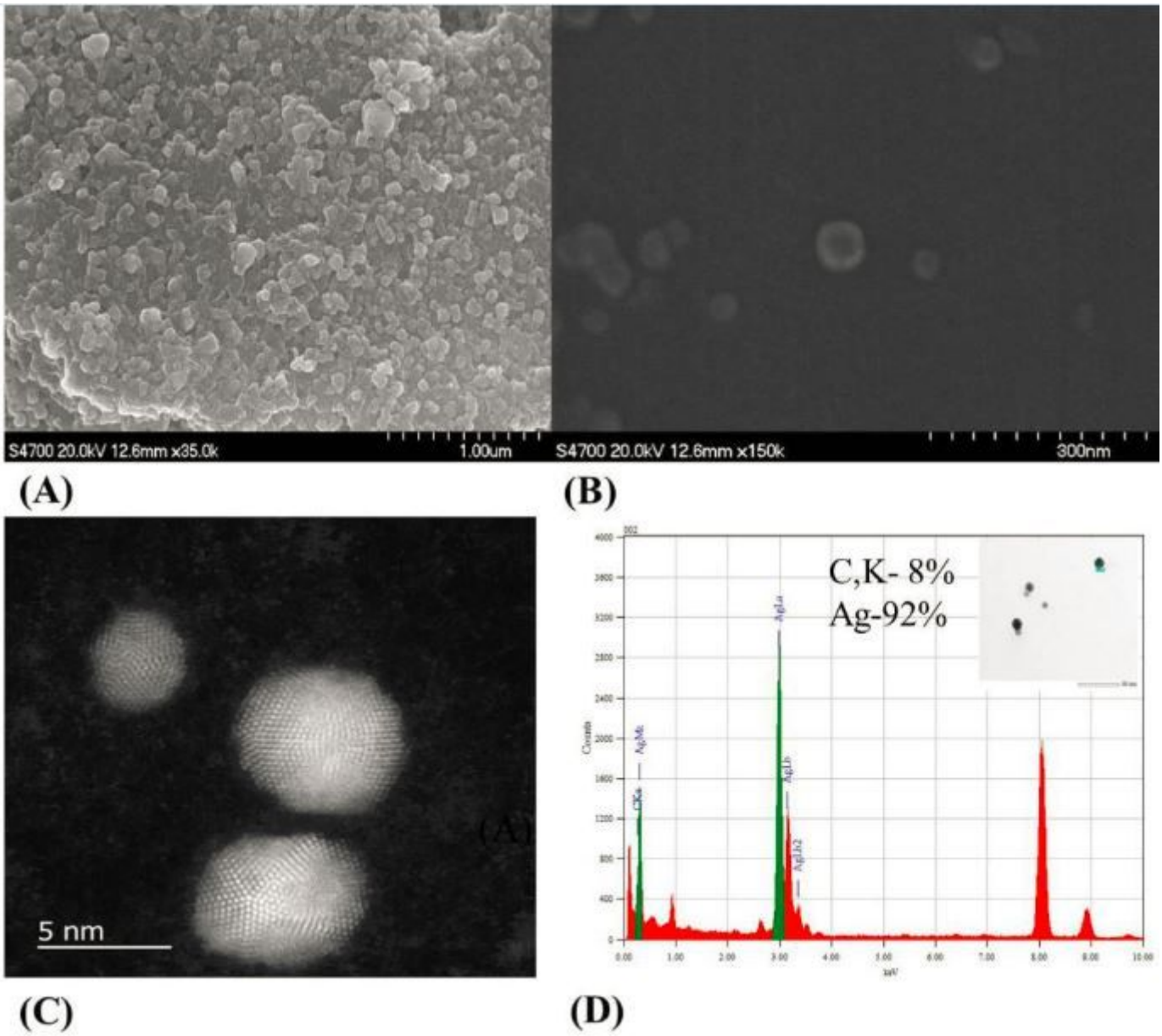

Figure 4

(A) SEM micrograph of AgNPs synthesized using Alternaria sp. (scale bar —1 µm), (B) STEM of AgNPs showing dispersed particles (scale bar—300 nm), (C) STEM image of the silver nanoparticle (scale bar— 5 nm) and (D) EDX spectrum of AgNPs confirming its composition.

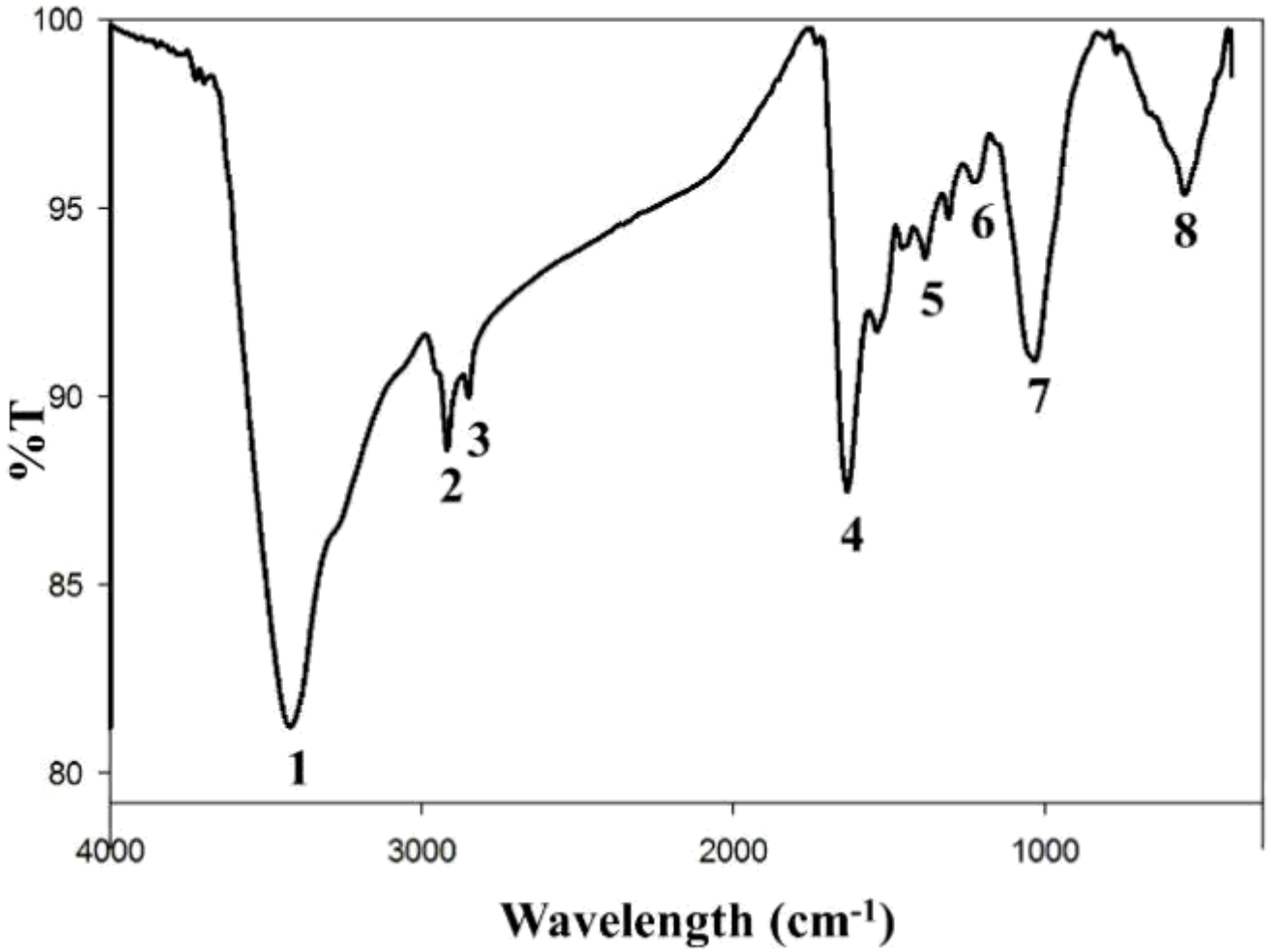

Figure 5

FTIR spectra of biosynthesized AgNPs using Alternaria sp. 1=Phenolic –OH group, 2=C-H stretching vibrating of alkyl group, 3=C-H stretching vibrating of alkane group, 4=Amide group, 5=Carboxylic acid, 6=C-C stretching aromatic group, 7=C-N stretching, 8=Alkyl halide.

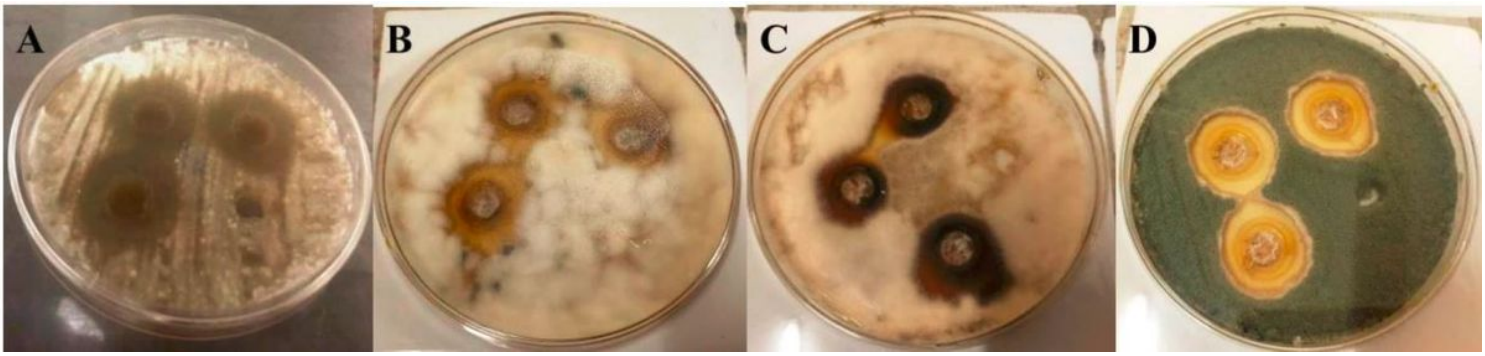

Figure 6

Antifungal activity of silver nanoparticles against (A) Fusarium oxysporum (B) Fusarium moniliforme (C) Fusarium tricinctum and (D) Alternaria sp.