

# Biogenesis of Bacterial and Fungal Endophytic Mediated Silver Nanoparticles

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## ABSTRACT

**Aim:** Nanoparticles play an important role to develop materials that are light, effective and ecofriendly with diverse applications. In this study, Bacterial and Fungal endophytes were screened for the synthesis of AgNPs. **Materials and Methods:** Endophytes for biogenesis of nanoparticles was isolated from various explants. Selected bacterial and fungal endophytes were identified by 18s rRNA and 16s rRNA sequencing. Endophyte mediated silver nanoparticles were characterized using different techniques like UV-visible spectroscopy, XRD, HRTEM, DLS, EDX and FTIR. **Results:** Optimization of AgNP synthesis by endophytic bacterial isolate *Bacillus cereus* showed optimum 5 mM silver nitrate concentration, reaction mixture temperature 37-40 °C, reaction time 30 min and PVP as stable surfactant. Characterization carried out using advanced analytical instrumentation showed sharp peak at 424 nm by UV-visible spectroscopy. According to XRD and HR-TEM data analyzed it was observed that average size of AgNP was 12.4 and 12.9 nm respectively. DLS and zeta potential showed nanoparticles are dispersed evenly, FTIR analysis confirmed coating with primary and secondary amino acids which is responsible or stability of nanoparticles. EDX confirmed elemental composition of silver nanoparticles. **Conclusion:** Synthesized nanoparticles are uniform in size, ecofriendly, good bio compatibility, easy processing and stable. We can further study potential biological activity like plant growth promotion, nano pesticide encapsulations, antifungal, anti-bacterial, antioxidant and cytotoxicity of these synthesised AgNPs.

**Keywords:** Silver Nanoparticles, XRD, HRTEM, DLS, FTIR.

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## INTRODUCTION

Due to the very small size, excellent magnetic properties and ability to change surface characteristics, high surface area to volume ratio nanoparticles possesses various physical, chemical and biological properties.<sup>[1]</sup> Silver nanoparticle possess diverse application in the field of optical, electronic, electrical, paint, textile, cosmetics, food industries, medical and environmental.<sup>[2]</sup> The common techniques used for the synthesis of nanoparticles includes chemical reduction, physical techniques and green synthesis.

All these techniques are expensive, time consuming, poor compatibility and use the hazardous chemicals. Nanoparticles synthesized by physical and chemical methods are unstable and possess toxic chemicals present over their exterior limits various applications.<sup>[3]</sup> Plants are good sources of microbial diversity that secrete bioactive compounds, which can be used for the synthesis of nanoparticles.<sup>[4]</sup> Biogenesis approach is fast, economically feasible, simple and nanoparticles synthesized are biocompatible.<sup>[5,6]</sup>

Several reports are available for the biogenesis of silver nanoparticles by plant, fungi and bacteria.<sup>[7]</sup> High amount of biomolecules are produced by both bacteria and fungi hence prevent the agglomeration, which helps in stabilization of nanoparticles.<sup>[8]</sup> Endophytic microbes are recognized as most valuable group of microbes in terms of diversity and remedial potential.<sup>[9]</sup> These microorganisms nurture in the intercellular

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spaces of host plants without causing any damage.<sup>[10]</sup> Endophytes are able to synthesize same chemical compounds as synthesized by the host plant, make a possible adaptation to the host's microenvironment.<sup>[11]</sup> In this study we have isolated endophytes from various remedial plants for biogenesis of silver nanoparticles. Process for biogenesis of silver nanoparticles was optimized under different conditions. Morphological and structural characteristics were identified using UV-visible spectroscopy, XRD, DLS, HR-TEM, EDX and FTIR.

## MATERIALS AND METHODS

### Isolation and Characterization of endophytes

The endophytes were isolated from leaves, barks and root sections of various medicinal plants.<sup>[12]</sup> Plants, *Withania somnifera* (Ashwagandha), *Aloebarbadensis miller* (Aloe vera), *Tinospora cordifolia* (Giloi), *Bryophyllum* (Patherchatt), *Anthocephalus cadamba* (Cadamb), *Phyllanthus emblica* (Amla) and *Melia azadirachta* (Neem) were taken from the Kurukshetra University. Endophytic mediated biogenesis of silver nanoparticles and cleaning for further study was performed.<sup>[13,14]</sup> Endophytic bacterial strain VXB10 was identification by using 16s rRNA sequencing from CSIR-IMTech, Chandigarh. The 16s RNA sequence attained from CSIR-IMTech were subjected to BLAST analysis. Similarity study on genetic level was performed by using nBLAST. Endophytic fungal strain VXF2 was identified by using 18s rRNA from Centre for Agriculture and Bioscience International, Bangalore.

### Optimization study for biogenesis of silver nanoparticles

#### Concentration of Silver nitrate

Study for biosynthesis of AgNP with different concentration of silver nitrate and supernatant of VXB10 endophytic strain. The silver nitrate concentration varied from 1mM to 10mM which was mixed with supernatant in ratios (1:1). Then resulting solution was incubated for 30 min under bright condition for biogenesis of silver nanoparticles. Observations were made using UV-visible spectrophotometry.

#### Temperature and time

Optimization study for biogenesis of silver nanoparticles under different temperature varying from 20 to 60°C. The silver nitrate with supernatant was incubated for 30 min at 20, 37, 40, 50 and 60°C. The effect of reaction time was checked for the biosynthesis of AgNPs by keeping reaction solution in direct sunlight

at different time (2, 5, 10, 15, 20, 30, 40 and 50 min). The effect of incubation temperature and time was observed by UV-visible spectrum analysis.

### Surfactant and stability

Surfactant acts as capping agent for nanoparticles, which increases stability and availability of nanoparticles. To study the effect of surfactants 0.1% of Polyvinyl Pyridone (PVP), Ethylenediaminetetraacetic Acid (EDTA), Sodium Dodecyl Sulfate (SDS), Trisodium citrate and glyconic acid were added to reaction solution. The stability study of biologically synthesized AgNP was checked for 30 days by using UV-Visible Spectrophotometer.<sup>[15]</sup>

### Characterization of synthesized AgNPs

Initially biologically synthesized AgNP were characterized using UV-visible spectrophotometry followed by advance instrumentation techniques like XRD, DLS, EDX, FTIR and HR-TEM.<sup>[13]</sup> XRD was done by comparing spectrum with standards in the Joint Committee of Powder Diffraction Standards issue 2010. Hydrophobic particle size, zeta potential and polydispersity index were predicted by DLS. Energy Dispersive X-Ray Spectroscopy was used to confirm elemental composition of AgNPs. The size, shape and arrangement of nanoparticles were confirmed by HR-TEM. Biomolecules and proteins on surface of AgNP were studied by using FT-IR spectrophotometer between 4000 - 400 cm<sup>-1</sup>.

## RESULTS

### Biogenesis of AgNP by endophytes

A total of 79 endophytes (30 fungi and 49 bacterial) were isolated from the various part of remedial plants. The endophytes were coded as VXF1- VXF30 for fungi and VXB1-VXB49 for bacteria. The 10 bacterial endophytes and 8 fungal endophytes were showing a change in colour after reaction of silver nitrate with culture filtrate. The silver nanoparticles synthesis by both bacterial and fungal endophytes were confirmed by UV-visible spectroscopy as shown in Figure 1. The peak between 400 to 450 nm absorbance confirms the synthesis of nanoparticles.

The fungal endophyte VXF2 was showing peak at 432 nm with absorbance value 1.158. The fungal isolates (VXF9, VXF13, VXF18, VXF20, VXF25, VXF26 and VXF30) showed broad peaks with low absorbance value. The bacterial endophyte VXB10 was showing peak at 424 nm with absorbance value 2.763. The bacterial endophytes (VXB5, VXB24, VXB29, VXB34,

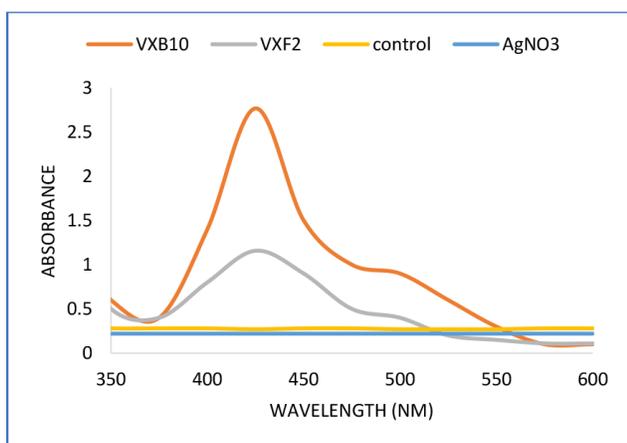


Figure 1: UV-vis analysis of endophytes VXF2 and VXB10.

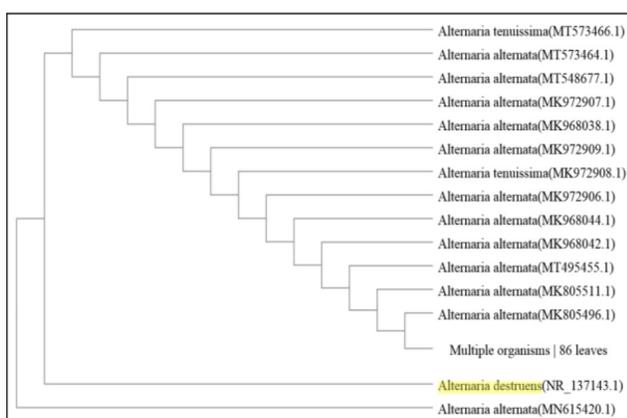


Figure 2: Phylogenetic tree constructed by neighbor joining method for endophytic fungi VXF2.

VXB35, VXB38, VXB39 and VXB40) showed broad peaks with low absorbance. Absorbance is co-related with the quantity of nanoparticles synthesized. The lower absorbance between 400-450 nm indicates the poor synthesis of nanoparticles.

To obtain the fungal biomass for the synthesis of nanoparticles is highly time consuming as compare to bacterial biomass. The synthesis of nanoparticles was slow when fungal biomass was used as compared to the use of bacterial supernatant. This is the reason only bacterial isolate VXB10 was used for the further study.

### Identification of selected microbial isolates

The fungal isolate was identified by with fungus database from EBI and BLAST algorithm as shown in Figure 2. Fungal endophyte VXF2 was showing 100% similarity to *Alternaria destruens* (ATCC 204363). The 16s rRNA sequence of bacterial isolate VXB10. On the basis of BLAST analysis, VXB10 showed was showing 100% identity to *Bacillus cereus*. The phylogenetic tree

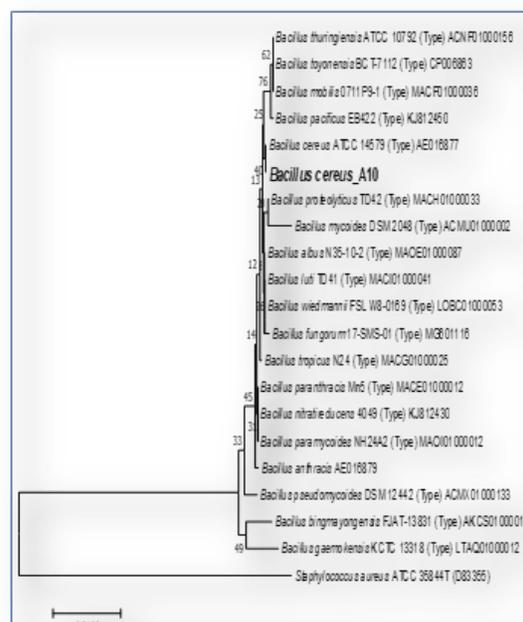


Figure 3: Phylogenetic tree constructed by neighbor joining method for endophytic bacterial isolate VXB10.

of bacterial endophyte VXB10 was constructed by Neighbor-Joining method was shown in Figure 3.

### Optimization of silver nanoparticles biosynthesis Silver nitrate concentration

The increasing concentration of silver nitrate resulted in higher synthesis of AgNP. A sharp peak around 420 nm was observed in case of 5 mM and 6 mM silver nitrate concentration as shown in Figure 4(a). The continuous rise in the absorbance peak intensities was observed with increasing concentration of silver nitrate (5 mM). It was observed that SPR peak after 5 mM concentration of silver nitrate became broad which depicts large size and agglomeration of nanoparticles. The UV-vis spectroscopy absorbance at 10 mM silver nitrate concentration was very less. The maximum absorbance was observed at 5 mM silver nitrate. All further experiments were carried out by mixing 5 mM silver nitrate with equal proportion of supernatant.

### Optimization of temperature

The change in temperature effect the synthesis of nanoparticles. At 20 and 30°C change in colour of reaction solution was very slow. The colour of reaction solution was dark and prominent at 40, 50 and 60°C. The change in colour was recorded by UV-visible spectrophotometer as shown in Figure 4(b). The absorbance was high at 40°C followed by no significant

change at 50°C and 60°C. Therefore, further study was conducted at 40°C.

### Time point for Biogenesis of silver nanoparticles

The change in colour was negligible up to 10 min. The prominent change in colour was seen after 15 min from pale yellow to dark brown. The significant increase in colour was not observed after 30 min of keeping the reaction solution. Spectrophotometrically, absorbance value increase consistently up to 30 min whereas, no significant increase in absorbance was recorded after 30 min as shown in Figure 4(c).

### Surfactants optimization

In UV-vis analysis, maximum surface plasmon resonance absorbance value was recorded with 0.1% PVP as compare to 0.1% EDTA and 0.1% SDS followed by minimum absorbance with 0.1% Trisodium citrate and 0.1% Glyconic acid as shown in Figure 4(d). The results inferred that 0.1% PVP was better surfactant for stabilizing nanoparticles and reduce agglomeration. The reaction mixture containing PVP was stored 4°C to check the stability of nanoparticles at different time interval (1, 15 and 30 days). The stability of nanoparticle remain same as no change in the absorbance was at different time interval as shown in Figure 4(e). The stable position of SPR absorbance peak indicates the stability

of nanoparticles. Hence, PVP was as most stable surfactant at low temperature.

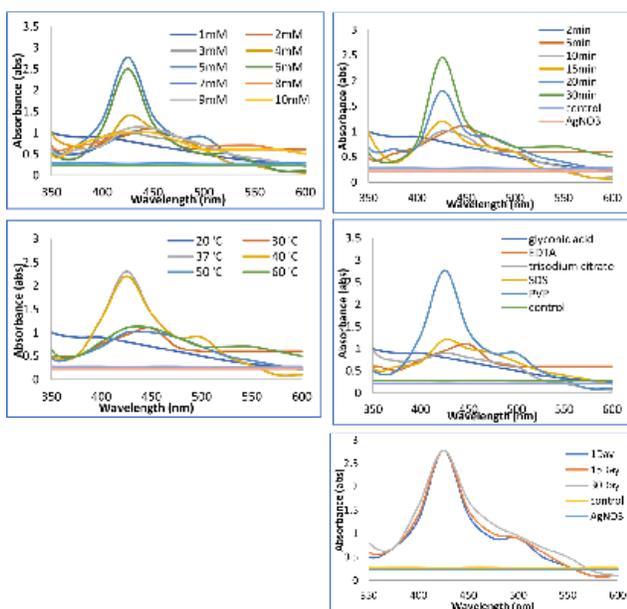
### Characterization of synthesised silver nanoparticles

The cell free supernatant of VXB10 bacterial endophyte was mixed individually with silver nitrate (5 mM) in equal volume with PVP as capping agent, the reaction mixture was incubated for 30 min. The synthesized nanoparticles were showing maximum absorbance at 424 nm as shown in Figure 5(a). In XRD peak analysis four diffractions patterns were observed at 38.18°, 44.39°, 64.55° and 77.45° consistent to (111) (200) (220) and (311) face centered cubic (fcc) planes for metallic silver as shown in Figure 5(b). The XRD spectrum analysed using Origin software for endophytic mediated nanoparticles was consistent with diffraction standard database JCPDS 04-0783. Usually high intensity is observed for fcc (111), which was similar to the data recorded in synthesized silver nanoparticles as shown in Table 1.

Hydrodynamic size of silver nanoparticles was estimated by using DLS. DLS analysis of synthesized silver nanoparticle was done to know the particle size, zeta potential and polydispersity index. Size of biologically synthesized silver was in range of 172-449 nm with average size of 226 nm as shown in Figure 5(c). The Polydispersity Index (PdI) of synthesized silver nanoparticles was below 0.474 with net surface charge of -35.75 mV which infers to the stability of nanoparticles.

EDX analysis revealed the strong signal in domain of silver which confirms the elemental composition of silver nanoparticles as shown in Figure 5(d). EDX spectrum showed presence of silver (62.7%), copper (25.96%) and carbon (11.33%) respectively. The crystals of metallic silver usually show optical absorption at 3 keV due to SPR of biologically synthesized silver nanoparticles. The copper was observed due to copper gride used in sample support during analysis.

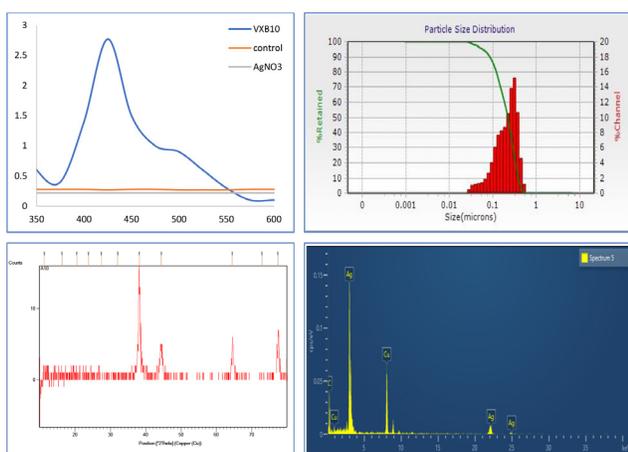
The HR-TEM images of biologically synthesized nanoparticles are shown in Figure 6. HR-TEM images when analysed using ImageJ software showed that nanoparticles were spherical and range from 2.5-30 nm with average size of 12.9 nm. These results are consistent with the shape as determined by the XRD analysis. The FTIR spectrum of biologically synthesized silver nanoparticles showed strong peaks at 3417.3, 2919.3, 2851.1, 1740.3, 1618.3, 1465.9, 1381.2, 1221, 1077.1, 827.3, 630 and 588.7  $\text{cm}^{-1}$  as shown in Figure 7.



**Figure 4: Silver nanoparticles biosynthesis at different a) concentrations of silver nitrate b) temperatures (20, 30, 37, 40, 50 and 60°C) c) time interval d) surfactants e) stability after 1, 15 and 30 days.**

**Table 1: Biologically synthesized AgNPs size calculated by Debye-Scherrer equation.**

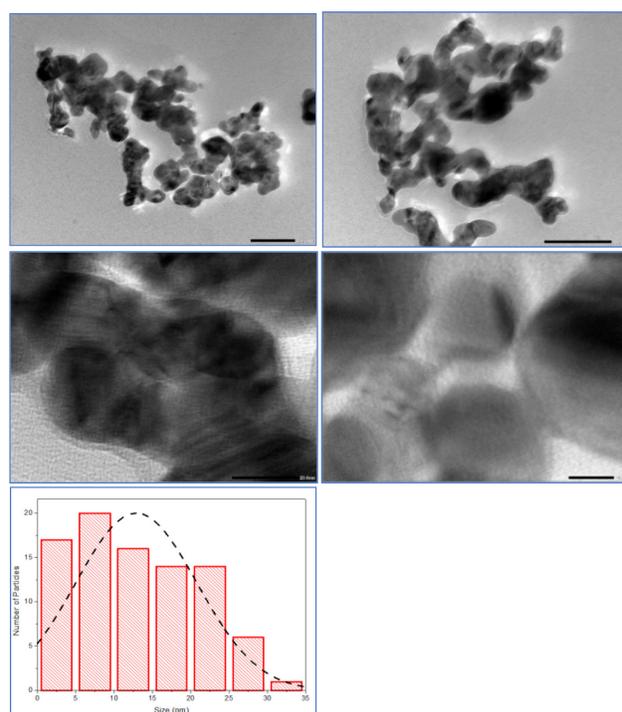
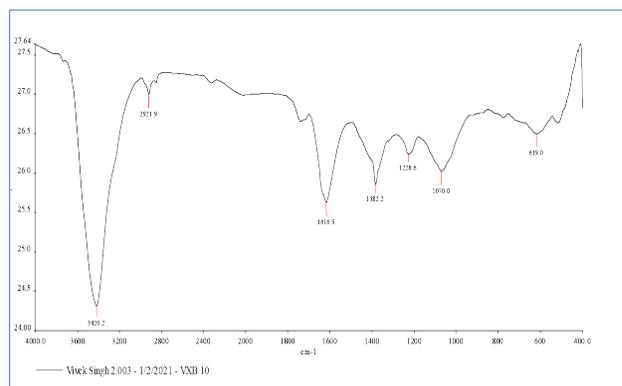
VXB10					
Position	FWHM	Size (nm)	d-space	Miller indices (h,k,l)	Avg. Size (nm)
38.18512	0.69274	12.1354788	2.354972	(1,1,1)	12.40
44.39455	0.87896	9.76173938	2.038927	(2,0,0)	
64.5515	0.66504	14.1286238	1.442524	(2,2,0)	
77.45878	0.74856	13.6041936	1.231214	(3,1,1)	

**Figure 5: Characterization of nanoparticles synthesized by bacterial isolate a) UV-visible spectroscopy b) XRD analysis c) DLS d) EDX.**

The FTIR peaks corresponding to different functional groups are shown in Table 2.

## DISCUSSION

The isolated bacterial endophytes VXB10 was identified by using 16s RNA sequencing as *Bacillus cereus* and fungal endophytes VXF2 identified by using 18s RNA sequencing as *Alternaria destruens*. Both endophytes VXF2 and VXB10 were showing silvernanoparticle synthesis from silver nitrate. The silver nanoparticles synthesised by endophytes *Cryptosporiopsis ericae*, *Aspergillus tamaris* and *Aspergillus versicolor*.<sup>[16-18]</sup> The bacterial isolate was easy to grow and showed faster reduction of silver nitrate as compared to fungi.<sup>[19]</sup> In fungal isolates due to high pigmentation the reduction of silver nitrate was taking more time as compared to bacterial isolates.<sup>[20]</sup> In this study bacterial endophytes were used for silvernanoparticle synthesis. The silver nanoparticles show strong absorption at 424 nm in the visible range due to SPR. High absorbance value shows quantitative analysis of AgNPs. XRD peak data was used for estimating size silver nanoparticles by using Debye-Scherer's formula. The average size calculated for silver nanoparticles was 12.4 nm, which was similar to

**Figure 6: HR-TEM analysis of silver nanoparticles synthesized by bacterial isolate.****Figure 7: FTIR analysis of silver nanoparticles synthesized by bacterial isolate.**

the nanoparticles size 12.9 nm calculated by HR-TEM image analysis. XRD spectrum broad peaks indicate the role of bacterial extract in particle formation and crystal nuclei growth.<sup>[21]</sup> Biogenesis of silver nanoparticles

**Table 2: Analysis of FTIR spectrum of silver nanoparticles synthesized by bacterial endophyte VXB10.**

Peak Value cm <sup>-1</sup>	Corresponding functional group
3417.3	N-H primary and secondary amino acids
3564 and 3584	O-H stretch
1465.9	C-C aromatic stretch
2929.3 and 2851.1	-CH hydrocarbons
1740.3	Carbonyl compound
1618.3	C=O amide stretch
1065	-CH <sub>3</sub> amino acid
2886	N-CH <sub>3</sub> bend
1381.2	C-O stretch
1221	free carboxylate group
827.3	C-H linkage
630 and 588.7	Disulphide

from rizobial-isolate *Arthroderma fulvum* and discovered particles which were spherical with average diameter  $15.5 \pm 2.5$  nm, with high uniformity and minor diameter distribution.<sup>[22]</sup> Energy Dispersive X-ray gives qualitative as well as quantitative status of elements involved in the formation of silver nanoparticles. The occurrence of primary silver was confirmed by EDX study, which confirms the silver element in reaction mixture was due to reduction of silver ions from silver nitrate.<sup>[23]</sup> In addition to size of metallic nanoparticles by XRD and HR-TEM, DLS also measures the size of stabilisers absorbed on the surface. As a result, DLS measurements of size are larger than TEM and XRD analysis measurements. PDI value  $\sim 0.4$  indicated that the nanoparticles have highly polydisperse and moderately disperse distribution.<sup>[24]</sup> Zeta potential showed negative surface charge which explains the long-term stability, dispersity and high colloidal nature of nanoparticles. The surface charge could avoid agglomeration of nanoparticles and thus provide stable particles.<sup>[25]</sup> The interaction of biomolecules present in cell free extract with AgNPs was evaluated by using FTIR measurement of dried AgNPs. FTIR analysis showed presence of primary and secondary amines, alcohol or phenol O-H, saturated hydrocarbons, carbonyl compound and amino acids respirable of reduction of silver nitrate and coating nanoparticles for stability and better biocompatibility.<sup>[26]</sup> According to a previous statement, proteins can connect to nanoparticles via free amine groups or cysteine residues and by the electrostatic attraction of negatively charged carboxylate groups. This could lead to the stability of the silver nanoparticles by proteins.<sup>[27]</sup> The

microbial strain used, temperature, capping agent and pH play an important role in synthesis of nanoparticles with specific size, shape and functional group.<sup>[28]</sup> The optimized condition for silver nanoparticle synthesis were 5 mM silver nitrate, bacterial supernatant in ratio 1:1, light exposure for 30 min, reaction temperature of 40°C and PVP as most suitable surfactant. Nanoparticles could be stored to more than 30 days at 4°C for more than 30 days. The optimum condition for the synthesis of nanoparticles by *Escherichia coli* were pH 5-6, temperature 30-37°C.<sup>[29]</sup>

## CONCLUSION

In this study fungal and bacterial isolates were used for the synthesis of silver nanoparticle. Bacterial endophytes have advantages over fungal endophytes for biosynthesis of silver nanoparticles due to their faster growth rates, easier culturing, and potential for genetic modification. Study has shown that bacterial endophytes produce smaller, more uniformly sized nanoparticles and can reduce silver ions more efficiently than fungal isolates. The bacterial endophyte was identified as *Bacillus cereus*. The synthesized silver nanoparticles were evenly distributed, small size, stable and crystalline in nature. We can further study potential biological activity (antifungal, anti-bacterial, antioxidant and cytotoxicity) of these synthesised AgNPs.

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## Statements and Declarations

The first author acknowledges World Bank TEQIP-III project for providing assistantship to carry out research work. We acknowledge CIL/SAIF facility, Punjab University, Chandigarh for providing with high end instruments.

## Author Contribution

Material preparation, data collection and analysis were performed by Vivek Singh and Deepak Kumar Malik. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**AgNP:** Silver nanoparticles; **HR-TEM:** High-resolution transmission electron microscopy; **XRD:** X-Ray Diffraction; **DLS:** dynamic light scattering; **EDX:** Energy Dispersive X-Ray Spectroscopy; **FTIR:** Fourier Transform Infrared; **PVP:** Polyvinyl Pyroidone **EDTA:** Ethylenediaminetetraacetic Acid; **SDS:** Sodium Dodecyl Sulfate.

## SUMMARY

Bacterial endophyte VXB10 identified as *Bacillus cereus* (*italics*) can be used for synthesis of silver nanoparticles with uniform size, ecofriendly, good bio compatibility, easy processing and stable. We can further explore potential biological activity like plant growth promotion, nano pesticide encapsulations, antifungal, anti-bacterial, antioxidant and cytotoxicity.

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