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A Study on Extracellular Synthesis of Silver Nanoparticles from Endophytic Fungi, Isolated from Ethanomedicinal Plants *Curcuma longa* and *Catharanthus roseus*

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Abstract. Biological method is considered as eco-friendly and reliable process for the synthesis of silver nanoparticles (AgNps) in the field of nanotechnology due to its tremendous applications in various fields. In this study we have isolated a total of twelve endophytic fungi from leaves of *Curcuma longa* (turmeric) and *Catharanthus roseus* out of which six endophytic fungi showed their ability to synthesized AgNps from silver nitrate ($AgNO_{3)}$ solution which splits into a positive silver ion (Ag^{+}) and a negative nitrate ion (NO_3^{-}) in order to turn the silver ions into solid silver (Ag^{o}). Of the six positive endophytic fungi VRD2 showed good and encouraging results and was identified as *Penicillium spinulosum* VRD2. UV-Visible Spectroscopy confirms the AgNps showing maximum peak at 425nm implying the bioreduction of $AgNO_3$. Transmission Electron Microscopy (TEM) revealed the particle are spherical and well dispersed without agglomeration size ranging from 25-30nm.

1. INTRODUCTION:

Nanotechnology is a promising field of research in the recent days. Nanotechnology provides a platform to modify and develop the important properties of metal in the form of nanopaticles having various applications in diagnostics, biomarkers, antimicrobial agent, antiplatelet, cancer and cytotoxic studies [1, 2]. Nanoparticles are quite unique in nature because of their properties due to their large surface area to volume ratio which is most important aspect of nanotechnology in synthesis of nanoparticles. The use of metal nanoparticles is gaining impetus in the present century due to their optical, electrical, biological and catalytic properties [3]. In the recent days much research is going on the metal nanoparticles, especially silver nanoparticles (AgNps) to solve the problems of emerging pathogens including Multi-Drug Resistant (MDR), Methicillin Resistant *Staphylococcus aureus (*MRSA) and Vancomycin Resistant *Staphylococcus aureus* (VRSA) bacteria [4]. Synthesis of AgNps by biological route is easy conventional method compared with chemical, physical, and photochemical methods. As these methods are capital intensive, toxic and non ecofriendly, a biological synthesis of AgNps with the help of plants and microorganisms including bacteria, actinomycetes, algae, and fungi, which has been realized globally due to its ecofriendly, cost effective and biocompatible synthesis, which has made the researchers to exploit the biological sources as nanofactories [5,6,7]. Fungal mediated synthesis of AgNps is determined nowadays because of their reception towards higher bioaccumulation, easy and simple scale up method, economic viability, compared to other synthesis procedures from bacteria, actinomycetes, algae, and plants. In recent days many researchers have worked on biosynthesis of AgNps using fungi. Krishnakumar et al., [8] reported extracellular biosynthesis of AgNps using *Fusarium oxysporium* and studied its antibacterial efficacy against human pathogens. Valentin et al., [9] revealed AgNps synthesis using marine fungus *Aspergillus oryzae* and their size ranged from 6-37nm. Gitanjali and Ashok, [10] also studied an mycosynthesis of AgNps from *Trichoderma*

harzianum, the particles formed are polydispersed spherical with a size ranging from 19-63nm. Ravindra and Rajasab, [11] also revealed an AgNps synthesis from different fungal species, such as *Fusarium oxysporum*, *Fusarium udum*, and *Stemphylium vrican* with different sizes. As such many reports have been demonstrated on fungi for the synthesis of AgNps. One such clique of microbes are the endophytes whose potential biosynthesis of AgNps has not been studied completely [12]. In the present study we have isolated endophytic fungi from healthy leaves of *Curcuma longa* (turmeric) and *Catharanthus roseus (*Madagascar rosy periwinkle) and screened for the potent AgNps Production further subjected for extracellular biosynthesis of AgNps and its characterization studies which have vast applications in medical field.

2. MATERIALS AND METHODS

2.1 Chemicals and Media

Potato Dextrose Agar (PDA) was used for the isolation of endophtyic fungi. Malt Extract Glucose Yeast Extract (MGYP) broth was used for screening of fungi for AgNps production. Whereas all the microbiological media were steam sterilized by autoclaving at 15 psi at 121ºC for 15 min. Silver nitrate and other chemicals were purchased from Himedia Laboratories Pvt. Ltd (Mumbai, India), all the solutions were freshly prepared, indicated elsewhere in the text.

2.2 Isolation of Endophytic Fungi

Healthy leaves of *Curcuma longa* (turmeric) and *Catharanthus roseus* were collected from the Department of Botany, Gulbarga University, Kalaburagi. The leaves brought to the laboratory were washed several times under running tap water and cut into small pieces following the method of Strobel et al., [13] and Maroof et al., [14] with slight modifications. These pieces were surface sterilized followed by sequential rinsing in 70% ethanol (C_2H_5OH) for 30 sec, 0.01% mercuric chloride ($HgCl_2$) for 5min, 0.5% sodium hypochlorite (NaOCl), and 2-3 minutes with sterile distilled water and then allowed to dry under sterile conditions. The cut surface of the segment was placed in petri dish containing PDA supplemented with streptomycin sulfate (250 mg/ml) at 28ºC for 3-4 days and monitored every day for the growth of endophytic fungal colony from leaf segment. Aliquots of 0.1 ml of the last washed distilled water were spread on PDA plates for evaluating the effectiveness of surface sterilization. The fungi which grew out from leaf segment were isolated and brought into pure culture onto other PDA plates. The fungal isolate was identified based on its morphological and reproductive characters using standard identification manual [15, 16].

2.3 Screening of Potent Fungal Isolate for Extracellular Synthesis of Silver Nanoparticles

All the isolated endophytic fungi were subjected for the biosynthesis of AgNps. A total of 12 endophytic fungi obtained from sterilized healthy leaves of *Curcuma longa* (turmeric) and *Catharanthus roseus* were screened for the synthesis of AgNps initially by visual observation of color change to reddish brown after addition of $AgNO_3$ to the fungal filtrate for the primary indication of silver nanoparticles and those isolates which showed color change to brown were further subjected to UV-Vis absorption spectroscopy for confirmation.

2.4 Molecular Identification of Endophytic Fungus

Molecular identification of potent endophytic fungus was carried out as per the standard procedures followed at Agharkar Research Institute, Pune. Genomic DNA was isolated in pure form, approximately ~529 bp rDNA fragments were successfully amplified using fungal universal primers ITS4 and ITS5. The sequencing PCR was set up with ABI-BigDye® Terminatory 3.1 cycle sequencing kit. The sequences obtained were analyzed using sequence scanner software and they were analyzed using online database NCBI BLAST (www.ncbi.nlm.nih.gov/blast) to find the closest match of the contig sequences. For phylogenetic study, related sequences were retrieved in FASTA format from Gen Bank. The closest homo-logues to the sequences were selected and the

multiple sequence alignments were carried out using the Clustal W program in the MEGA 5 software, phylogenetic tree was constructed and rDNA sequences have been deposited in Gen Bank for the accession number.

2.5 Characterization Studies for Silver Nanoparticles

2.5.1 UV –Visible Spectroscopy

The formation of AgNps was preliminarily confirmed by visual observation of colour change from pale white to reddish brown, after adding 1mM $AgNO_3$ solution to fungal filtrate. Further confirmed by sharp peak given by AgNps in the visible region using UV-visible spectroscopy (T90+UV-Vis Spectrophotometer) which confirms AgNps at the absorption range between 390 to 440nm due to Surface Plasmon Resonance (SPR) which is considered to be a reliable and accurate analytical laboratory assessment procedure for the analysis [17].

2.5.2 Transmission Electron Microscopy (TEM)

Characterization of AgNps was done by TEM (Hitachi-H-7500) to know the particle size, shape of a material in nano dimension and study the crystal structure meticulously, TEM is a microscopic technique wherein beam of electron is transmitted through an ultra thin specimen and interacts as electrons waves exiting from the sample to form an image. The samples were prepared by drop-coating the AgNps solution onto the carbon–coated copper grid and kept under vacuum before loaded onto a specimen holder. TEM micrographs were taken and then sizes, shape of AgNps were confirmed [18].

3. RESULTS AND DISCUSSION

3.1 Isolation, Screening and Identification of Endophytic Fungi for the Synthesis of AgNps

A systematic study about the isolation of endophytic fungi from two medicinal plants, *Curcuma longa* (turmeric) and *Catharanthus roseus* was carried out to evaluate their capacity to produce the AgNps extracellularly. The leaf samples of *Curcuma longa* (turmeric) and *Catharanthus roseus* were collected from the Department of Botany, Gulbarga University, Kalaburagi. From the surface sterilized leaf segments of these medicinal plants, a total of 12 endophytic fungi were isolated on PDA medium (Fig.1 and Fig.2). The fungi grown out from tissue were brought into pure culture, and were subjected to screen for the synthesis of AgNps. Amongst endophytic fungal isolates VRD-1, VRD-2, VRD-4, VRD-6, VRD-8, and VRD-9 showed positive for their ability to synthesize AgNps (Table-1). Of the six positive endophytic fungi VRD-2 showed good and encouraging results for their ability to produce AgNps based on the colour change to reddish brown after addition of $AgNO_3$ solution to endophytic fungal filtrate within 1h. The colour change was caused by SPR of silver nanocrystals in visible region as also observed by Varshney et al., [19]. Only metals (essentially Au, Ag, Cu and alkali metals) with free electrons possess Plasmon resonance in the visible spectrum which gives rise to such intense colours [20] and also by UV-vis spectroscopy techniques to verify the formation of AgNps providing a sharp plasmon absorbance peak at 425 nm which confirms as a potent isolate for biosynthesis of AgNps, Hence, further work was carried out with endophytic fungus, VRD-2. Based on morphological and microscopic identification studies VRD-2 endophytic fungus, was identified as *Penicillium* sp. (Fig.3.).

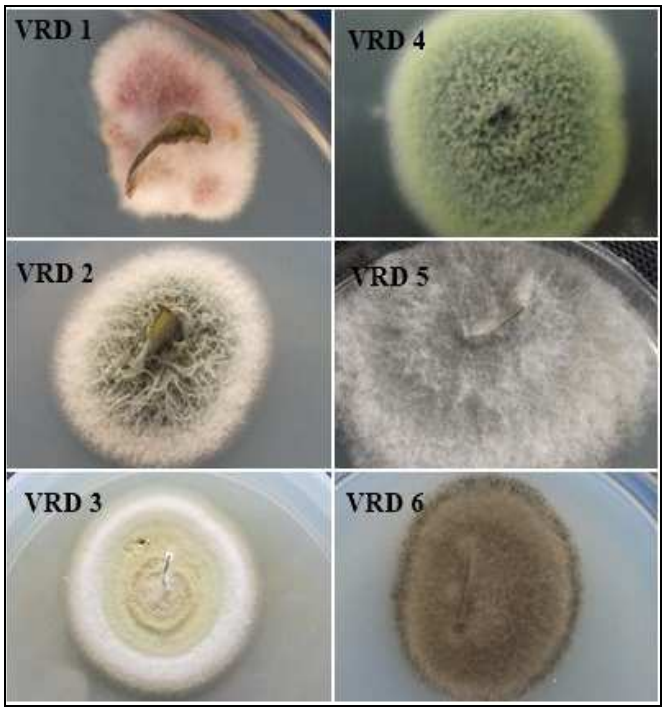

Fig.1: Endophytic fungi grown from sterilized leaf segment of *Curcuma longa* (turmeric)

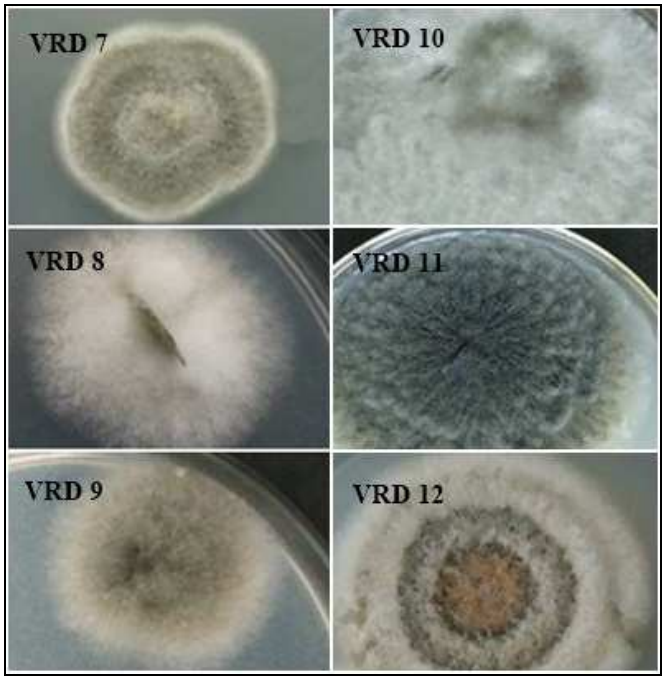

Fig.2: Endophytic fungi grown from sterilized leaf segment of *Catharanthus roseus*

Table-1: Screening of endophytic fungi for biosynthesis of AgNps

Endophytic Fungi Culture Code	UV-Vis spect	Synthesis of AgNps
Endophytic fungi isolated from leaf segment of *Curcuma longa*		
VRD 1	380 nm	Positive
VRD 2	**425 nm**	**Positive**
VRD 3	-	Negative
VRD 4	357 nm	Positive
VRD 5	_	Negative
VRD 6	390 nm	Positive

Endophytic fungi isolated from leaf segment of *Catharanthus roseus*		
VRD 7	_	Negative
VRD 8	379 nm	Positive
VRD 9	382 nm	Positive
VRD 10	_	Negative
VRD 11	_	Negative
VRD 12	_	Negative

(-) No Peak

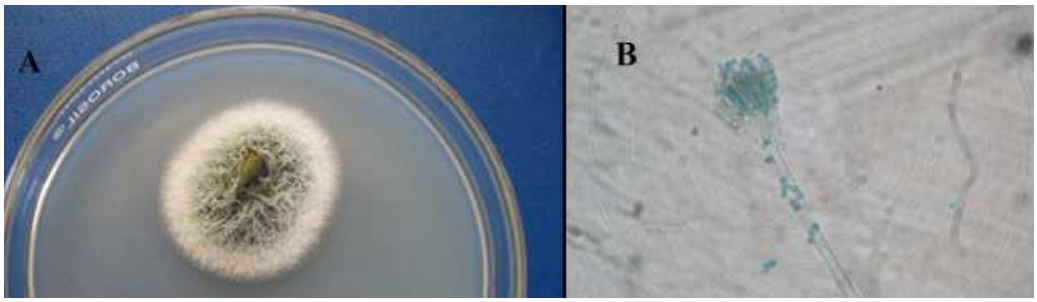

Fig.3: A) Endophytic fungi grown from sterilized leaf segment of *Curcuma longa* on PDA. (B) Microscopic image of endophytic fungi, *Penicillium* sp.

3.2 Molecular Identification of Endophytic Fungus

Of the promising fungal isolates, based on the morphological and microscopic observation viz., VRD-2 was tentatively identified as *Penicillium* sp. This was further confirmed as *Penicillium spinulosum* based on ITS sequences. Amplification and sequencing of ITS region of the fungal rDNA resulted in ~ 529bp long nucleotide sequences. The nucleotide sequences obtained were deposited in NCBI Gen Bank (Accession number KM063185). The closest homologues to the sequences were carried out using the clustal W programme in the MEGA5 software. The tested endophytic strain VRD 2 isolate showed 99% significant similarly with *Penicillium spinulosum,* based on nucleotide homology and phylogenitic analysis (Fig.4).

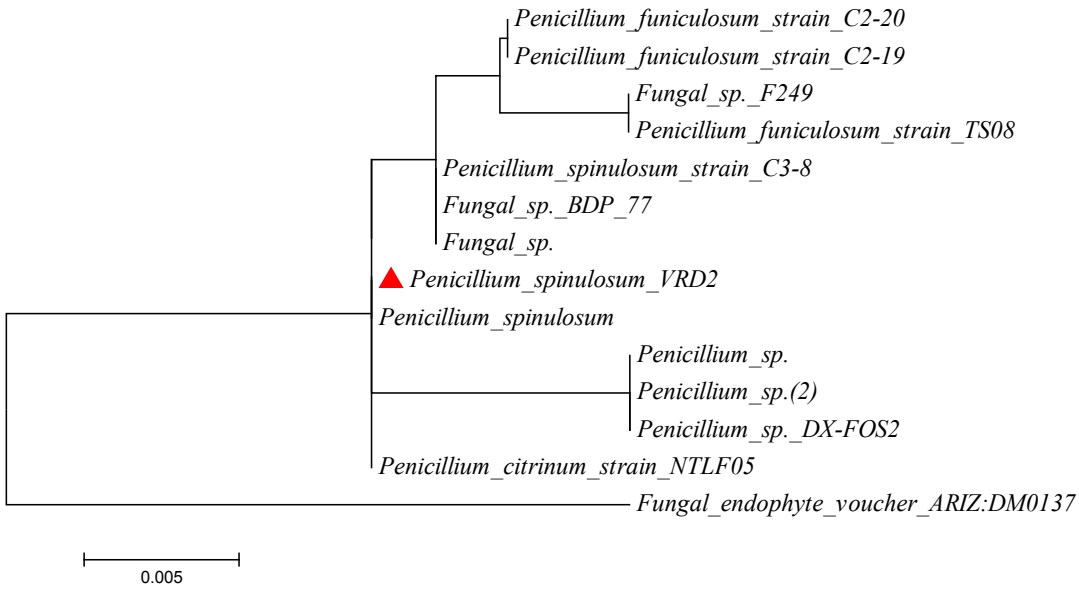

Fig.4: Based on nucleotide homology and phylogenetic analysis VRD-2 showed similarity with *Penicillium spinulosum*

3.3 Extracellular Synthesis of Silver Nanoparticles

Fungal filtrate was treated with equal volume of 1mM $AgNO_3$ solution which splits into a positive silver ion (Ag^+) and a negative nitrate ion (NO_3^-) in order to turn the silver ions into solid silver (Ag^o); the appearance of colour change from pale white to brown which is a clear indication for the formation of AgNps in the reaction mixture (Fig.5). The intensity of the colour was increased with the period of incubation. The appearance of the brown colour was due to the excitation of SPR [5]. Shivaraj et al., [2] reported biosynthesis of AgNps by change in colour to deep brown using the fungus *Aspergillus flavus* culture filtrate with $AgNO_3$ solution. Afreen et al., [5] also reported synthesis of AgNps by *Rhizopus stolonifer* and also reported the increase in colour intensity of AgNps was due to increased number of nanoparticles formed in the reaction mixture. Verma et al., [21] reported biosynthesis of AgNps by *Aspergillus clavatus* an endophytic fungus isolated from sterilized stem tissues of *Azadirachta indica*. Sophiya Devi et al., [22] also used endophytic fungi *Alternaria solani* GS1 and *Penicillium funiculosum* GS2 isolated from the ethno medicinal plant *Gloriosa superb* L for extracellular biosynthesis of AgNps.

Fig.5: A) Enzyme filtrate of Endophytic fungus, *Penicillium spinulosum* (VRD-2). B) Colour change to reddish brown after treating with 1mM Silver nitrate solution

3.4 Characterization Studies of Silver Nanoparticles

3.4.1 UV-Visible Spectroscopy

The extracellular synthesis of AgNps using endophytic fungus, *Penicillium spinulosum* involves the bioreduction of silver ions in the filtrate. Reaction solution was monitored using UV-visible spectroscopy. Synthesized AgNps from *Penicillium spinulosum* showed maximum absorbance peak at 425 nm after 24 h of incubation (Fig.6). Farkanda et al., [4] reported sharp peak of SPR at 415 nm from the endophytic fungus, *Pestalotia* sp isolated from leaves of *Syzygium cumini* (L). Swetha and Nachiyar [12] reported the absorption peak at 400 nm and 423 nm using endophytic fungi isolated from leaf samples of *Garcinia Xanthochymus* and *Aravae lanata*. Nirjanta Devi et al., [17] also revealed SPR band for AgNps in the range of 390-440nm by endophytic fungus, *Penicillium* sp isolated from *Centella asiatica* plant. Verma et al., [21] also reported SPR peak at 415 nm by endophytic fungus *Aspergillus clavatus* isolated from the surface sterilized stem tissues of *Azadirchta indica*. While Sophiya Devi et al., [22] revealed SPR sharp peak at 415nm and 403nm by endophytic fungi *Alternaria solani* and *Penicillium funiculosum* respectively isolated from the ethno medicinal plant *Gloriosa superb* L. All these reports suggest that the peaks shown by almost all the endophytic fungi as per the literature lie in the range of 390-440 nm. Our results correlate with all these above said authors.

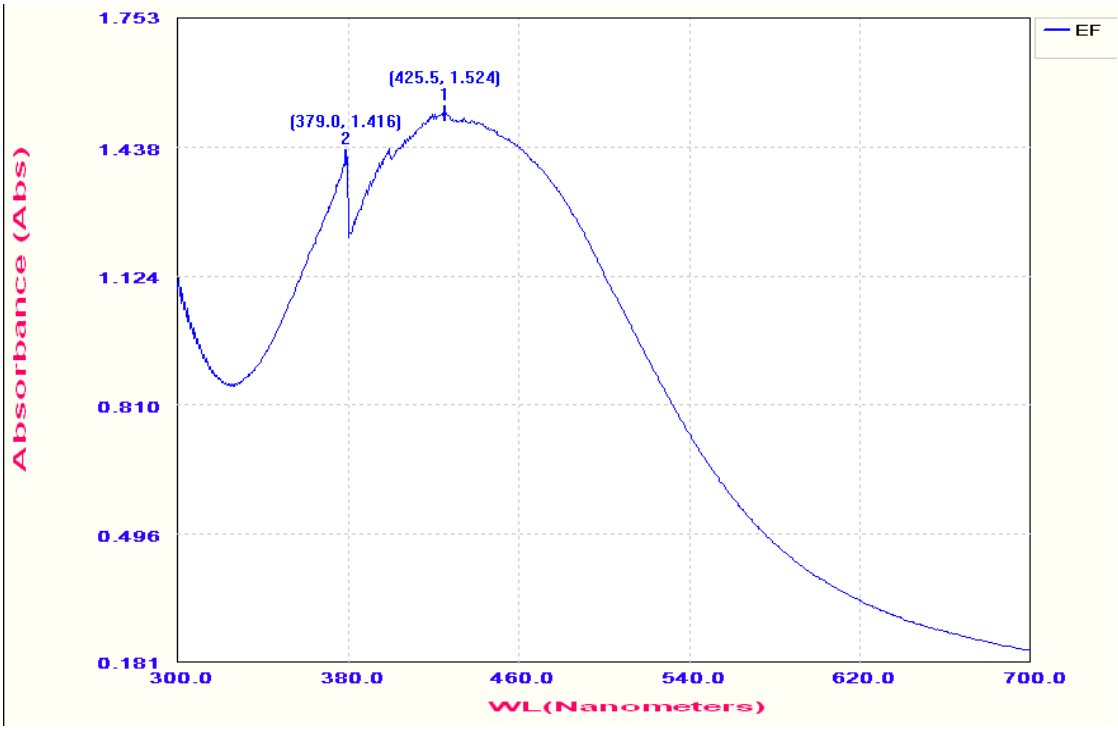

Fig.6: UV-Visible Sepctra of AgNps of Endophytic fungus, *Penicillium spinulosum*

3.4.2 Transmission Electron Microscopy (TEM)

TEM measurements were carried out to determine the morphology and shape of the AgNps. TEM micrograph (Fig.7) revealed that the particle is spherical and well dispersed without agglomeration. The particle size of AgNps synthesized by endophytic fungus, *Penicillium spinulosum* ranges from 25 to 30nm. Various reports have provided evidence of extracellular synthesis of AgNps by TEM images.

Farkanda et al., [4] revealed spherical and polydispersive AgNps ranging from 10 to 40 nm with an average diameter by endophytic fungus *Pestalotia* sp. isolated from leaves of *Syzygium cumini.* Afreen et al., [5] revealed spherical shaped AgNps with the size ranging between 3 and 20 nm by *R. stolonifer*. Sophiya Devi et al., [22] revealed synthesis of AgNps with distinct shape and size. They reported particles are spherical in shape and uniformly distributed without significant agglomeration from the synthesized AgNps by *Alternaria solani* (GS 1) and *Penicillium fumiculosum* (GS 2) isolated from medicinal plant *Gloriosa superb* (L) with size ranging from 5-20 nm and 5-10 nm respectively. Sharanabasava et al., [23] reported well-distributed spherical shaped AgNps in the range of 5-50nm by *Penicillium diversum* and Whereas Shivaraj et al., [24] also revealed the particles are spherical in shape and size to be 20-55 nm by fungus *Aspergillus niger.*

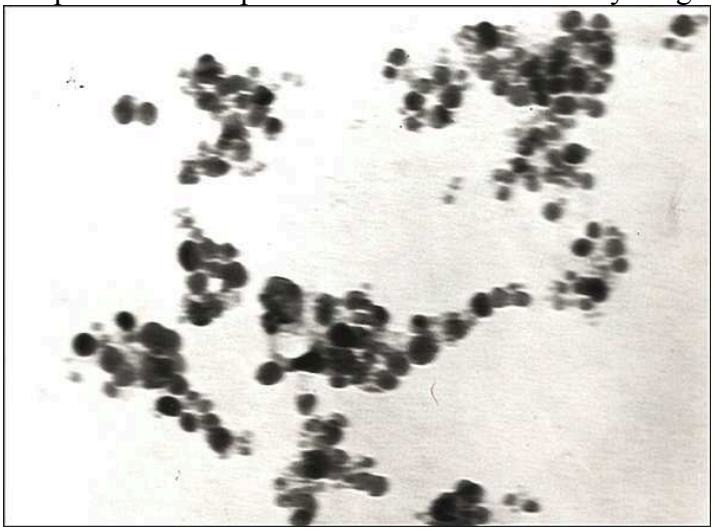

Fig.7: TEM Image shows biosynthesized AgNps by endophytic fungus, *Penicillium spinulosum*

CONCLUSION

Biological Synthesis of nanoparticles has proved as better methodologies and approaches in the Medical field. These methods are environmental friendly and commercially economic. It has been demonstrated that the Endophytic fungus, *Penicillium spinulosum* (VRD2) is capable of producing AgNps which remained untouched with size ranging from 25-30nm which is proven to be powerful weapon in medicine. It is noteworthy that apart from being producing rich sources of secondary metabolites, these endophytic fungi also have the ability to reduce metals. Apart from this endophytes also play an important role in agriculture by providing plant growth, provide protection from environmental stress and are also a source of important metabolites. Hence, AgNps are regarded as important addition in the area of nanoscience because of their diversified properties they provide in terms of applications in various walks of life.

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