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Research Article

SCREENING THE SILVER NANOPARTICLE SYNTHESIS POTENTIAL OF ENDOPHYTIC FUNGI WITH SPECIAL REFERENCE TO THEIR ANTIBACTERIAL ACTIVITY

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ABSTRACT

Nanobiotechnology is the innovative field of science which involves biological synthesis, characterization and application of numerous nanoparticles. The present study was planned to explore potential of endophytic fungi for eco-friendly synthesis of silver nanoparticles with antibacterial activity. In this study 8 endophytic fungal isolates were obtained from different parts of medicinal plants. These isolates were identified belonging to different genera namely, *Penicillium*, *Aspergillus*, *Alternaria* etc. All endophytic fungi were subjected to extra cellular biosynthesis of silver nanoparticles. Out of these *Penicillium sp.*,*Aspergillus sp*., *Alternaria sp.,* isolated from healthy plant parts of *Aegle marmelos, Dalbergia sissoo and Lantana camara* respectively, gave positive results. The antibacterial activities of these nanoparticles were determined against some pathogenic bacteria by Agar Well Diffusion assay. During which it was found that the silver nanoparticles sample exhibiting highest antibacterial potential was characterized by UV-Visible Spectroscopy, Dynamic Light Scattering (DLS), Zeta Potential analysis and Transmission Electron Microscopy. UV-Visible spectroscopy analysis gave surface plasmone resonance peak alternatively 416nm, 444nm and420nm.The average size range of silver nanoparticles as determined via, dynamic light scattering analysis is: 126nm, 88nm, 141nm. The Zeta potential of three samples as determined by zeta sizer showed a high negative surface charge on them which confers stability to them. The zeta potentials of these particles are: -19.1 mV, -22.0 mV, -16.2 mV. Silver nanoparticles produced by endophytic fungi displays considerable antibacterial agent and hence the study would prove to provide novel antibacterial agents.

Keywords: Antibacterial Activity, Biosynthesis, Characterization of Nanoparticle, Endophytic Fungi.

INTRODUCTION

Nanotechnology is the innovative field of science which involves synthesis of numerous nanoparticles. Nanoparticles (NP) are normally clusters of atoms in the size range of 1-100 nm. As compared to distinct heavy metal nanoparticles, silver nanoparticles are very significant because of their special properties (Kero Jemal *et al*., 2017) such as good catalytic activity, conductivity, chemical stability, antibacterial, antifungal, antiviral, and anti-inflammatory activities which are determined by their shape, size, composition, structure and crystallinity (Li *et al*., 2011). They are now considered as a usable alternative for solving the problem of Multi Drug Resistant (MDR) pathogens because of their extraordinary antibacterial and antifungal properties (Rai *et al*., 2012). Besides this nanoparticle have variety of applications in different areas like electronics, optics, bio-medicine, magnetics, catalysis, and energy science etc (Chokriwal *et al*., 2014). This has resulted in an increased demand for novel approaches of silver nanoparticles synthesis. Biological methods particularly those involving the use of endophytic fungi have emerged as an attractive approach over chemical and physical methods (Sandhu and Shukla 2017). Endophytic fungi show endo-symbiotic relationship with plants and live without causing any evident harm (Petrini *et al*., 1992). These fungi synthesize nanoparticles possessing variety of

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bioactivities by producing huge number of organic chemicals like proteins, enzymes, carbohydrates, fats, alkaloids, flavonoids, terpenoids, phenols, saponines etc. that are capable of donating electrons for reduction of Ag^+ to Ag^0. The active components responsible for reduction of Ag^+ ions vary depending upon the species (Jha *et al*., 2009). Thus, realizing the need and importance of biologically synthesized silver nanoparticles in the present scenario this research work was undertaken with the aim of using endophytic fungal flora for biosynthesis of silver nanoparticles.

MATERIALS AND METHODS

Isolation of Endophytic Fungi

Plant parts collected from various regions of Jabalpur city were tagged and placed in separate zip lock polythene bags, brought back to the lab and processed within 24 hrs of collection. The plant samples were collected according to National and International Guidelines. These plants were identified by the taxonomist at the Bio-Design Innovation centre. These plant samples were washed in running tap water for 15 minutes (Bussaban *et al*., 2001). Different plant parts were cut into small pieces (1mm-2mm), surface disinfected by sequential washes in 70% ethanol (1min), and 4.0% NaOCl (1min), then rinsed with sterile distill water and allowed to dry on sterilized Whatman filter paper No.1. (Schulz *et al*., 1993). All operations were carried out inside the laminar hood. Pieces of plant parts were placed on Potato dextrose agar (Potato-200 gms, Dextrose-20gms, Agar-20gms) medium supplemented with streptomycin (Himedia 100mg/ L) and were incubated at 28 ± 2ºC for 7 days for growth of endophytic fungi. Growing fungi were sub-cultured on PDA plates/slants for further use and maintained in refrigerator at 4ºC (Rodriguez *et al*., 2008).

Identification of Endophytic Fungal Isolates

Slide Culture technique

All isolated fungal strains were identified on the basis of morphological characteristics by using slide culture technique. In this method Potato dextrose agar medium was poured on a sterilized glass plate kept in a sterilized moist chamber in the form of a thin film (upto 5mm). After solidification, the film was cut into small cubes with flamed scalpel. These cubes were placed on slides inside the moist chamber and inoculated with fungal spores separately. Inoculated slides were incubated at 28 ± 2ºC for 5 days. After sporulation culture was stained with lacto phenol cotton blue staining technique and observed under light microscope. Further confirmation of the fungal species was made by comparing the microscopic images with those available in the standard manual (Manogaran *et al*., 2015).

Screening of Endophytic Fungi for Extracellular Synthesis of Silver Nanoparticle

Biomass preparation

Isolated fungi were inoculated in 250ml conical flask containing 100 ml potato dextrose broth at 28±2ºC for 7 days (Devi *et al*., 2012). After 7 days of incubation, fungal mycelium mats were separated with the help of Whatmann filter paper No-1 and washed with sterile distilled water. These mycelia mats were weighed and used for synthesis of silver nanoparticles (Netala *et al*., 2016).

Biosynthesis of Silver Nanoparticles

The fungal mycelia as harvested above were firstly washed thrice with sterilized distilled water. They were then inoculated in separate 250 ml conical flask containing 100 ml of sterilized distilled water and incubated at 28±2ºC for 96 hrs. After incubation these suspensions were filtered through Whatmann filter paper No. 1 and filtrates (Mycelia Free Water Extracts) so obtained were mixed with 1mM silver nitratefor reduction of Ag^+ ions into Ag^0 and incubated at 28 ± 2ºC for 72 hrs. These reaction mixtures were regularly observed for a change in the colour to brown (Ninganagouda *et al*., 2013).

Evaluation of Antibacterial Activity of Silver Nanoparticles

The brown colloidal solution of silver nanoparticles synthesized by endophytic fungi were tested for antibacterial activity *via.*, agar well diffusion method against pathogenic bacteria.

Agar Well Diffusion Assay

In this method 20 µl bacterial cultures were seeded on the surface of Nutrient Agar Media plates by spread plate technique. Media was punched with a sterile cork borer to make open wells (4 mm) in all plates. Different colloidal solutions of silver nanoparticles (80µl) synthesized above were poured in separate wells. Then these plates were incubated at 37°C for 24 hrs. After 24 hours incubation antibacterial activities of different nanoparticles sample were recorded in terms of diameter of inhibition zone measured in mm by using HiMedia Scale (Rajeshkumar and Malarkodi, 2014).

Characterization of Mycofabricated Silver Nanoparticles

The brown colloidal solution containing silver nanoparticles obtained in above experiments is further subjected to following characterization techniques for determination of their physical, chemical properties viz., size, stability etc.

UV-Visible Spectroscopic Analysis

Change in colour of the mycelium free water extract incubated with 1 mM silver nitrate solution visually observed over a period of time indicates the bio reduction of silver ions to silver nanoparticles. The colloidal brown solution was monitored by absorption measurements carried out on UV–Visible Spectrophotometer (UV 1800 spectrophotometer Schimadzu) at a resolution of 1 nm between 300 to 800 nm (which is a characteristic wavelength absorption range for silver nanoparticles)

wavelength range for confirming the synthesis of silver nanoparticles in the solution (Devi and Joshi 2015). For absorption measurements, different brown colloidal solutions were poured in cuvette and placed in sample holder where wavelength of specific range is passed through it and absorption values are displayed in the form a spectra. Maximum absorption at a particular wavelength was depicted as a peak.

Zeta Potential Analysis

Zeta potential is a physical property which gives the net surface charge of the nanoparticles which in turn determines the criteria of stability of NPs. Surface zeta potentials were measured using the laser Zeta Sizer (Malvern ZS 90, Malvern). Liquid samples of the nanoparticles (5ml) were diluted with double distilled water (50ml) using NaCl as suspending electrolyte solution. The *p*H was then adjusted to the required value. The samples were shaken for 30 minutes. After shaking, the equilibrium *p*H was recorded and the samples were subjected for zeta potential analysis of the metallic particles. Zeta potential of silver nanoparticles was determined from the graphical result displayed by the instrument (Haider and Mehdi, 2014).

Dynamic Light Scattering Analysis

DLS can probe the size distribution of small particles a scale ranging from submicron down to one nanometre in solution or suspension. Dynamic light scattering is a method that depends on the interaction of light with particles. This method can be used for the measurement of narrow particle size distributions, especially in the range of 2-500 nm. Among the techniques for the characterization of nanoparticles, the most commonly used is DLS. DLS measures the light scattered from a laser that passes through a colloid, and mostly relies on Rayleigh scattering from the suspended nanoparticles. Next, the modulation of the scattered light intensity as a function of time is analyzed, and the hydrodynamic size of particles can be determined.

To evaluate the toxic potential of any nanomaterials, its characterization in solution is essential. Therefore; DLS is mainly used to determine particle size and size distributions in aqueous or physiological solutions. The size obtained from DLS is usually larger than TEM, which may be due to the influence of Brownian motion. DLS is a non-destructive method used to obtain the average diameter of nanoparticles dispersed in liquids. It has the special advantage of probing a large quantity of particles simultaneously; however, it has a number of sample-specific limitations. The sample was loaded into quartz micro cuvette, and subjected to light scattering analysis. The result was displayed in the form of spectra having two peaks, for which the mean was calculated and average size range of silver nanoparticles was determined (Zhang *et al*. 2016).

Scanning Electron Microscopic Analysis

The size and surface morphology of the biosynthesized silver nanoparticles was characterized using ZEISS EVO 18SEM (Germany) at 15 kV. For this the silver nanoparticles sample was air dried prior to use after centrifugation (Devi *et al*., 2012).

RESULTS AND DISCUSSION

Plant parts were collected from various regions of Jabalpur city viz., campus of R.D. University, Dumna forest, and Bhedaghat. These plant samples were washed in running tap water for 15 minutes (Bussaban *et al*., 2001) and then surface sterilized (Schulz *et al*., 1993). These pieces were placed on PDA plates and incubated for growth of endophytic fungi. After incubation endophytic fungal isolates growing out form different parts of plants were separated and maintained in pure form on PDA slants for further use. As depicted in Table 1 a total of 8 endophytic fungal isolates (Figure 1) were obtained from different parts of four medicinal plants. Several researchers have isolated endophytic fungi from different medicinal plants for synthesis of silver nanoparticles. Tiwari *et al*. (2016) isolated endophytic fungi *Alternaria alternate* from medicinal plant *Pongamia pinnata* and used them for synthesis of silver nanoparticles. Similarly, an endophytic fungus *Fusarium solani* isolated from *Withania somnifera*was used for the synthesis of silver nanoparticles (Vijayan *et al*., 2016). On the basis of microscopic study of slides prepared by slide culture technique endophytic fungal isolates belonging to different genera namely, *Penicillium*, *Aspergillus*, *Alternaria* etc. were identified (Table 1). During identification help of some experts and available literature was undertaken. Images of pure culture of *Aspergillus* sp. (DSL#44) is depicted in Figure 2. In another study, endophytic fungus *Fusarium* sp. isolated from leaves of *Withania somnifera* was identified morphologically and used for silver nanoparticle synthesis (Singh *et al*., 2015).

Mycelia free water extracts of 8 endophytic fungal isolates were challenged with 1 mM silver nitrate solution and incubated for 72 hrs. After incubation a visible colour change in the water extracts of 3 endophytic fungal isolates namely, *Alternaria* sp. (LCL#20), *Aspergillus* sp. (DSL#44), *Penicillium* sp. (AML#75) was observed out of 8 reaction mixtures (Table 2) (Figure 3). These fungi were isolated from host plants *Lantana camara*, *Dalbergia sissoo* and *Aegle marmelos* respectively. The colour of these three reaction mixtures changed from pale yellow to reddish brown giving the first indication of synthesis of silver nanoparticles in the reaction mixture. The intensity of brown colour of above 3 samples varied from each other depending upon the difference in silver nanoparticles synthesis potential of different fungi. All brown colloidal solutions obtained above were then subjected to further characterization techniques for confirmation of the synthesis of silver nanoparticles.

Table 1. Isolation and Identification of Endophytic Fungal isolates by Slide Culture Technique.

S.No.	Host Plant	Endophtic Fungal Isolates	Identified Endophytic Fungi
1.	*Dalbergia sissoo*	DSL#44	*Aspergillus sp.*
		DSL#45	*Fusarium sp.*
2.	*Lantana camara*	LCL#20	*Alternaria sp.*
		LCL#21	*Curvulariasp.*
3.	*Aegle marmelos*	AML#75	*Penicillium sp.*
		AMS#76	*Nigrosporasp.*
4.	*Eucalyptus globules*	EGL#10	*Phomasp.*
		EGS#11	*Fusarium sp.*

Table 2. Biosynthesis of Silver Nanoparticles by Isolated Endophytic Fungi.

Host Plant	Endophytic Fungi	Silver Nanoparticles Sample
Dalbergia sissoo	*Aspergillus* sp. (DSL#44)	Sample 1
Lantana camara	*Alternaria* sp. (LCL#20)	Sample 2
Aegle marmelos	*Penicillium* sp. (AML#75)	Sample 3

Table 3. Antibacterial activity of synthesized silver nanoparticles.

S.No	Name of sample	Antibacterial activity in terms of zone inhibition (mm)			
		Escherichia coli(EC)	*Bacillus subtilis*(TCBS)	*Klebsiella pneumonia*(YC)	*Salmonella typhimurium*(XLD)
1.	Sample 1	4	6	9	5
2.	Sample 2	5	5	6	4
3.	Sample 3	6	7	7	5

Figure 1. Pure cultures of isolated Endophytic Fungi maintained on PDA slant.

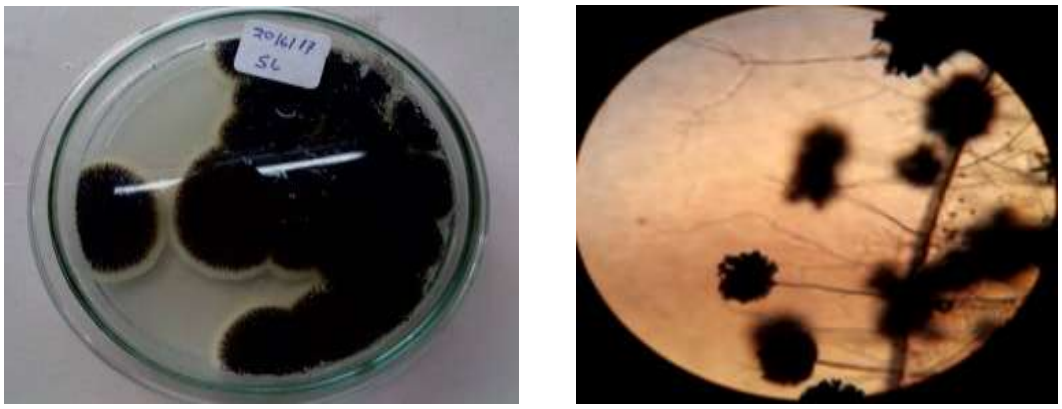

Figure 2. Pure culture and Microscopic images of the most potent endophytic fungus *Aspergillus sp.* (DSL44#).

Figure 3. Brown Coloured Reaction Mixtures of 3 Endophytic Fungal Isolates obtained during screening for Biosynthesis of Silver Nanoparticles.

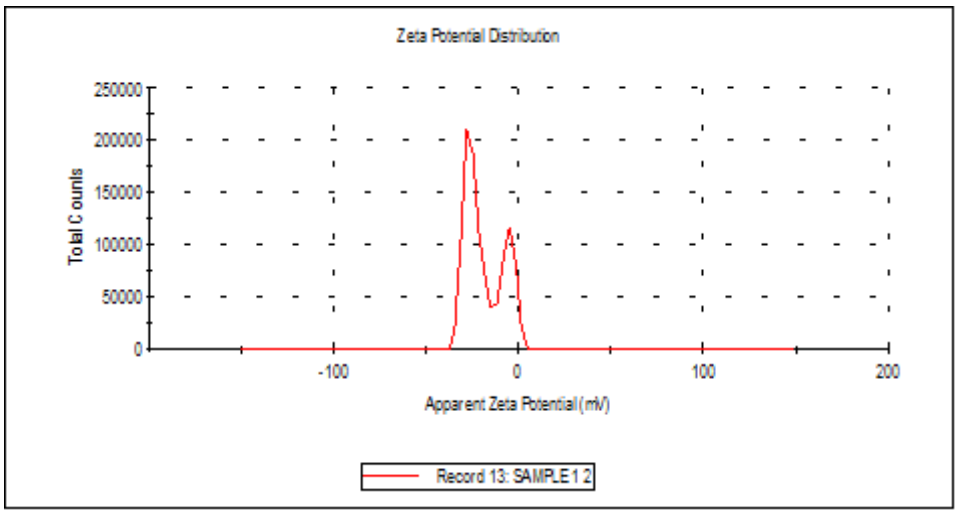

Figure 4. Zeta Potential Analysis of sample 2.

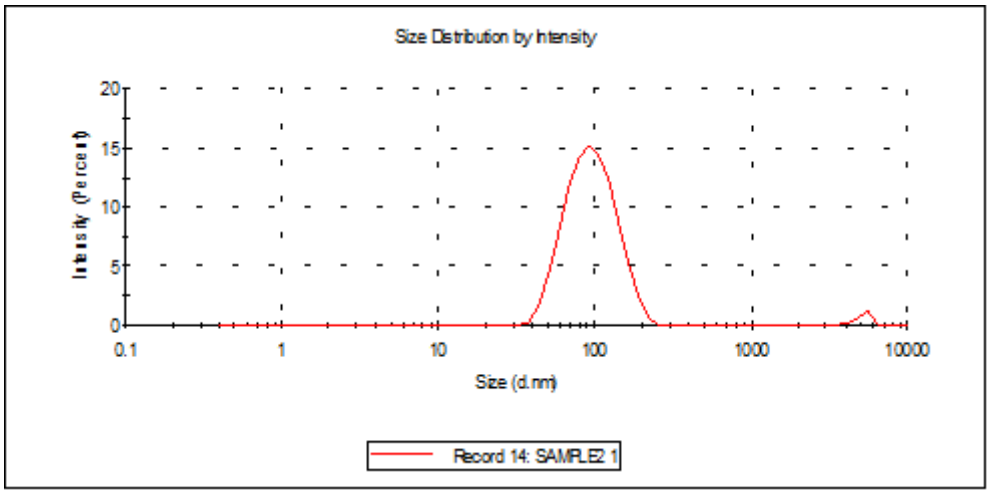

Figure 5. Dynamic Light Scattering (DLS) Analysis of sample 2.

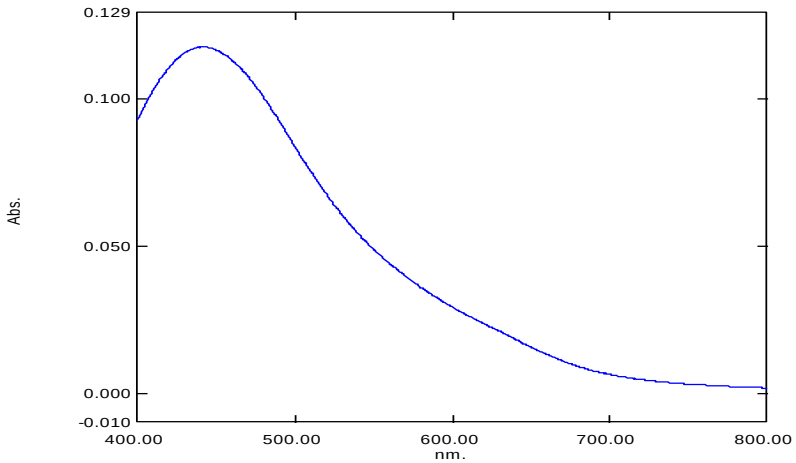

Figure 6. UV- Visible Spectroscopic Analysis of sample 2.

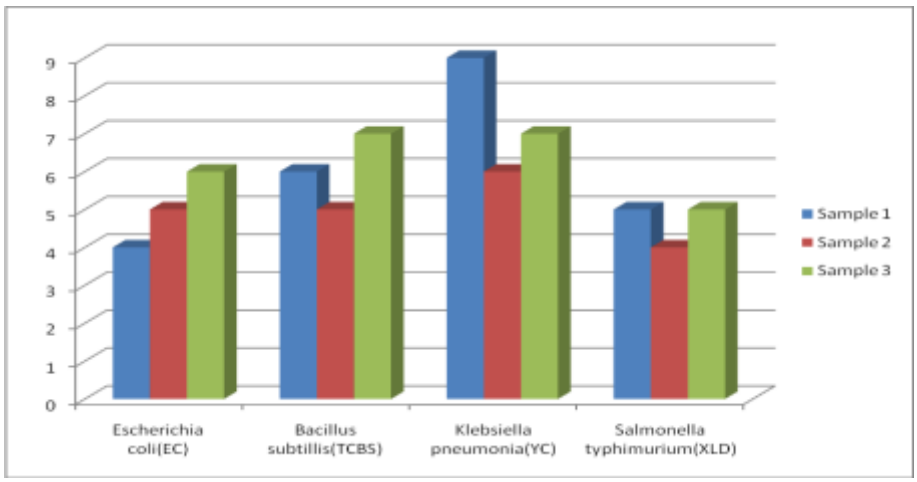

Figure 7. Graphical representation of antibacterial activity of samples.

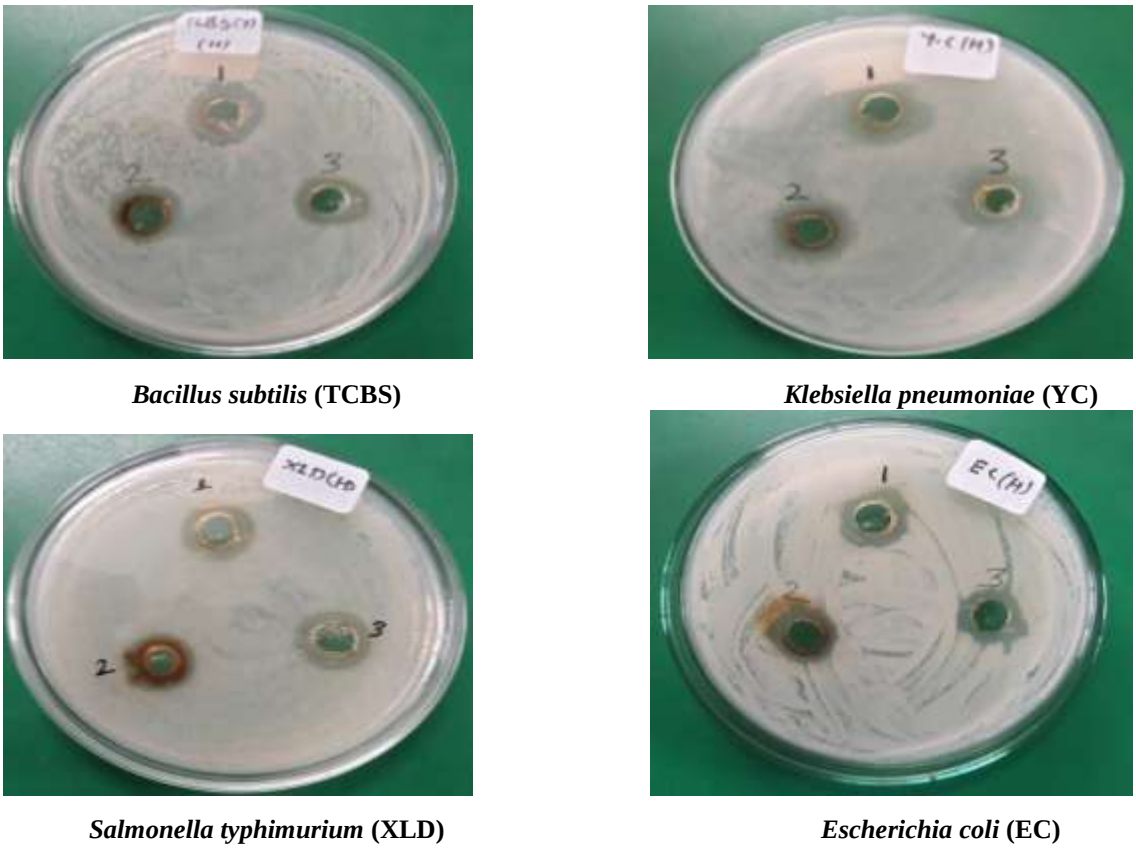

Figure 8. Antibacterial Activity Test of Synthesized Silver Nanoparticles.

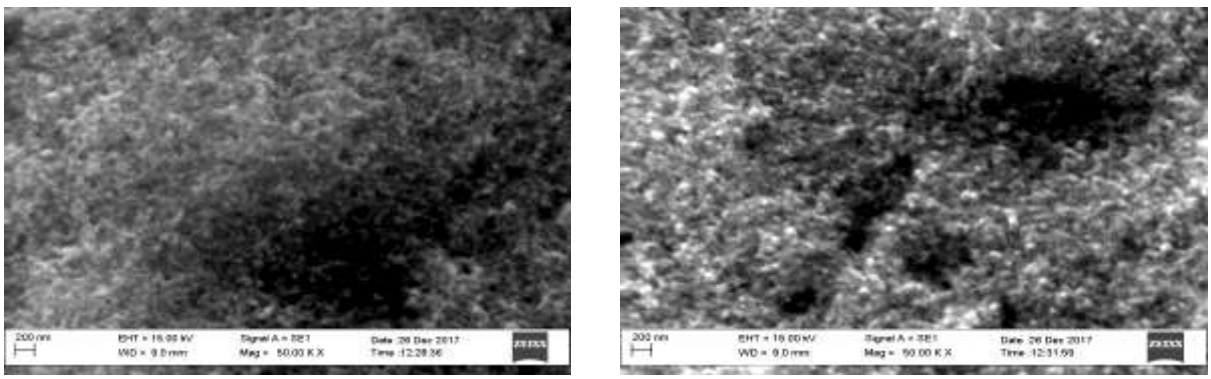

Figure 9. Scanning Electron Microscopic Images of Biosynthesized Silver Nanoparticles.

A control containing mixture of sterilized distilled water and 1mM silver nitrate was also incubated along with the reaction mixtures of the endophytic fungal isolates. No change in the colour of the control was observed after completion of incubation period thus indicating the presence of certain reducing agents in the water extracts of endophytic fungal isolates which were responsible for the reduction of silver nitrate in those reaction mixtures resulting in a visible colour change to brown solution. Patil *et al.* (2011) was studied on the extracellular biosynthesis of silver nanoparticles by a fungus, *Aspergillus flavus*on basis of change in colour (Pale yellow) of the filtrate. Similarly, Vardhana and Kathiravane, (2015) also observed synthesized silver nanoparticles by Endophytic fungi *Pestaloptiopsis pauciseta* with a change in colour of the medium from yellow to brown colour.

All brown colloidal solutions obtained in above experiment were examined for their antibacterial activity against 4 pathogenic bacteria viz., *Bacillus subtilis* (TCBS),

Escherichia coli (EC), *Klebsiella pneumoniae* (YC), *Salmonella typhimurium* (XLD). All samples exhibited considerably good activity against all test pathogenic bacterial strains (Plate 4). According to table 3 (Figure 4 and Figure 5) Sample 1 showed highest activity against all pathogenic bacterial strains. It gave maximum activity (9mm zone) against *Klebseilla pneumoniae* (YC) and lowest activity (5mm zone) against *Salmonella typhimurium* (XLD). Its activity against *Bacillus subtillis* (TCBS) and *Escherichia coli* (EC) was 6 mm and 5 mm respectively. On the other hand Sample 2 followed Sample 1 by showing maximum activity (6mm zone) against *Klebseilla pneumoniae* (YC) and lowest activity (4mm zone) against *Salmonella typhimurium* (XLD).

Its activity against *Bacillus subtillis* (TCBS) and *Escherichia coli* (EC) was 5mm and 5mm respectively. Sample 3 gave maximum activity (7mm zone) against *Bacillus subtilis* (TCBS) and lowest activity (5mm zone) against *Salmonella typhimurium* (XLD). Its activity against *Bacillus subtillis* (TCBS) and *Escherichia coli* (EC) was 7mm and 6mm respectively. Since highest activity was given by silver nanoparticles Sample 1 synthesized by *Aspergillus* sp. (DSL#44) isolated from *Dalbergia sissoo*, it was further subjected to various techniques for complete characterization. Singh *et al*. (2014) reported significant antibacterial activity of synthesized silver nanoparticles from *Tinospora cordifolia* against multi drug resistant strains of *Pseudomonas aeruginosa.* Similar study done by Shukla and Sandhu, (2017) were tested antibacterial potential of silver nanoparticles synthesized by different fungal strains of Alternaria species against *Escherichia coli, Bacillus subtilis*, *Salmonella typhimurium, Enterococcus* sp. and *Klebsiella pneumoniae* also reported maximum activity against *E. coli, B. subtillis* and *S. typhimuriun.*

The sample 1 was subjected to characterization techniques as stated below for further confirmation and determination of physicochemical properties of the sample. Absorption spectra's obtained after UV-Visible spectroscopic analysis of brown colloidal solution contained a maximum absorption peak at a wavelength falling in the characteristic wavelength absorption range (300-800 nm) of silver nanoparticles, thus confirming the presence of silver nanoparticles in the sample. This characteristic absorption range is due to the Surface Plasmon Resonance Phenomenon (SPR) exhibited by silver nanoparticles on exposure to UV-VIS light. This peak is therefore called as SPR peak. The maximum absorbance of 0.117 at a wavelength 444 nm was observed (Figure 6). Netala *et al*. (2016) confirmed synthesis of silver nanoparticles by an endophytic fungus *Aspergillus versicolor* by obtaining a surface plasmon resonance peak at 429 nm. On the other hand silver nanoparticles synthesized by using endophytic fungus *Alternaria sp.* exhibited an intense peak at 426 nm during the UV-VIS Spectroscopic analysis (Singh *et al*., 2017). DLS was employed to determine average size distributions and a more precise quantity of monodispersity in the colloidal solution. The differential intensity, number and zeta potential related to particle size distributions of the biosynthesized silver nanoparticles were obtained from DLS analysis. As depicted in the graph obtained after DLS analysis of sample 1, two peaks were observed. The mean value of these two peaks was calculated to determine the average size distribution of silver nanoparticles present in all samples. The average particle size distribution of Sample 1 was 88nm (Figure 7).

In another study by Ramalingam *et al*. (2016) the average size of silver nanoparticles synthesized by endophytic fungus *Curvularia lunata* was revealed to be 64.3 nm. Similarly, Anandalakshmi *et al.* (2016) analyzed the average size of silver nanoparticles synthesized by *Pedalium murex*was revealed to be 73.14nm. It is used to determine the surface charge/stability of silver nanoparticles present in brown colloidal solution. As shown in graphical result the zeta potential value of sample 1 was in negative range which clearly reveals that silver nanoparticles synthesized are stable. The zeta potential of Sample 1 was -22.0 mV (Figure 8). Zeta potential of silver nanoparticles synthesized by using aqueous silk fibroin obtained from *Bombyx mori* was -33.6 mV (Shivananda *et al*. 2016). In a similar study by Ramalingam *et al.* (2015) the zeta potential of silver nanoparticles synthesized by an endophytic fungus *Curvularia lunata* was found to be -26.6 mV.

According to SEM images obtained, silver nanoparticles synthesized in the present study were of irregular morphology with many shapes like square, rod and spherical. The SEM image also showed aggregation of silver nanoparticles. Their size was estimated to range between 25 to 35nm (Figure 9). Similar study done by Ramalingam *et al* 2015 obtained the spherical shape silver nanoparticles between the average size of 26nm synthesized by Endophytic Fungus, *Curvularia lunata.* SEM analysis done by Govindappa *et al* 2016 shows uniformly distributed silver nanoparticles synthesized by endophytic fungi, Penicillium species of *Glycosmis mauritiana*. The AgNPs were in spherical shape with particle size was observed from two locations at 50 K magnification and they are 65.92 and 64.64 nm.

CONCLUSION

Thus from the present study it could be inferred that endophytic fungal flora has great potential for the synthesis of silver nanoparticles having efficient antibacterial activity because of their unique physical and chemical properties. Silver nanoparticles synthesized in this work showed considerable activity against all pathogenic bacterial strains, hence could be developed as novel broad spectrum antibiotics.

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