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Isolation and Identification of Thermo Tolerant Endophytic Fungi from Melia dubia and Synthesis of Zinc Nano Particles

Sri vidhya S[1]* and Kalyanaraman Rajagopal[2]

[1]Department of Biotechnology, School of life sciences,
Vels University (VISTAS), Chennai-600117, India.

Abstract

In this study the synthesis of zinc nano particles by thermo tolerant endophytic fungi Cladosporium sp is reported. The endophytic fungus was isolated from the leaf of Melia dubia. The ZnO nano particles have been synthesized by precipitation method UV-vis absorption spectroscopy, FTIR, X-ray diffraction and Scanning Electron Microscopy were used to characterize the zinc nano particles. XRD patterns confirm the crystalline nature of ZnO nano particles with the particle size around 1.4511nm with the intense peak located at 39.93°. The SEM result confirms that the nano particles were ellipsoidal, rod shape; irregular and the particle size were within 50-100nm. The uv-vis spectro photometry shows the maximum absorbance at 230nm with the optical density of 1.50. The obtained FTIR results shows that the proteins present in the fungal filtrate played main role in both bio-reduction and stabilization of zinc nanoparticles with the maximum peak observed at 2301.08 cm^{-1} due to O-H stretching and deformation.

Keywords: Thermo tolerant endophytic fungi, ZnO nano particles

1. INRODUCTION

Endophytes are microorganisms (bacteria and fungi) that are present in living tissue of various plants like root, fruit, stem, seed, leaf etc. establishing mutual relationship without apparently any symptoms of diseases. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites. Medicinal plants are known to harbor endophytic fungi that are believed to be associated with the production of pharmaceutical products. Therefore, it is important to explore endophytic mycoflora in the medicinal plants. (Sardul Singh

Sandhu *et al* 2014). Heat tolerant endophytic fungi are currently classified into two sub groups, namely thermophilic and thermo tolerants. (Cooney and Emerson 1964). A thermo tolerant fungus is the one that has a thermal maximum near 50°c and a minimum well below 20°c. (Jean Mouchacca., 2007).

Nanoparticles are the clusters of atoms in the size range of 1–100nm. In this size range, materials often develop useful attributes that are distinct from the properties of the bulk material. Metal particles in the nanometre size exhibit unique physical properties that are different from both the ion and the bulk material. (Devi *et al* 2015). Recent studies on the use of microorganisms in the synthesis of nanoparticles are a new and exciting area of research with considerable potential for development. A variety of techniques have been developed to synthesize metal nanoparticles. Zinc nanoparticles have wide applications in Textile fibers, Energy conservation, Electronics Chemical gas sensing, Sunscreen, Paints, Catalyst and in Fuel cells. (Pavani et al., 2011). ZnO has been one of the most promising materials for electrical devices, including transparent conductive films, light emitting diodes and photocatalyst. ZnO can be synthesized practically into different nano forms (Latif et al.,2012).The preparation of ZnO nanopowders have been developed, namely, sol-gel, microemulsion, thermal decomposition of organic precursor, spray pyrolysis, electrodeposition, ultra- sonic, microwave-assisted techniques, chemical vapor deposition, and hydrothermal and precipitation methods. Talam *et al*., 2012. The main objective of this research is to find the biological method or synthesis of Zinc oxide nanoparticles from thermo tolerant endophytic fungi and characterize using UV-VIS spectrophotometry, SEM, XRD and FTIR.

2. MATERIALS AND METHODS

2.1 COLLECTION OF PLANT MATERIAL*:*

The *Melia dubia* plant leaves were collected from Madhavaram and Hasthinapuram in Government Park in Tamilnadu. Each sample was tagged and placed in separate sterile zip lock polythene bags, brought back to the lab and processed within 24 hours of collection.

2.2 STERILIZATION OF SELECTED PLANT PARTS:

The Plant samples were washed in running tap water for 15 minutes (Bussaban *et al.,* 2001). The leaf segments were cut into small pieces (5mm-8mm) and surface disinfected by sequential washes in 70% (v/v) ethanol (1min) and 4.0% (v/v) NaOCl (2min) and then rinsed with sterile distilled water and allowed to dry under sterile condition (Schulz *et al.,* 1993). Final sterilization of explant was performed individually using 0.1% mercuric chloride for 3 min and rinsed thoroughly with sterile distilled water. All operations were carried out inside the laminar hood.

2.3 INOCULATION OF STERILIZED LEAF SEGMENTS IN TO THE MEDIA*:*

The leaf segments were plated on Potato dextrose agar (39g/l) medium supplemented with streptomycin (Himedia 100mg L-1). 4 to 6 segments were placed on each plates were incubated in a light chamber for two weeks at 12 hours light/dark cycles at 23°c (Rodrigues.,2008). The incubation temperature is maintained at 45±2°**c.** The inoculated plates were incubated for 2-3 weeks for endophytic growth. The fungal growth is monitored and frequently colonized fungi were slotted. (Redman *et al*., 2001; Marquez *et al*., 2007; Rodriguez *et al*., 2008).

2.4 ISOLATION OF ENDOPHYTIC FUNGI:

After incubation for 2-3 weeks individual fungal colonies were picked from the edge of an advancing colony with a sterile fine tipped needle under sterro-binocular microscope and transferred onto potato dextrose agar (Suryanarayanan, 2003).

2.5 IDENTIFICATION OF ENDOPHYTIC FUNGI:

Species identification was carried out based on the morphological and taxonomic keys provided by (*Hyde, 2004*). Isolations of endophytic thermo tolerant were maintained in slants on PDA (potatoes in fusion form 200g/litre, dextrose 20g/litre, agar 15grams and pH 5.1) of the medium and the mass culture was raised in PDB (potatoes in fusion form 200g/litre, dextrose 20g/litre and pH 5.1) for further studies. (Krings., 2007).

2.6 BIO SYNTHESIS OF ZINC NANOPARTICLES:

For the synthesis of zinc nanoparticles, the biomass of *Cladosporium* sp were grown aerobically in potato dextrose broth containing infusion of 250 g potato and 20 g dextrose per litre of distilled water. The inoculated flasks were incubated on orbital shaker at 25±2°C and agitated at 120 rpm for 96 h. The biomass was harvested after incubation by filtering through filter paper followed by repeated washing with distilled water to remove any medium component from the biomass. 10 g (wet weight) was brought in contact with 100 mL of sterilized double distilled water for 48 h at 25 ± 2 ° C in a 250 mL Erlenmeyer flask and agitated again at 120 rpm. After the incubation, the cell filtrate was obtained by filtering it through Whattman filter paper. The filtrates were treated with 1 mM of zinc oxide (Sigma Aldrich) solution in an Erlenmeyer flask and incubated at room temperature in dark. Control containing cell-free filtrate without zinc oxide solution was also run as standard. (Pavani., 2012, Refaz Ahmad Dar., 2015).

2.7 UV–VISIBLE SPECTROSCOPY ANALYSIS:

Change in colour of the mycelium free filtrate incubated with 1 mM zinc oxide solution visually observed over a period of time indicates the bio reduction of zinc ions to zinc nanoparticles. The zinc nanoparticles formed in the mycelium free fungal filtrate were monitored by sampling of aliquots (1mL) at different time intervals (24hrs, 48hrs, 72hrs and 96hrs). Absorption measurements were carried out on UV–visible spectro photometer (CARY-100 BIO UV–vis spectrophotometer; Varian Inc.,

USA) at a resolution of 1 nm between 200 and 800 nm ranges. (Pavani.,2011, Lamabam Sophiya Devi ., 2015).

2.8 SEM STUDIES:

In order to carry out SEM analysis, zinc nanoparticles solutions were centrifuged for 20 min at 10,000 rpm and drop coated on to thin glass film. Compositional analysis and the conformation of presence of elemental zinc was carried out through Energy dispersive X-ray Spectroscopy (EDS) using the SEM equipped with an EDAX attachment (Oxford, London). The endophytic fungi was grown in 1.0mmol of zinc oxide and after 48 hrs, the filtrate embedded with zinc nanoparticles was dried under vacuum at 80°**c** for 3 hours. Samples were then examined using the Scanning Electron Microscope (JSM-6360, JEOL). The Presence of extracellular Zinc nanoparticles were analyzed (Lamabam Sophiya Devi; 2015).

2.9 X-RAY DIFFRACTION (XRD) STUDIES:

XRD analysis was carried out to reveal the crystalline nature of zinc nanoparticles. The crude extract of endophytic fungi *cladosporium sp* with zinc oxide of 1mM was dried at 80°C in oven for 2-3 hours and the yield was about 91%. The crystalline structure and morphology of fungal zinc oxide powder was assessed by XRD. The distance between the crystal planes (d) is analysed using the Bragg's law $\mathbf{d = n\lambda/2\sin\theta}$**.** (Shimadzu XRD-6000) was with copper radiation (Cu K_{α}, 1.5406 Å) as incident radiation and with Atomic Force Microscopy (AFM). (Satyanarayana Talam; 2012).

2.10 (FTIR)-FOURIER TRANSFORM INFRARED SPECTROSCOPY STUDIES*:*

The crude extracts of endophytic fungus *cladosporium sp* with 1mM of zinc oxide was analysed by FTIR to know the different functional groups present in the fungal extracts of *cladosporium sp* which is dried at 80°C and then subjected to analyze in FTIR in which the diffuse reflectance technique was followed. The samples were irradiated by a broad spectrum of infrared light and the level of absorbance at a particular frequency was plotted after Fourier transformation of the data. The resulting spectrum was characteristic of the organic molecule present in the sample. The absorbance was measured at 400-600 nm for the identification and quantification of organic species. (Shakeel Ahmed *et al*; 2016, Latif *et al;* 2011).

3. RESULTS AND DISCUSSION:

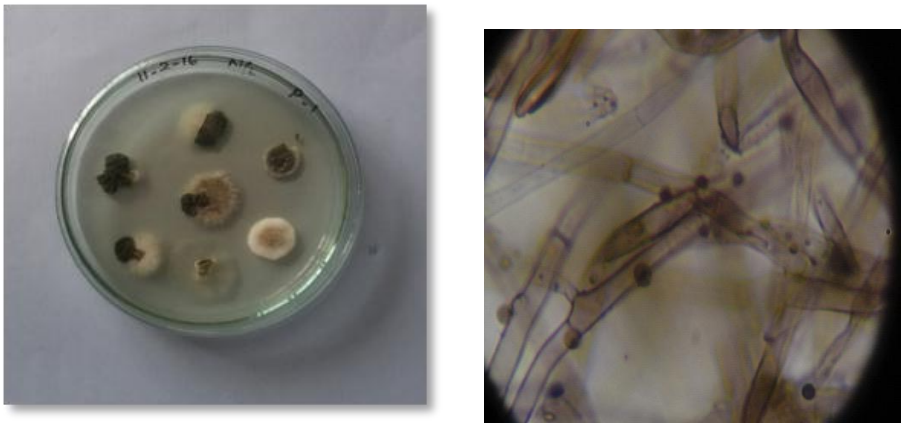
3.1 ISOLATION OF THERMO TOLERANT ENDOPHYTIC FUNGI FROM *MELIA DUBIA*

200 leaf segments were surface sterilized and screened for thermo tolerant endophytes as mentioned in materials and methods. The incubation temperature is maintained under 45±2°c. A total number of 7 isolates of the thermo tolerant fungal endophytes belonging to 4 species were isolated as shown in (Table 1). Most of the studies on endophytic fungi were carried out at the room temperature. Since no report has been

carried out on the thermo tolerant endophytic fungi associated with angiosperms. This study was carried at elevated temperature at 45ºc. To our knowledge for the first time endophytes are isolated at 45ºc.

Table 1: Colonization Frequency of Thermo Tolerant Endophytic Fungi

NAME OF THE ENDOPHYTIC FUNGI	NO OF ISOLATES
Cladosporium sp	23
Nigrospora oryzae	12
Sterile form I	10
Sterile form II	5



(a) (b)

Figure 1. (a) Microscopic observation of Cladosporium SP (b) Growth of Endophytic fungi on leaf segments

3.2 UV VIS SPECTROSCOPY ANALYSIS

UV stability of the nanoparticles formed in the broth was confirmed by using UV-VIS Spectroscopy. When the filtered broth sample was analyzed in the wavelength range between 200nm to 260nm. The spectral analysis was performed at different concentrations of zinc oxide ranging from (0.1mM-2mM). After 24hrs, 48hrs, 72hrs, and 96 hrs of incubation. The maximum absorbance was observed at 230nm after 96hrs of incubation. The absorbance above 260nm was found to be negative. The

sample was read at 200-300nm. The zinc nano particles have been synthesized by Beer-Lambert law.

$$A = -\log (\%T/100\%)$$

Mathematically, absorbance is related to percentage transmittance T by the expression:

$$A = \log10 (Io/I) = \log10 (100/T) = kcL$$

Where L is the length of the radiation path through the sample, c is the concentration of absorbing molecules in that path, and k is the extinction coefficient, a constant dependent only on the nature of the molecule and the wavelength of the radiation. The transmittance of our sample fell from 75 to 56.25% when the incubation period is increased from 24 hours to 96 hours. The (OD) optical density of the zinc nano particles synthesized by endophytic thermo tolerant fungi *Cladosporium sp* is 1.50 at 230nm (Fig 2). UV- visible absorption spectroscopy is widely being used technique to examine the optical properties of nano sized particles (Talam *et al.,* 2012). The spectroscopic analysis in this study showed maximum peak at 230nm indicating the presence of nano particles in the broth, since variation was not observed at maximum absorbance, the zinc nano particles were confirmed and appeared to be stable.

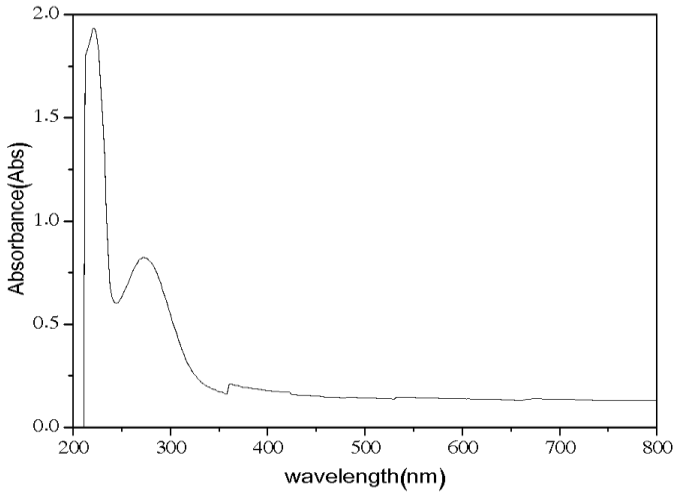
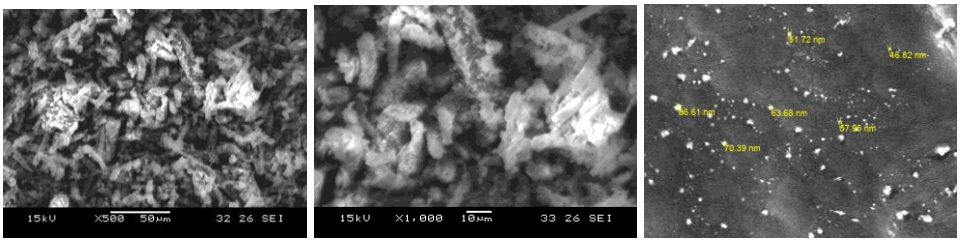


Figure 2. UV-VIS absorption spectrum of zinc nano particles

3.3 SEM ANALYSIS

The morphology and size of the synthesized nano particles was analysed using (SEM) Scanning electron microscope. The results are presented in (Plate 2a-2c). This study also confirms the formation of zinc nano particles. The study also indicates that the shape of the nano particles is irregular and some of them are rod like. (Plate 2a). The study also confirms that the size of the nano particles is around 50-100nm. The zinc nano particles synthesized by endophytic fungi *Cladosporium sp* has the micron

markers of 1µm to 10µm with the magnification ranging from 500X to 10000X. Scanning Electron Microscopy performed in the present study has provided further insight into the morphology and size details of the synthesized nano particles. SEM micrograph revealed the formation of nano particles in the size range of 50-100nm. The picture confirms the formation of zinc nano particles (Fig 3). Significant improvement in the mono dispersity of gold nano particles is achieved using Actinomycetes (Mukherjee *et al.,* 2002). The size and dispersity control may be due to interaction of different proteins with metal nano particles. It is evident from reports that reduction of zinc ions is due to the metabolites excreted extracellularly by *Klebsiella pneumonia, Escherichia coli and Enterobacter cloacae* (Minaeian., 2008). The morphology of the nano particles is variable with majority of them being rod, similar observations were made in other studies, however the particle size and shape varies (Vipul Bansal *et al*., 2004; Talam *et al*., 2012).



(a) (b) (c)

Figure 3. Sem analysis of zinc nano particles synthesized by *cladosporium* sp. (a) Sem pictures of zinc nano particles at 500X magnification; (b) Sem pictures of zinc nano particles at 1000X magnification; (c) Sem pictures of zinc nano particles at 500X magnification with particle ranging from 50-100nm.

3.4 XRD ANALYSIS

The X-ray diffraction pattern of zinc oxide Nano powder synthesized by endophytic fungi *Cladosporium sp* is represented in figure 3. A definite line broadening of the XRD peaks indicates that the prepared material consist of particles in nano scale range. The diffraction peaks located at 32.84◦(220), 34.52◦(220), 36.33◦(221), 39.93◦(311), 48.21◦(400), 57.96◦(331), 59.13◦(420), 63.51◦(422) and 69.18◦(422) have been keenly indexed as hexagonal wurtzite phase of Zinc oxide. Some characteristic peaks of impurities were detected, suggesting that ZnO NPs were little impure. The average crystallite size (*d*) of ZnO NPs was estimated by Scherrer's formula:

$$d = n\lambda/2\sin\theta$$

Here **Θ** is the Bragg diffraction angle *n* is an integer representing the number of wavelengths required for constructive interference to occur. At the smallest angle of incidence (θ) for a maxima *n* = 1, the average particle size of the sample was found to be 1.4511nm which is derived from the more intense peak corresponding to 311 plane located at 39.93◦ using Scherrer's formula. The XRD analysis was carried out to find

the nano scale size of the particles based on peak intensity. Similar studies were made for zinc nano particles (Talam *et al.,* 2012). By considering the dominant diffraction peak of zinc oxide nano particle the crystalline size of zinc oxide was calculated as 1.4511nm corresponding to 311 plane located at 39.93º with the lattice point at 4.0541Å (Fig 4). The agglomerations of smaller nano particles occur due to the fact that we are dealing with the biological material show such agglomeration. (Dobrucka and Dlugaszewska., 2015). Similar situation was observed in the present study.

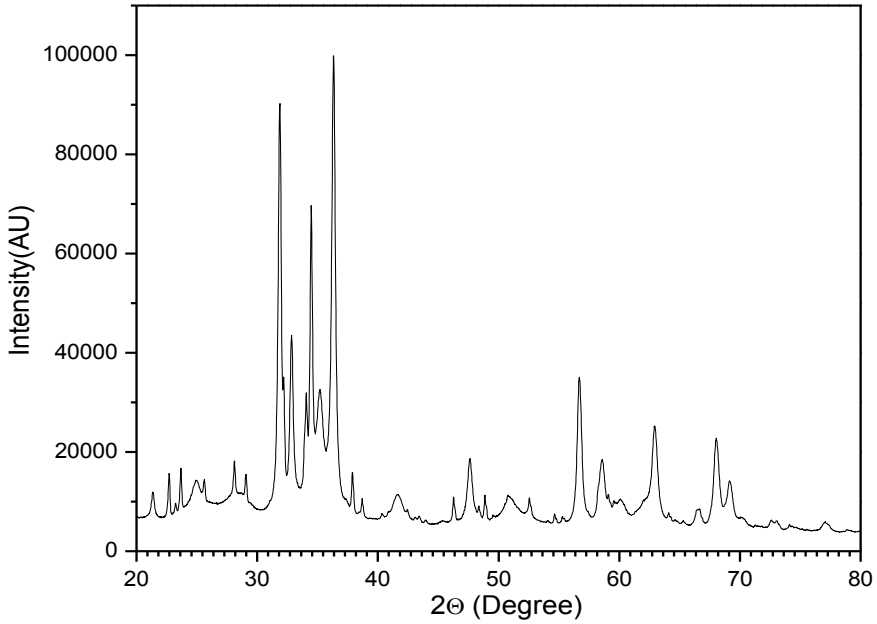


Figure 4. XRD pattern of synthesized nano particle by *Cladosporium* sp

3.5 FTIR SPECTROSCOPY

FTIR studies were carried out in order to ascertain the purity and nature of the metal nanoparticles. The dual role of the zinc oxide nano particles synthesized by *Cladosporium sp* as a reducing and capping agent and presence of some functional groups was confirmed by FTIR analysis of zinc nano particle. The FTIR spectrum of the ZnO nanoparticles synthesized by endophytic thermo tolerant fungi *Cladosporium sp* was aquired in the range of 500-4000 cm^{-1}. From this FTIR we can also observe that increasing the annealing temperature sharpens the characteristic peaks for metal oxide, sugesting that, the hexagonal nature of ZnO increases on increasing the temperature. The peaks in the range of 2301.08-1986.05cm^{-1} corresponds to the C=O bonds. The adsorbed band at 1892.02 cm^{-1} is assigned to O-H bending vibrations. The peak at 2301.08 cm^{-1} corresponds to C=O and O-H bending vibrations diminishes gradually for sample annealed at higher temperature. Similar observation was made in

other investigations (Parthasarathi and Thilagavathi 2012; Harish and Renu., 2013). Therefore the synthesized nano particle was surrounded by metabolites and protein moieties. This phenomenon is called capping of nano particle which may perform dual functions of formation and stabilization of zinc oxide nano particles.

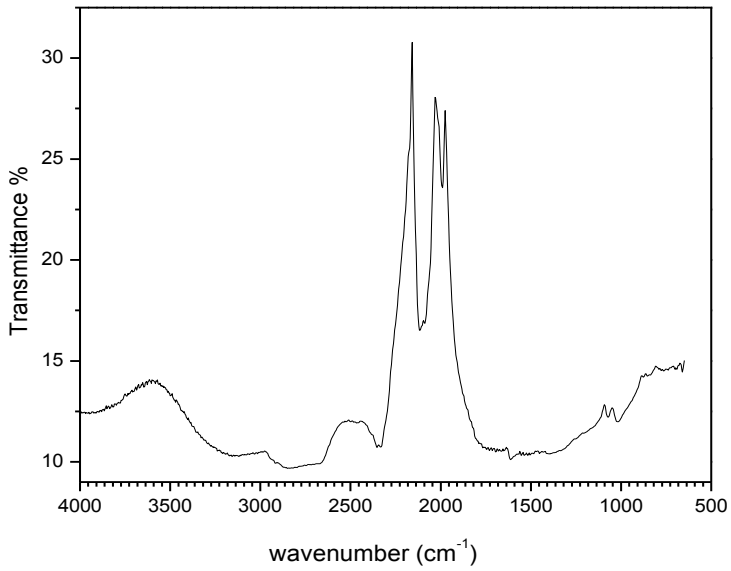


Figure 5. FTIR pattern of zinc nano particles synthesized by *Cladosporium* sp

CONCLUSION

To conclude, *Melia dubia* leaves were the first time screened for the thermo tolerant endophytic fungi and synthesis of ZnO nano particles. The nano particles were synthesized using the mycelial free filtrate of *Cladosporium* sp. The study proved the formation of ZnO nano particle by an reduction method. However the molecular mechanism of formation of nano particle and their interaction needed to be analyzed for the exact mechanism of nano particle synthesis.

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