Study of the Antimicrobial activity of Trichoderma-Silver fused Nanoparticles (TR-Ag Np^0) against pathogenic bacterial and fungal strains

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***Abstract*-** The genus, *Trichoderma* and its metabolites were meant for antimicrobial activity against the microbial strain and thus it is considered to be natural antimicrobial agents. Silver is also known as an antimicrobial agent and is utilized in several antimicrobials and medications. In the present investigation, the nano-particles were prepared both of *Trichoderma* and Ag^+ separately and further after fusing *Trichoderma* and Ag^+. *Trichoderma harzianum* secretes secondary metabolites which act as a capping and reducing agent. The biosynthesized silver nanoparticles (AgNPs) were characterized by UV–Vis spectroscopy and Transmission electron microscopy (TEM). UV–Vis spectra of silver and *Trichoderma* filtrate showed absorption spectra at 450 nm and 430 nm respectively while fused nanoparticles showed absorption spectra at 415 nm corresponding to the surface plasmon resonance of silver nanoparticles. The size and morphology of the fused nanoparticles was determined by TEM, which shows the formation of spherical nanoparticles in the size range of 8–24 nm. The antibacterial activity of biosynthesized AgNPs, *Trichoderma* filtrate and fused TR-Ag^0 nanoparticles were evaluated by measuring the diameter of zone of inhibition against pathogenic microbial strains and drug resistant *Staphylococcus aureus*. It was also observed that, fused nanoparticles of Trichoderma-Ag^0 were having prominent antimicrobial activity in comparison to individual silver nanoparticles.

***Index Terms*-** Nanoparticles, antimicrobial activity, silver, Trichoderma, secondary metabolites.

I. INTRODUCTION

The anamorphic fungal genus *Trichoderma* (*Hypocreales*, *Ascomycota*) is cosmopolitan in soils and on decaying wood and other forms of plant organic matter [1]. *Trichoderma* species were among the most widely distributed and common fungi in nature and exist in climates ranging from the tundra to the tropics. This may be attributable to their diverse metabolic capability and aggressively competitive nature [2, 3]. Rapid growth rates in culture and the production of numerous spores (conidia) that were mostly varying shades of green characterize fungi in this genus. A growing number of teleomorphs in *Hypocrea* have been linked to commonly occurring *Trichoderma* anamorphs, but most strains of *Trichoderma* were classified as imperfect fungi because they have not been associated with a sexual state. Interestingly, *Hypocrea/Trichoderma* spp. were even able to induce systemic resistance, which is characterized by the occurrence of disease control in the plant at a site distant from the location of *Hypocrea/Trichoderma*. Trichoderma species have been described as biological control agents against fungal and bacterial pathogens [4, 5]. They stimulate the production of low-molecular weight compounds that have antimicrobial activity like e.g. phytoalexins which were normally produced by plants in response to an attack by pathogens. A large were a of interest in biocontrol is the reduction of plant diseases caused by soil-borne and foliar plant pathogenic fungi. Roughly 70% of all the major crop diseases were caused by fungi, or the fungus-like Oomycota. Notorious examples were species belonging to the genera *Rhizoctonia*, *Botrytis*, *Phytophthora*, *Pythium*, *Sclerotinia* and *Fusarium*. Most of the formulations of commercially available biocontrol products against plant pathogenic fungi contain the bacteria *Pseudomonas* and *Bacillus* or fungi belonging to the genus *Hypocrea/Trichoderma. Hypocrea/Trichoderma* spp. produces a wide range of enzymes for degradation of homo- and hetero-polysaccharides, which were designative for their broad spectrum of substrate utilization and their ubiquitous occurrence in nature. Furthermore they possess a wide spectrum of proteases which help them in the defense of their habitats and the competition for nutrients with other microorganisms. Biological synthesis of metal nanoparticles involving the use of microbes is easy, cost-effective and eco-friendly technique. These nano-particles were effective, bio-compatible and bio-degradable [6]. Silver compounds have been used to treat burns, wounds and infections. Various salts of silver and their derivatives were used as antimicrobial agents [7, 8]. Recent studies have reported that nano-sized silver particles exhibit antimicrobial properties [9, 10]. Since *Trichoderma* sp. as a natural source and Ag+ as synthetic source both have antimicrobial effect against the variety of pathogens. The present study described the effect of Trichoderma-Ag fused nanoparticles (TR-Ag Nps) on the variety of bacterial and fungal pathogens.

II. MATERIALS AND METHODS

Isolation of Trichoderma species from soil samples

To isolate *Trichoderma* from soil, selective medium was prepared [11]. The basal medium consisted of 0.2 g MgSO4 (7H2O), 0.9 g K2HPO4, 0.15 g KCl, 1.0 g NH4NO3, 3.0 g D-

glucose anhydrous, 0.15 g Rose Bengal and 20g agar. These constituents were added to 950 ml of distilled water and autoclaved at 121°C for 30 minutes. The biocidal ingredients, 0.25g tetracycline were mixed in 50 ml of sterilized distilled water and added to the autoclaved basal medium where it cooled to 40 to 50°C. 10 grams of soil were suspended in 50 ml of sterile distilled water and agitated for 30 minutes at 200 rpm in a rotary shaker. Serial dilutions were made and 0.1 ml of each was spread on the *Trichoderma* selective medium plates with a glass rod. Three plates of each sample were prepared and incubated for 5 days at 30°C. *Trichoderma* isolates were collected and transferred onto potato dextrose agar (PDA) plates for maintaining pure culture [12].

Cultivation and culture conditions

Trichoderma cultures obtained were cultivated and maintained on slants of potato dextrose agar for 5 days at 28°C. Three hundred milliliters flasks were incubated for 14 days at 28°C on a laboratory incubator. The fungal biomass was collected for further use.

Microscopic and taxonomic identification of Trichoderma culture

Two techniques, visual observation on petri dishes and micro-morphological studies in slide culture, were adopted for identification of Trichoderma species. For visual observation, the isolates were grown on PDA agar for 3-5 days. The mode of mycelia growth, colour, odour and changes of medium colour for each isolate were examined every day. For micro-morphological studies, a slide culture technique was used [13]. Examination of the shape, size, arrangement and development of conidiophores or phialides provided a tentative identification of Trichoderma spp. Samples were compared to a taxonomic key for the genus Trichoderma [14] and further verified and confirmed by National Centre of Fungal Taxonomy, New Delhi, India.

Preparation of Trichoderma filtrate

Trichoderma was inoculated in liquid nutrient medium containing consisted of 0.2 g MgSO4 (7H2O), 0.9 g K2HPO4, 0.15 g KCl, 1.0 g NH4NO3, 3.0 g D-glucose anhydrous, 0.15 g Rose Bengal and 0.25 g tetracycline per 50 ml of sterilized distilled water and added to the autoclaved basal medium where it is cooled to 40 to 50°C. The culture was then after placed in incubator shaker at 200 rpm at 28^0C for 5 days. Further filtered by Whatman's filter paper No. 42 to obtain filtrate. Then the mycelium and filtrate were separately subjected to solvent extraction.

Extraction of the filtrate

The filtrate of each fungus was extracted several times with ethyl acetate (*v/v*) in a separating funnel. The extracts from both mycelia and filtrate were evaporated under vaccum at 50^0C till dryness. The obtained solid material was dissolved in ethyl acetate to form the crude extract and tested for antimicrobial activity. For day optimization the fungus was grown in the malt extract medium at pH 6.2. Inoculated flasks were incubated at 27^0C on an incubator shaker for 8 days. The biomass production was determined each day for antibacterial activity.

Preparation of silver nanoparticles

For the preparation of silver nanoparticles two stabilizing agents, sodium dodecyl sulphate (SDS) and sodium citrate were used. For the synthesis of silver nanoparticles, silver nitrate solution (from 1.0 mM to 6.0 mM) and 8% (w/w) sodium dodecyl sulphate (SDS) were used as a metal salt precursor and a stabilizing agent, respectively. Hydrazine hydrate solution with a concentrate ranging from 2.0 mM to 12 mM and sodium citrate (1. 0 mM to 2.0 mM) were used as reducing agents. Citrate of sodium was used as stabilizing agent at room temperature. The transparentcolorless solution was converted to the characteristic pale yellow and pale red colour, when citrate of sodium was used as stabilizing agent. The occurrence of colour was indicated by the formation of silver nanoparticles. The silver nanoparticles were purified by centrifugation. To remove excess silver ions, the silver colloids were washed at least three times with deionized water under nitrogen stream. A dried powder of the nanosize silver was obtained by freeze-drying [15-19].

Preparation of Trichoderma fused Silver nanoparticles

The silver nitrate (1 mM) solution was prepared in 50 ml deionized water. Fungal biomass (5 g) was brought in contact with the silver nitrate solution in a 200 ml Erlenmeyer flask. The solution was then kept in dark condition at 29±1 ^{0}C under continuous shaking at 200 rpm for 72 h. After 72 h of reaction time the colour change was observed [20-23].

Characterization of prepwered nanoparticles via UV-absorption spectra and Transmission electron microscopy (TEM)

The formation of AgNPs and TR-AgNPs by the bioreduction of Ag+ to Ag^0 was easily monitored using UV–Vis spectroscopy. The scanning was performed in the range of 200–700 nm. The morphology and size were determined by TEM [24-28].

Determination of antimicrobial activity of Trichoderma-Ag* fused nanoparticles against the pathogens

The antimicrobial activity of Trichoderma extract and Trichoderma fused silver nanoparticles was determined at different concentrations against local isolated pathogenic cultures viz. *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Micrococcus luteus*, *Leuconostoc mesentroides*, *Aspergillus niger* and *Aspergillus ochraceous* by well diffusion method [29].

III. RESULTS AND DISCUSSION

Isolation of Trichoderma species from soil samples

The present study showed the isolation and characterization of Trichoderma spp. culture isolated from soil. Further the culture was determined taxonomically and was determined as *Trichoderma harzianum* and was denoted as per the accession no- NCFT.9153.17. The culture photograph is shown in **Figure 1**.

Figure 1: Isolated Trichoderma cultures

Microscopic and taxonomic identification of Trichoderma culture

The colony color was initially watery white and turned bright green to dark green and dull green with compact conidiophores throughout the petriplates. Mycelium were not typically obvious on CMD, conidia typically form within one week in compact or loose tufts in shades of green or yellow or less frequently white. A yellow pigment may be secreted into the agar, especially on PDA. Septate hyaline hyphae were determined. Conidiophores were found to be hyaline, branched phialides were hyaline, flask-shaped, and inflated at the base. The colour of the conidia was mostly green. The colony produces a characteristic sweet or 'coconut' odor. Conidiophores were highly branched and thus difficult to define or measure, loosely or compactly tufted, often formed in distinct concentric rings or borne along the scant aerial hyphae. Main branches of the conidiophores produce lateral side branches the longest branches distant from the tip and often phialides arising directly from the main axis near the tip. The branches were branched, with the secondary branches often paired and longest secondary branches being closest to the main axis. All primary and secondary branches arise at or near 90° with respect to the main axis. The typical *Trichoderma* conidiophore, with paired branches assumes a pyramidal aspect. Phialides were typically enlarged in the middle but may be cylindrical or nearly subglobose. Phialides were held in whorls, at an angle of 90° with respect to other members of the whorl. Phialides were densely clustered on wide main axis. The culture was identified as *Trichoderma harzianum*. The microscopic slide images are shown in **Figure 2**.

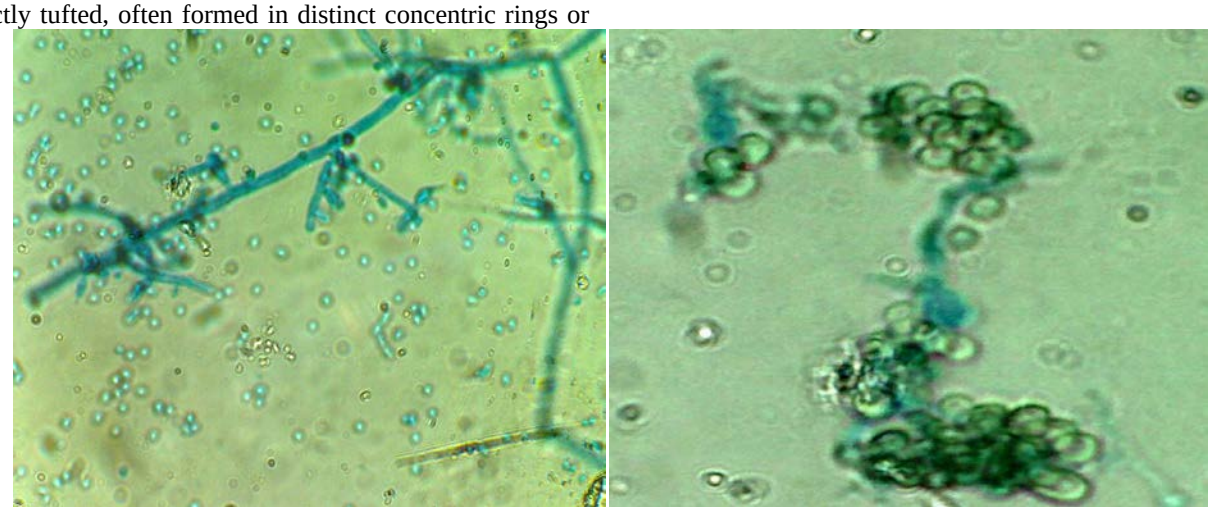

Figure 2: Conidia and spores in *Trichoderma harzianum*

Preparation and extraction of the filtrate

Trichoderma extracts were prepared and filtered. Further solvent extraction was performed in order to determine the presence of secondary metabolites if any. The solvent extract was further vaccum dried in order to evaporate the solvent and to obtain the powder having secondary metabolites for further identification and preparation of nanoparticles in fusion with silver. The results are shown in **Figure 3.**

Figure 3: Preparation of extract and solvent extraction of *T. harzianum*

Preparation and characterization of Trichoderma fused silver nanoparticles (TR-Ag Nps)

In the present investigation, Trichoderma fused silver nanoparticles (TR-Ag Nps) were produced **(Figure 4 and 5).** The particle size was determined by SEM and their absorption spectrum was determined at 200-700 nm. It was observed that the TR-Ag Nps produced were of very fine shape and size having optimal 20 nm size. These nano particles were having slightly rough spherical structures which were observed in free and interconnected form **(Figure 6)**. The UV absorption spectra of the fused nanoparticles recorded the maximum wavelength at 415 nm.

Figure 4: Preparation of silver nanoparticles

Figure 5: Preparation of Trichoderma fused silver nanoparticles (TR-Ag Nps)

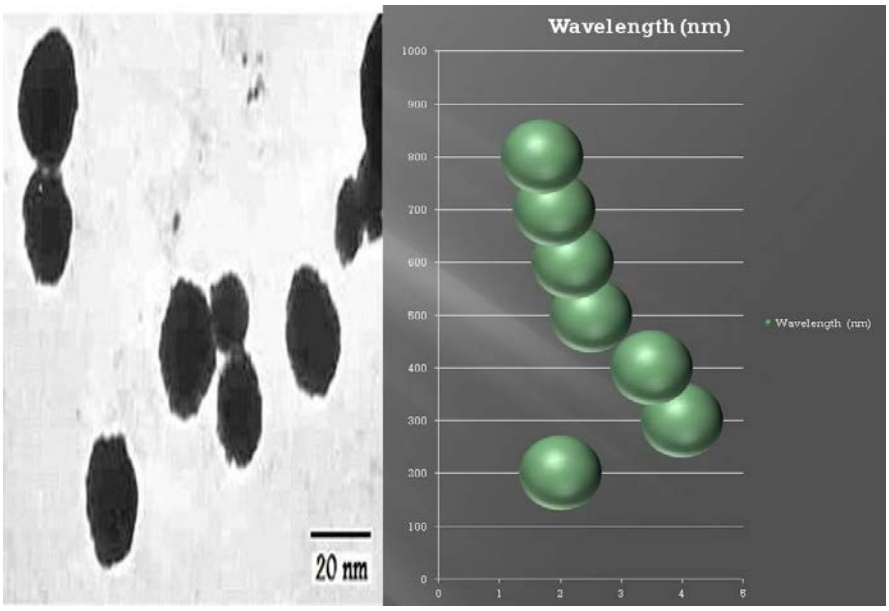

Figure 6: Characterization of TR-Ag Np by SEM and determination of absorption maxima

Determination of antimicrobial activity of Trichoderma solvent extract and Trichoderma-Ag* fused nanoparticles against the pathogens

The antimicrobial activity of Trichoderma solvent extract and Trichoderma-Ag fused nanoparticles was evaluated against the bacterial and fungal pathogens viz. *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Micrococcus luteus*, *Leuconostoc mesentroides*, *Aspergillus niger* and *Aspergillus ochraceous* by well diffusion method. The results were found to be surprising as both Trichoderma solvent extract and TR-Ag fused nanoparticles were having significant antimicrobial activity against the pathogens studied. The antimicrobial activity of Trichoderma solvent extract was determined at 10 µl, 20µl, 5 ppm, 10 ppm, 25ppm and as such (1000 µl) while antimicrobial activity of Trichoderma fused silver nanoparticles (TR-Ag Np) was determined at 20 µl, 50µl, 5 ppm, 10 ppm, 25ppm and as such (1000 µl). The results of both were found to be in correlation as at ppm level doses, none of the tests viz. Trichoderma solvent extract and Trichoderma fused silver nanoparticles (TR-Ag Np) showed any antimicrobial activity. Also no antifungal activity of each of these was found against *Aspergillus niger* and *Aspergillus ochraceous* at small doses but at 1000 µl were found to be effective against *Aspergillus niger*. The results of antimicrobial activity of Trichoderma extract are shown in **Table 1**; **Figures 7 (a) & (b)** while that of Trichoderma fused silver nanoparticles are shown in **Table 2**; **Figures 8 (a) & (b)**.

Table 1: Antimicrobial activity of solvent extracts of Trichoderma

Trichoderma solvent extract	**Diameter of zone of inhibition (mm)**					
	PA	**BS**	**ML**	**LM**	**AN**	**AO**
10 µl	25.0	26.0	29.0	32.0	NA	NA
20 µl	30.0	29.0	33.0	36.0	NA	NA

5 ppm	NA	NA	NA	NA	NA	NA
10 ppm	NA	NA	NA	NA	NA	NA
25 ppm	NA	NA	NA	NA	NA	NA
As such (1 ml)	45.0	38.0	47.0	42.0	43.0	NA
Ethyl acetate	NA	NA	NA	NA	NA	NA

*NA, No activity; PA, *Pseudomonas aeruginosa*; BS, *Bacillus subtilis*; ML, *Micrococcus luteus*; LM, *Leuconostoc mesentroides*; AN, *Aspergillus niger*; AO, *Aspergillus ochraceous*

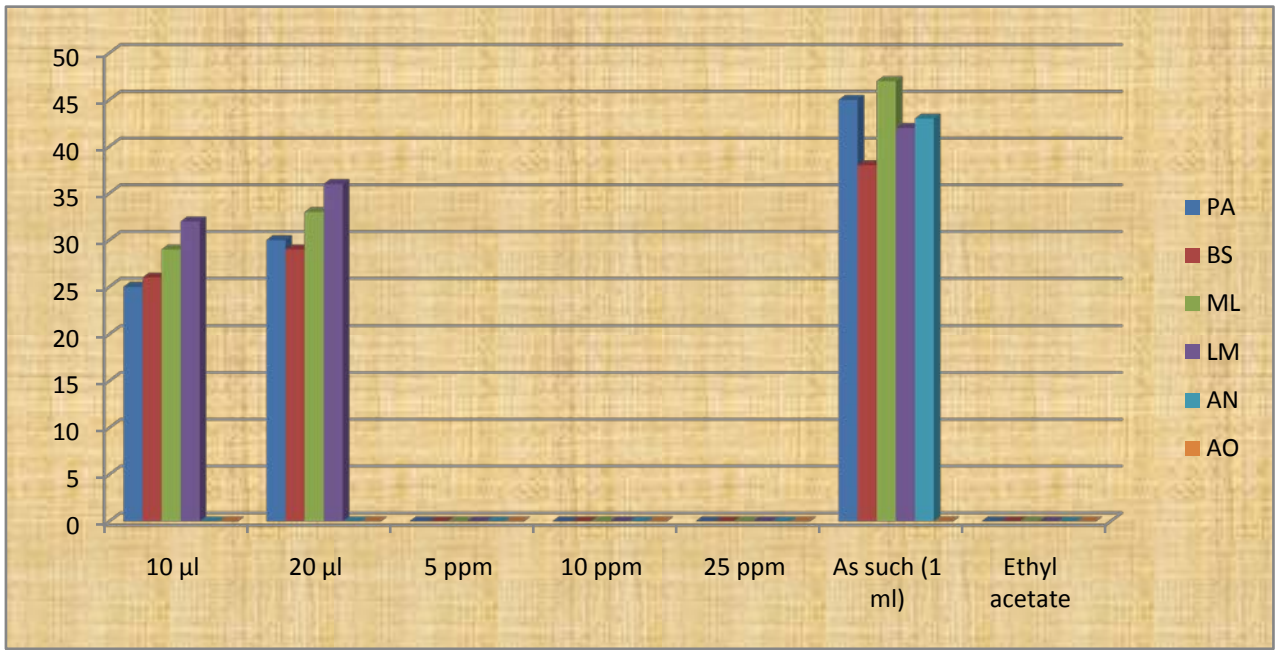

Figure 7 (a): Antimicrobial activity of solvent extracts of Trichoderma

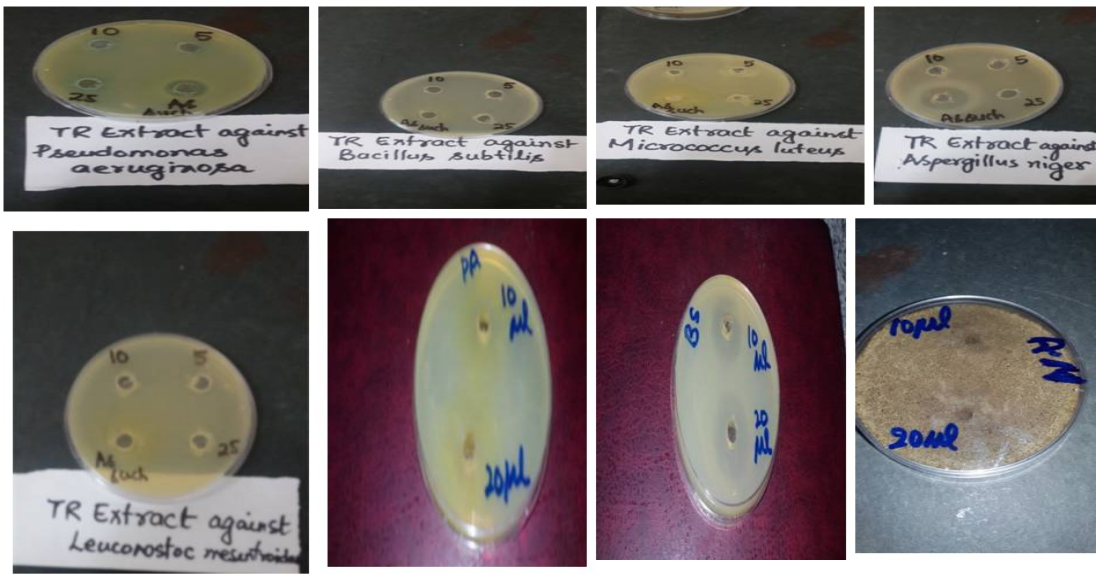

Figure 7 (b): Antimicrobial activity determination by well diffusion method of solvent extracts of Trichoderma

Table 2: Antimicrobial activity of solvent extracts of Trichoderma fused silver nanoparticles (TR-Ag Np)

Trichoderma-Ag* fused nanoparticles	Diameter of zone of inhibition (mm)					
	PA	**BS**	**ML**	**LM**	**AN**	**AO**
20 µl	40.0	37.0	39.0	39.0	NA	NA
50 µl	54.0	52.0	45.0	46.0	NA	NA
5 ppm	NA	NA	NA	NA	NA	NA
10 ppm	NA	NA	NA	NA	NA	NA
25 ppm	NA	NA	NA	NA	NA	NA
As such (1 ml)	57.0	58.0	49.0	48.0	48.0	NA
N-saline	NA	NA	NA	NA	NA	NA

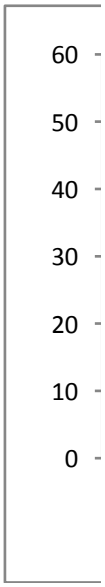

Figure 8 (a): Antimicrobial activity of solvent extracts of Trichoderma fused silver nanoparticles (TR-Ag Np)

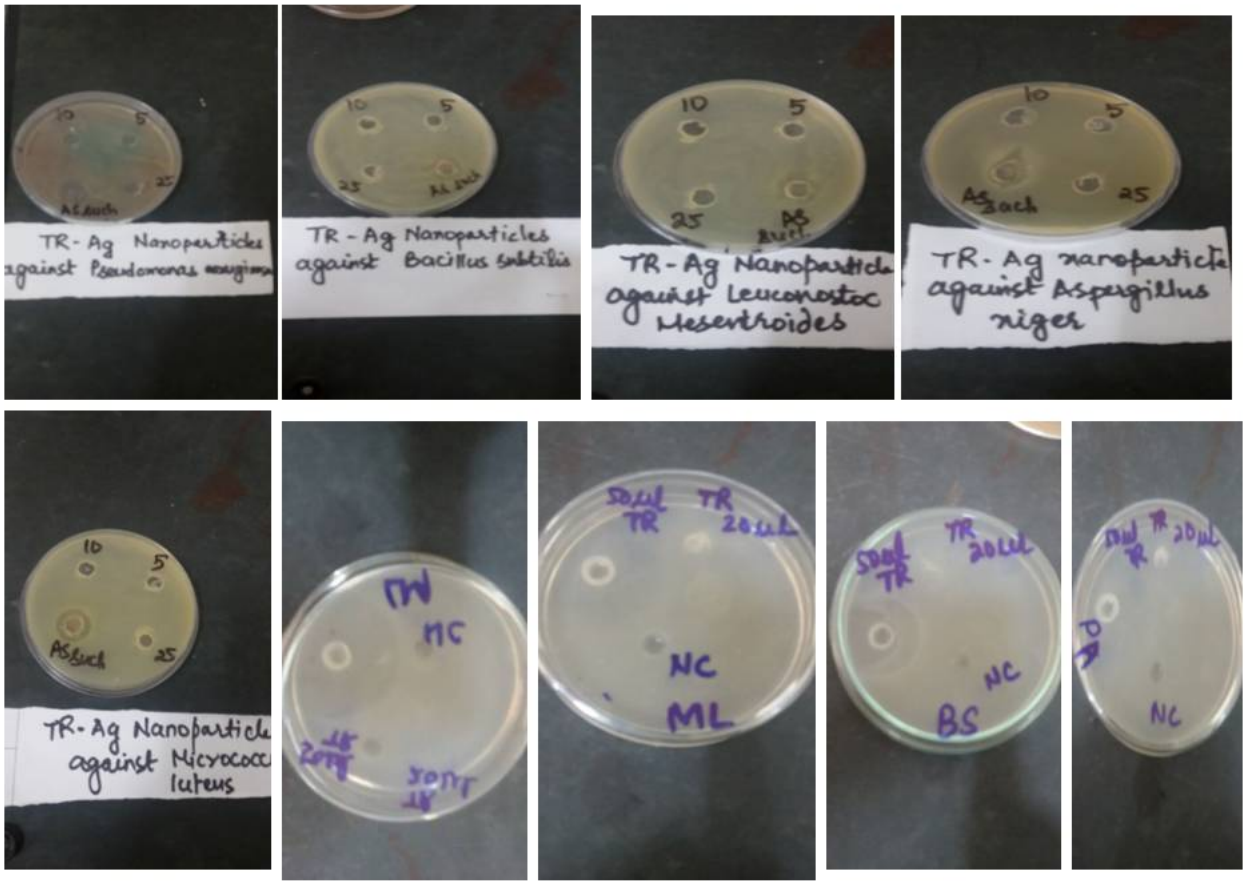

Figure 8 (b): Antimicrobial activity determination by well diffusion method of Trichoderma fused silver nanoparticles (TR-Ag Np)

Trichoderma is known as an important and significant bio-control agent. The classical mechanisms of control have included antibiosis, mycoparasitism, and competition for nutrients. The significant combination of Trichoderma with synthetic salts/metallic ions can be utilized in treatment of different infections within the plants and animals both. These can be utilized as alternatives to different antibiotics etc. The present study thus concludes that, Trichoderma secretes some potent secondary metabolites responsible for antimicrobial activity against opportunistic and nosocomial infections causing pathogens. Both Trichoderma (biological) and silver (Inorganic metallic ions) are known to have significant antimicrobial potency. But fusions of both the biological and inorganic metallic ion have not been studied. The production of fused nanoparticles is a first kind of study done ever to determine the antimicrobial spectrum against opportunistic pathogens. Previous studies reported by our group have already described the antifungal behavior of Trichoderma fused silver nanoparticles against fungal phyto-pathogens [30].

IV. CONCLUSION

The combination of secondary metabolites from Trichoderma and Ag ions in terms of fused nanoparticles can results in preparation of potent antimicrobial agents. The study may thus lead to the isolation and identification of significant antimicrobial molecule (s) from Trichoderma which can be utilized in preparation of different types of nanoparticles. These nanoparticles can thus be utilized as a potent biocide against pathogens. However, further study is required to isolate and characterize the secondary metabolites from Trichoderma solvent extract and to determine the antimicrobial behavior of the same against other pathogenic and drug resistant microbes.

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