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SYNERGISTIC OLIGODYNAMIC EFFECT OF MYCOGENIC BIMETALLIC NANOPARTICLES WITH DAPTOMYCIN FOR CONTROLLING PATHOGENS

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ABSTRACT: Bimetallic nanoparticles have several useful opto-electronic and catalytic properties that are different from those of monometallic nanoparticles. The development of non-toxic and eco-friendly protocols for the synthesis of bimetallic nanoparticles is a still less explored area. In the present study, bimetallic silver-gold nanoparticles were synthesized using *Trichoderma reesei* (NCIM 992). An effective approach for co-reduction of metal salts was adopted wherein the fungal biomass was grown under non-limiting conditions, followed by the addition of metal salts. The bio fabricated bimetallic nanoparticles were characterized using UV-Vis Spectroscopy, ICP-MS, SEM equipped with an EDS, FTIR and XRD. The fungal biomass color changed from colorless to dark purple within 2 days of incubation period. UV-Visible spectrum of the intracellularly produced bimetallic nanoparticles showed characteristic peak between 420 nm and 540 nm. ICP-MS of the residual salt concentration depicted the percentage of bioconversion of metal salts to metal nanoparticles. SEM-EDS studies confirmed the presence of intracellular silver-gold bimetallic nanoparticles. FT-IR analysis revealed the existence of peaks in the amide regions, a characteristic of chemical functional groups that are responsible for the reduction of metal ions and nanoparticles stabilization. XRD results determined that the nanoparticles ranged between 25 - 40 nm in size and were crystalline in nature with the face centered cubic symmetry. The synergistic oligodynamic effect of bimetallic nanoparticles along with Daptomycin showed enhanced antimicrobial activity against methicillin-resistant *Staphylococcus* and *Micrococcus* sp.

INTRODUCTION: Noble metal nanoparticles have gained prominence in therapeutic and diagnostic applications [1]. Many researchers have now devoted their efforts to the fabrication of bi-metallic nanocomposites like nano - alloys, core-shell and mixed metal nano-particles, owing to their valuable applications [2 - 3].

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Bimetallic nanoparticle synthesis involves simultaneous or sequential reduction of two metals resulting in to either core - shell or alloy form of nano-arrangement. Sequential reduction refers to the reduction of shell metal over the pre-formed seed of core metal.

The formation of bimetallic nanoparticles depends on purity, non-reactivity and reducibility of the metal salts. Previous literature has focused on the physical and the chemical synthesis of bimetallic nanoparticles like sol-gel method, sono-chemical method, micro - emulsion technique, aerosol technology, *etc*. Nucleation and transition phases of ions to nanoparticles need to be controlled

especially in chemical synthesis [4 - 7]. Zhang *et al*., have reported hollow Ag - Au bimetallic nanoparticles formed by galvanic replacement reaction [8]. To overcome the drawbacks of the chemical and physical methods, biogenesis of bimetallic nanoparticles seems to be an eco-friendly and sustainable approach towards nanoparticle development. Biological sources of bimetallic salts reduction have been gaining tremendous attention as a viable alternative to the hazardous conventional techniques. Biological systems like bacteria, fungi, actinomycetes and plants have the ability to produce nanoparticles. Nanoparticles synthesized by microbes have gained popularity due to their innate potential, eco-friendly and stabilized nature [9 - 12].

Fungi are reliable sources for large scale metal nanoparticle production as they offer less stringent handling, simple purification and feasible scale-up. Different fungal species have been reported to synthesize NPs either intracellularly or extra-cellularly. The Cell wall plays a vital role in intracellular nanoparticle formation. The negatively charged cell wall attracts the positively charged metal ions electrostatically and traps the ions [13]. The physical parameters like temperature and pH influence the intracellular nanoparticle formation [14 - 15]. Fungal synthesis of bimetallic nanoparticles is still less explored [16 - 17]. These bimetallic NPs offer the versatility of combining the antimicrobial silver activity with the presence of gold, stabilized with biomolecules.

Bimetallic nanoparticles comprising of gold and silver have been reported to possess greater antimicrobial activity than monometallic ones [18 - 20]. Ag-Au bimetallic nanoparticles were synthesized by a chemical method and assayed for their antibacterial activity against *S. aureus* [21]. Ag-Au alloy nanoparticles antibacterial activity was increased when combined with penicillin G and piperacillin.

Bimetallic Au-Ag nanoparticles were synthesized by using *Ocimum basilicum* aqueous leaf and flower extracts and revealed a high antibacterial activity against many pathogenic microorganisms [22]. Our previous work was limited to the production of gold nanoparticles by different fungal species like *Fusarium oxysoprum*, *Trichoderma reesei*, *Verticillium luteum*, *Aspergillus niger*, *Aspergillus versicolor etc*. and their varied applications [23 - 25].

We were now interested to utilize the potential of fungal strain for simultaneous bio-reduction of metal salts to form bimetallic nanoparticles. Therefore, the potential of *Trichoderma reesei* was used to form silver-gold bimetallic nanoparticles. Daptomycin, an anti-MRSA antibiotic which treats complicated skin and skin structure infections was combined with non-functionalized bimetallic nanoparticles which were biologically synthesized and are non-toxic [18 - 19]. The antimicrobial activities of the Daptomycin, bimetallic nanoparticles and the combination of antibiotic and nanoparticles were then evaluated against methicillin-resistant *S. aureus*, *S. epidermis* and *M. luteus* strains. Thus, promising its potentiality for therapeutic purposes, especially; skin infection treatments in future endeavors.

MATERIALS AND METHODS:

Growth of Microorganism: The microbial strain of *Trichoderma reesei* (NCIM 992) was obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The microorganism was subcultured in modified mold growth medium containing (g/L): sucrose 20, sodium nitrate 3, yeast extract 1, peptone 1, dipotassium hydrogen phosphate 1, potassium chloride 0.5, ferrous sulphate 0.01 and magnesium sulphate 0.5. The strain was grown aerobically in 250 ml flasks in an incubator shaker at 200 rpm and 28 °C for 72 hours, and initial pH was maintained at 6.8 ± 0.2.

The strain was then adapted to different concentrations of metal salts (0.05 mM to 1.5 mM) each of $HAuCl_4$ and $AgNO_3$ solution. The adaptation studies were conducted for continuous six months. The total cultivation time was reduced from seven days to five days and finally up to three days of incubation.

Production of Bimetallic (Silver and Gold) Nanoparticles by Co-reduction: Under aseptic conditions, sufficient cell mass of *Trichoderma reesei* was separated and washed thrice with distilled water. 20 grams of wet biomass was taken and suspended in to a 500 ml Erlenmeyer flask containing 100 ml of each 10^{-3} M $HAuCl_4$ and

10^{-3}M $AgNO_3$ solution. Above culture was incubated at 28 °C at 200 rpm for 2 days. For control, fungal biomass was suspended in distilled water and incubated at 28 °C at 200 rpm for two days. The amount of bimetallic nanoparticles produced was measured using ICP-MS.

Characterizations: The fabrication of bimetallic nanoparticles by *Trichoderma reesei* was analyzed by various analytical techniques. The fungal biomass without nanoparticles and with nanoparticles was centrifuged at 10,000 rpm for 15 minutes respectively. The obtained biomass was freeze dried at -54 °C, followed by lyophilisation. The lyophilised samples were placed on pin stubs and coated with gold under vacuum as they were non-conducting and examined by Scanning Electron Microscopy (SEM) on ZEISS: EVO18 equipped with an Energy Dispersive Spectrometer (EDS) on OXFORD INSTRUMENTS. Similarly, the samples were prepared for FTIR analysis. The samples were ground with KBr pellets and examined on Shimadzu FTIR-8400 in the range of 4000 - 400 cm^{-1}. For XRD analysis, the sample was cast onto the glass slide and measurements were carried out using Rigaku Ultima IV instrument at the two-theta angle in the range of 20° to 80°.

Antimicrobial Assay: To determine the antibacterial activity of synthesized gold nanoparticles, standard disk diffusion method was carried out against Multi Drug Resistant Strain (MRSA) *Staphylococcus aureus.* The agar plates having suitable nutrient media (LB media) was prepared, sterilized and allowed to solidify. After solidification, the agar plates were inoculated with bacterial cultures. 6 mm disks were impregnated with required quantity of antibiotic and nanoparticles in petri-plates containing suitable nutrient agar medium seeded with 120 µL of 36 h of each pathogen.

20 µg/ml (Minimum Inhibitory Concentration (MIC) of Daptomycin antibiotic, 20 µg/ml (equal amount as that of antibiotic) of nanoparticle and a mixture of both Daptomycin and lyophilized nanoparticle was formulated. 150 µl of each of these was impregnated on the corresponding disks and incubated at 28 °C for two days. The diameter of zones of inhibition was measured using a ruler and mean value was recorded for each pathogen and expressed in millimetre (mm). All the above experiments were carried out in duplicate.

RESULTS AND DISCUSSION:

Nanoparticle Formation: The visual changes observed after the bioconversion of metal salts is depicted in **Fig. 1** and it was noticed that the color varied for blank, gold chloride supplemented broth, silver nitrate supplemented broth and for the combination of both metal salts.

Gold nanoparticles **Silver nanoparticles** **Bimetallic nanoparticles**

FIG. 1: FORMATION OF SILVER NANOPARTICLES, GOLD NANOPARTICLES AND BIMETALLIC GOLD AND SILVER NANOPARTICLES

UV-Visible Spectroscopy studies of blank media revealed no characteristic peak near the 450 and 520 nm range indicating no extracellular synthesis (as per no change in media color). After two days,

the biomass in the blank was white while it turned to yellowish color for silver nitrate supplemented broth. This indicated the formation of silver nanoparticles. Also, gold nanoparticles showed ruby-red color and the bimetallic nanoparticles showed dark purple color. The morphology of the cells and the metal salt concentration were two essential prerequisites for bimetallic nanoparticles formation. The optimum concentration of metal salts for the nanoparticles synthesis was found to be 1 mM (threshold level). Metal salt concentrations lesser than 1 mM did not give significant results and the cells died at concentrations beyond this level. Biomass supplemented with 1 mM of both metal salts showed color changes after 48 hours of incubation in dark conditions. The color change indicated the Nanoparticles' formation. The bimetallic nano-particles produced were kept in the dark at 4 °C and even after one month, no major changes were observed (color or any physical appearance) making it stable [26 - 27]. ICP-MS of the residual salts revealed that more than 75% of the metal salts were converted to nanoparticles as shown in **Table 1**.

TABLE 1: ICP-MS OF THE RESIDUAL SILVER AND GOLD SALTS

Metal Salt	Original Salt Concentration	Residual Salt Concentration	Percentage of Bioreduction of Metal Salt
Silver Nitrate	30 ppm	6.1259	79.5
Gold Chloride	70 ppm	12.5432	82.08
Bimetallic Gold and Silver Salt	30 ppm and 70 ppm	7.0415 and 16.4610	77 and 76.48

Characterization of Nanoparticles:

UV-Vis Spectroscopy: UV-Vis spectrum for Au, Ag and Au/Ag nanoparticles after separation and purification from the fungal biomass showed peaks at 550, 450 and 480 nm, respectively **Fig. 2**.

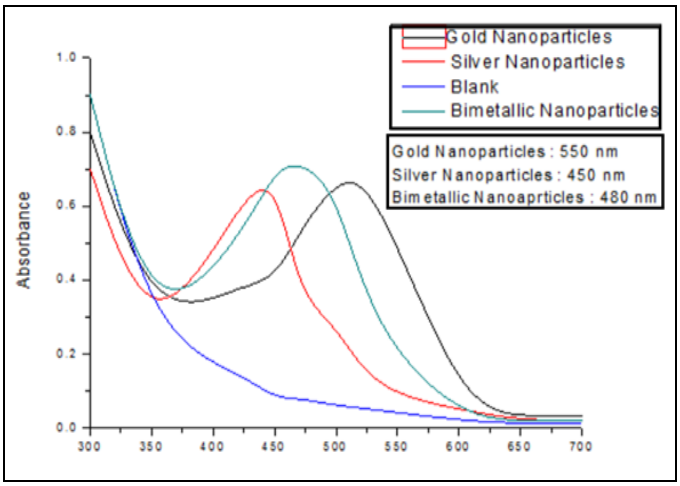

FIG. 2: UV-VIS SPECTRUM FOR Au, Ag AND Au / Ag NANOPARTICLES AFTER SONICATION

SEM - EDS Analysis: Fig. 3 and **5**, represent the SEM images of the fungal biomass samples before and after treating with metal salt solutions.

The SEM instrument equipped with EDS studies were carried out for the composition analysis. EDS spectrum of fungal biomass samples before and after treating with Gold-Silver salt solutions are displayed in **Fig. 4** and **6**.

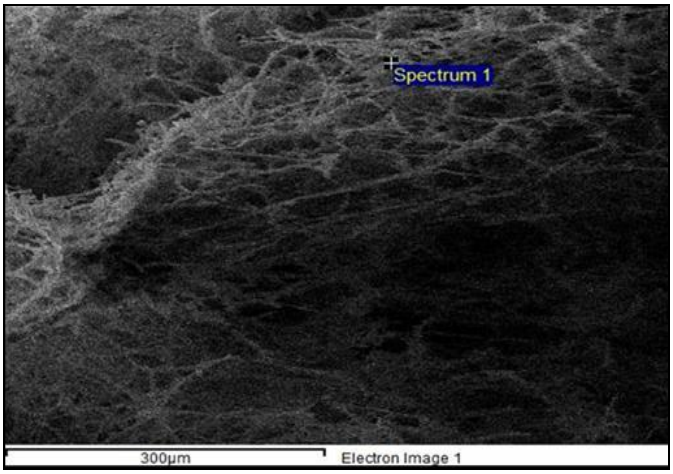

FIG. 3: SEM IMAGES DEPICTING THE ONLY FUNGAL BIOMASS

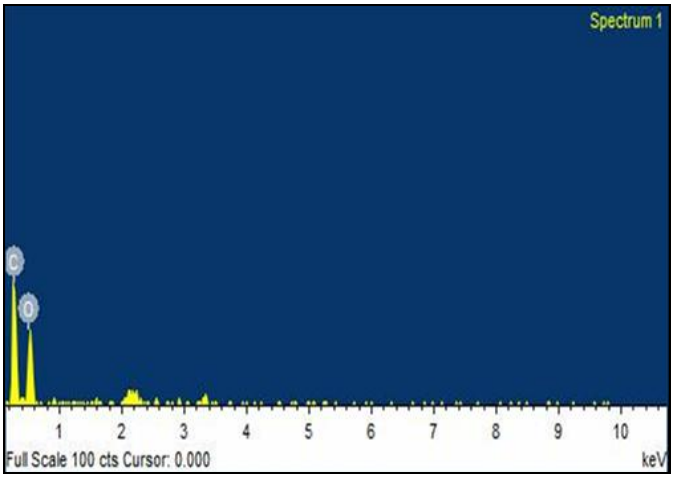

FIG. 4: EDS IMAGE OF THE FUNGAL BIOMASS SHOWING ONLY CARBON AND OXYGEN PEAKS

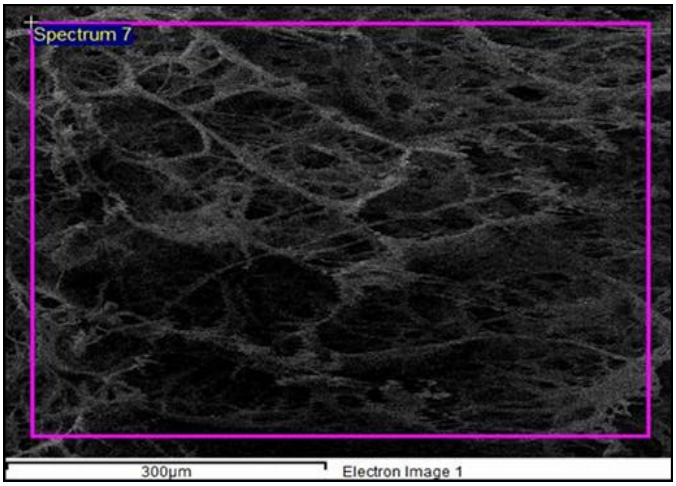

FIG. 5: SEM IMAGE DEPICTING THE FUNGAL BIOMASS TREATED WITH THE GOLD AND SILVER SALTS

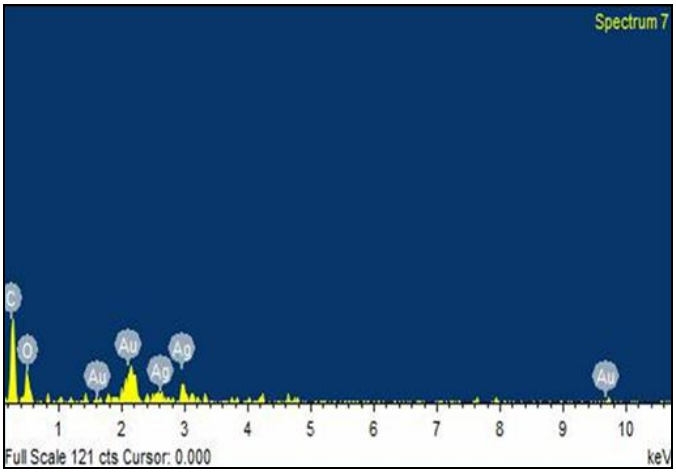

FIG. 6: EDS IMAGE OF THE FUNGAL BIOMASS TREATED WITH GOLD AND SILVER SALTS SHOWING THE GOLD AND SILVER PEAKS ALONG WITH CARBON AND OXYGEN PEAKS

Fig. 4 shows strong carbon and oxygen peaks, which could be attributed to the bio-molecules that are bound to the nanoparticles which are acting as stabilizing agents. In **Fig. 5**, the existence of bimetallic nanoparticles as Ag-Au alloy was confirmed in the EDS spectrum at 1.5 - 3 keV. These results were used as a quick test to confirm the presence of bimetallic nanoparticles in the fungal biomass [28].

Fourier Transform Infrared Spectroscopy (FTIR) Studies: FTIR measurements were carried out to identify the possible (protein) bio-molecules responsible for the capping and efficient stabilization of the metal nanoparticles synthesized by *Trichoderma reesei*. FTIR studies were conducted to determine the various chemical functional groups present in the sample containing the nanoparticles [17]. FTIR spectra of untreated fungal biomass and fungal biomass supplemented with gold and silver metal salts were shown in Figure 7 and 8. The IR peak shifts shown at 3421, 2854, 2341, 1659, 1473 and 1035 cm^{-1} indicated the changes in the functional groups. The peak shift from 1458 to 1473, an amide II region, is due to the -NH- bending vibrations from ketone to amine group. The shifting of peak to 1035 can be attributed to the amide bond of C-O stretching mode to N-O vibration mode indicating the presence of carboxylic group and amide groups in the material bound to the synthesized Ag-Au nanoparticles. The peaks at 2854 and 2341 can be attributed to the –C-H groups where the carbon is of sp^3 hybridization and stretching of –O-H bond of carboxylic acids respectively. The band at 1659 cm^{-1}, an amide I region, corresponds to carbonyl (–C=O) stretch in the proteins.

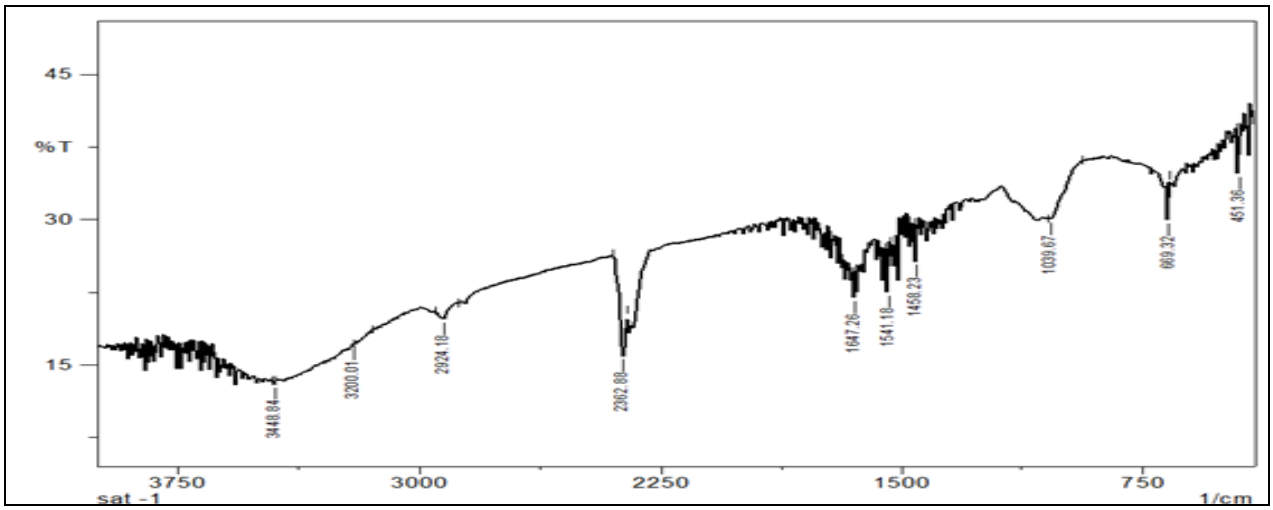

FIG. 7: FTIR SPECTRA OF ONLY LYOPHILISED FUNGAL BIOMASS

This was further confirmed by band at 3421 cm^{-1}. Similarly, Honery *et al.,* [28] and Sandt *et al.,* [29] pointed out similar chemical changes in functional groups resulting in nanoparticle formation in their FTIR studies. Further, the FTIR studies indicated that the secondary structure of the proteins was not affected because of their interaction with Ag-Au nanoparticles. The appearances of peaks in the amide regions were characteristic of proteins / enzymes that were found to be responsible for reduction of metal ions indicated the binding of nanoparticles with proteins. FTIR spectroscopy studies confirmed that the carbonyl group of amino acid residues and peptides of proteins has a stronger ability to bind the metal, so that the most proteins could most possibly form a coat covering the metal nanoparticles (*i.e.* capping) to prevent agglomeration of the particles and thus, the nanoparticles are stabilized in the medium [30 - 31].

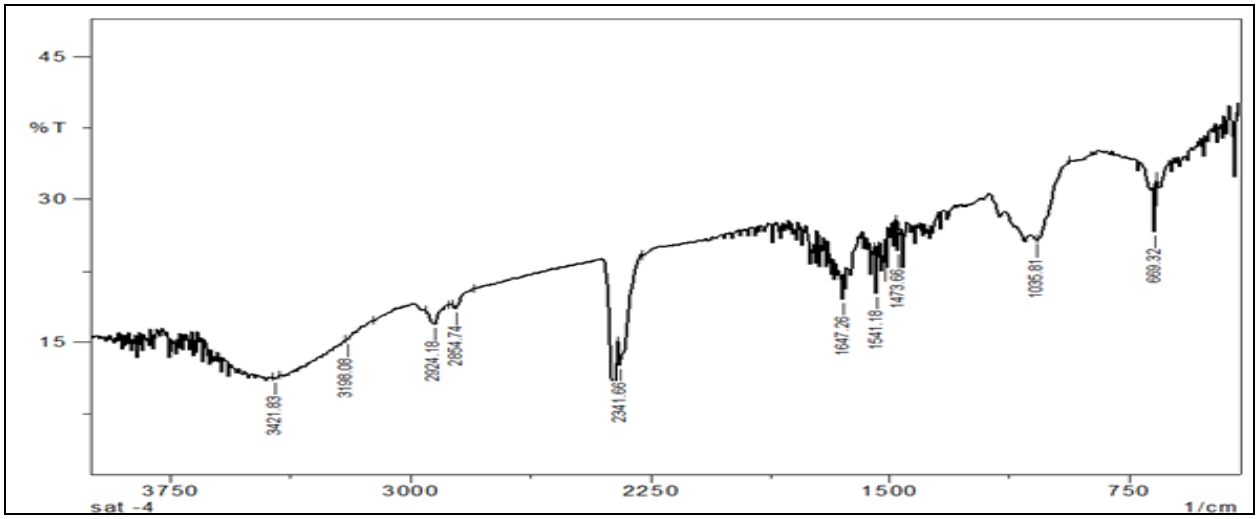

FIG. 8: FTIR SPECTRA OF FUNGAL BIOMASS TREATED WITH GOLD AND SILVER SALTS

X-ray Diffraction Studies: Further the evidence for the formation of Gold Silver Bimetallic Nanoparticles was provided by the X-Ray Diffraction analysis. The crystalline nature of bi-metallic nanoparticles synthesized by *Trichoderma reesei* was determined by Bragg's diffraction peaks evident from XRD pattern as shown in **Fig. 9**. XRD pattern of bimetallic Ag-Au NPs, the Bragg's reflection peaks at 2θ values of 38.4° (111), 44.6° (200), 46.3° (200), 64.6° (220) and 77.3° (311) were observed. All these values showed the Face Centred Cubic (FCC) structure of the obtained nanoparticles having different lattice planes.

The Bragg's peak positions in the spectrum and their intensities were compared with standard JCPDS files [JCPDS file nos 04-0783 and 01-1174, respectively]. The fraction of the intensities of the (200), (220), (311) peaks were found to be much lower, suggesting 111 is the predominant orientation. The remaining unidentified peaks in XRD pattern including a sharp peak at 27.9°, 54.9° and 57.4° can be attributed to the crystalline nature of capping and stabilizing proteins present over the surface of synthesized Gold-Silver bimetallic nanoparticles from *Trichoderma reesei* [32]. Likewise, similar values of Bragg's diffraction peaks for the Ag-Au bimetallic nanoparticles were reported by Sawle *et al*., Basavaraja *et al*., and Shankar *et al.,* [33 - 36].

The mean diameter of the Au-Ag NPs was calculated from the XRD pattern according to the line width of the maximum intensity reflection peak using the Debye Scherrer's equation:

$$D = K\lambda / \beta_{1/2} \cos\theta$$

Where K is a dimensionless shape factor with a value close to unity, λ is the X ray wavelength in angstrom, $\beta_{1/2}$ is the width of the XRD peak at half height and θ is the Bragg's angle. The calculated average particle sizes of the Au-Ag NPs were found to be 25 - 40 nm.

Thus, the XRD pattern clearly shows that the Au-Ag NPs were formed by the reduction of metal ions and are in crystalline in nature.

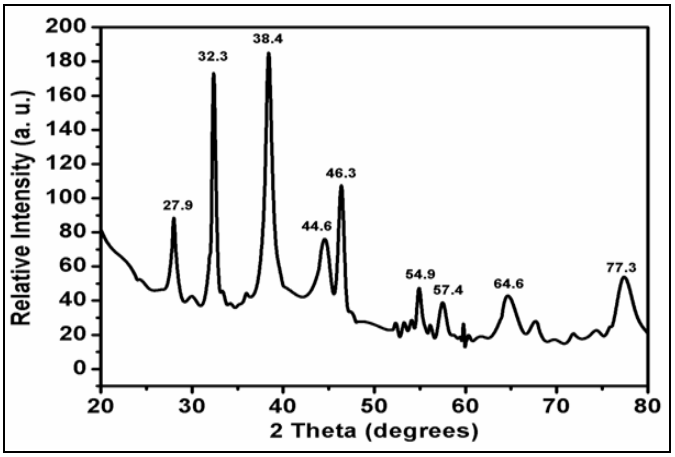

FIG. 9: XRD SPECTRA OF FUNGAL BIOMASS TREATED WITH GOLD AND SILVER SALTS

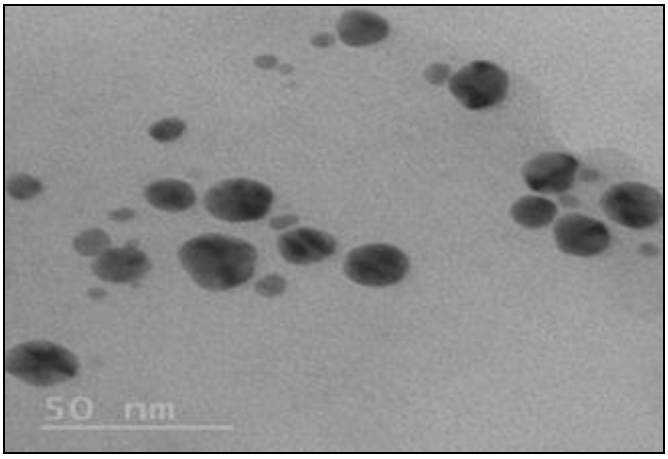

FIG. 10: TEM IMAGE OF BIMETALLIC SILVER-GOLD NANOPARTICLE

Transmission Electron Microscopy (TEM) Analysis: Transmission electron microscopy study shown in **Fig. 10** confirmed that bimetallic silver-gold nanoparticles formed by *Trichoderma reesei* were ranged between 20 - 50 nm sizes which were in sync with the results of XRD. The homogeneity in nanoparticles was evident in case of bimetallic nanoparticles produced. No particular core-shell arrangement was seen for the silver-gold bimetallic nanoparticles. All the characterization results concluded alloy (mixtures of silver and gold nanoparticles in random configuration) sort of bimetallic nanoparticles.

Antimicrobial Assay: Bimetallic nanoparticles have already proved their mettle as good antimicrobial compounds in previous studies, over the monometallic compounds [21 - 22]. Now, the *in vitro* bactericidal activity of Daptomycin (antimicrobial agent for skin infections) was combined with bimetallic nanoparticles. This combined therapeutic agent was found to be more effective as compared to their respective free forms. Daptomycin combined with bimetallic nanoparticles and free Daptomycin were tested on bacterial strains by comparing the corresponding zone of inhibition (mm) as shown in **Table 2**.

TABLE 2: ZONE OF INHIBITION (mm) FOR FREE DAPTOMYCIN AND DAPTOMYCIN COMBINED WITH BIMETALLIC NANOPARTICLES

Name of the bacterial strain	Zone of Inhibition (mm)			Increased Zone Size (mm) (b-a)	Fold increase (%) ((b-a)/a)*100
	Free Form of Bimetallic Nanoparticles	Free Form of Daptomycin (a)	Combined Form of Daptomycin and Bimetallic Nanoparticles (b)		
S. aureus	7.65 ± 0.28	8.67 ± 0.45	12.32 ± 0.20	3.65	42
M. luteus	8.12 ± 0.4	9.45 ± 0.1	12.95± 0.43	3.50	37
S. epidermis	8.00 ± 0.1	10.34 ± 0.2	13.5 ± 0.12	3.16	30
Average synergistic antimicrobial effect (%)					37

The process was non-toxic and eco-friendly. The combinatorial effect led to the higher binding affinity of the drug and enhanced antimicrobial efficacy. The antibiotic combined with bimetallic nanoparticles showed improved bactericidal activity than free form [37 - 40] of antibiotic. This was evident from the increased zone of inhibition of antibiotic combined with bimetallic nanoparticles. There was 37% increase in efficacy of Daptomycin due to the synergistic effect with bimetallic nanoparticles. The synergistic form of the drug prompted that the toxicity of high drug dosage can be mitigated in an economical way.

CONCLUSION: In the present study, silver and gold bimetallic nanoparticles were synthesized using *Trichoderma reesei*. The synthesized nanoparticles were confirmed using SEM - EDS, and XRD and their stability was analyzed by FTIR. Production of bimetallic silver and gold nanoparticles were successfully achieved using *Trichoderma reesei* and the change of biomass color from colorless to dark purple within 48 hours of incubation period indicated it to be intracellular bimetallic nanoparticle synthesis. Characteristic peaks near the 420 nm - 540 nm range were revealed by UV-vis Spectroscopy studies indicating

intracellular nanoparticle formation. No extracellular synthesis (as per no change in media color) was observed. The stabilization of the nanoparticles by the carbonyl group of amino acid residues and peptides of proteins was confirmed by the FTIR spectroscopy studies.

The antimicrobial properties of the synthesized bimetallic nanoparticles in combination with antibiotic were found to be promising for treating infections caused by pathogenic bacteria. Though, the mechanism of action of this combinatorial drug is yet not deciphered. Future prospects of this work would involve extensive research on the exact mechanism involved and other relevant clinical applications.

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COMPETING INTEREST: The authors declare that have no competing interests.

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