Biosynthesis of bimetallic nanoparticles by algae, fungi and higher plants as reducing agents have been gaining immense attention as a viable alternative for the hazardous physicochemical techniques. The molds are capable of biogenic inorganic nanoparticles (gold, silver & bimetallic) formation and their biomass is easier to handle, hence, they are favored over bacteria and other micro-organisms.

Noble metal nanoparticles have gained prominence in therapeutic and diagnostic applications [Rai, M. *et al.* (2016)]. 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 [Feng *et al.* (2010), Roopan (2014)].

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 [Wang *et al.* (2011), Gumeci *et al.* (2013), Chen (2002), Byeon (2012)]. Zhang *et al*., have reported hollow bimetallic (Au-Ag) nanoparticles formed by galvanic replacement reaction [Zhang *et al.* (2013)]. 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 [Hamid (2013), Tamuly *et al.* (2013), Salunke *et al.* (2014), Garcia *et al.* (2014)].

Molds are reliable sources for large scale metal nanoparticle production as they offer less stringent handling, simple purification and feasible scale-up. Different mold 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 [Duran *et al.* (2011)]. The physical parameters like temperature and pH influence the intracellular nanoparticle formation [Liu *et al.* (2017), Chitra *et al.* (2014)]. Mold synthesis of bimetallic nanoparticles is still less explored [Roopan *et al.* (2014), Tripathi *et al.* (2015)]. These bimetallic NPs offer the versatility of combining the antimicrobial silver activity with the presence of gold, stabilized with biomolecules.

6.1 Strain Selected

Aspergellus niger NCIM 616 and *Trichoderma reesei* NCIM 992 microbial mold strain was acquired from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India.

6.2 Growth characteristic comparison of control and experimental Bimetallic (with Gold Chloride & Silver Nitrate) media

Growth patterns of the mold strains were studied to establish the effect of the salt on the growth of the microorganism. The growth study was done in normal nutrient media as well as in the presence of Bimetallic (Gold chloride and Silver nitrate) salt.

Mold culture shows normal growth pattern in normal media. However, growth was limited in case of cultures grown in presence of metal salts, including toxicity of the salt towards the strains. In addition, the mold biomass acquired a colour such as Gold chloride shows lavender colour, Silver nitrate shows yellow colour and mixture of both shows red colour. This suggests that through the presence of metal salts is inhibitory to mold growth, the limited growth is associated with reduction of the salt. Inoculation and the growth of the fungal strains in metal salts containing media results in limited growth and associated slow reduction of metal salts. Growth associated nanoparticle reduction is not very efficient. Hence, for the purpose of nanoparticle formation a more efficient approach would be to first grow the fungal biomass in normal non-limiting condition, followed by the addition of the metal salt solution.

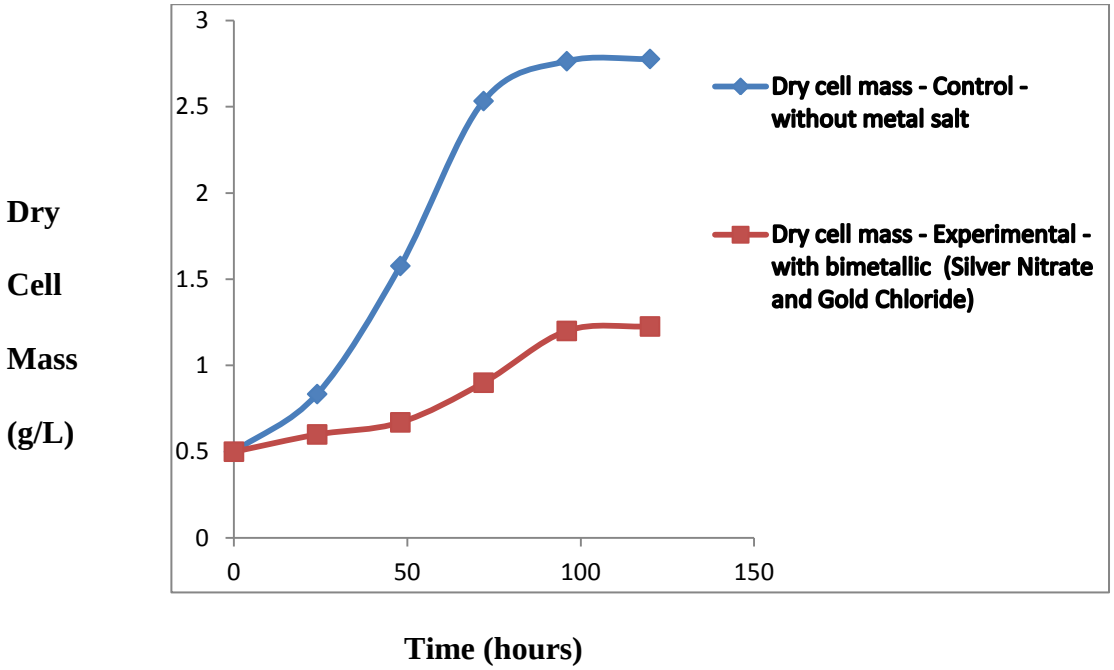
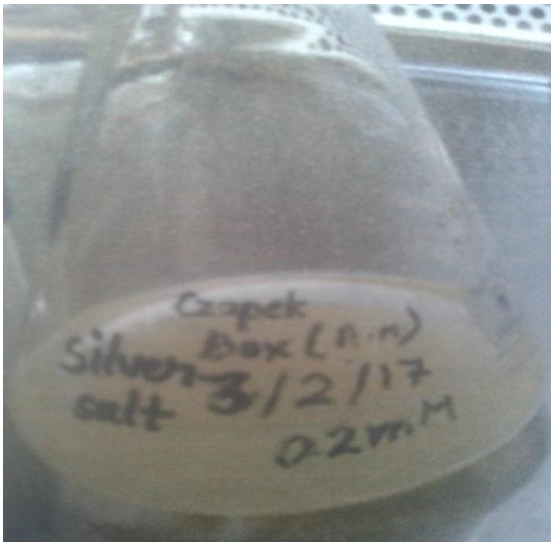


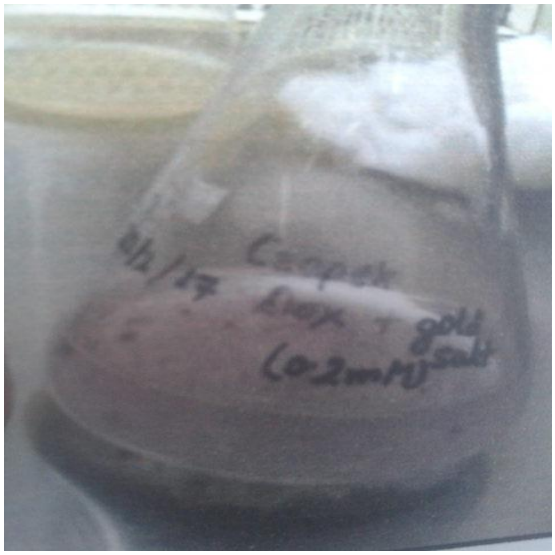
Figure 6.1: Comparative growth pattern of two different *Trichoderma reesei* NCIM 992 strains i.e. without and with Bimetallic (Gold Chloride and Silver Nitrate) salt

6.3 Synthesis of Bimetallic (Gold-Silver) Nanoparticles

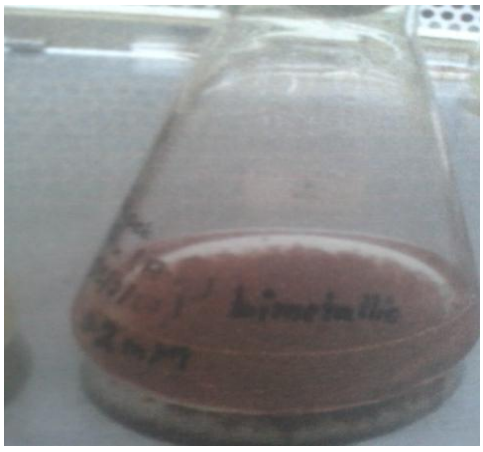
By using *Aspergillus niger* NCIM 616

The mold culture of *Aspergillus niger* NCIM 616 showed visual changes within 4-5 hours. Within 8 hours in orbital shaker, culture with gold nanoparticles showed purple colour, silver nanoparticles showed yellow colour and bimetallic nanoparticles showed lavender colour.





Silver Nanoparticles Gold Nanoparticles



Gold-Silver Bimetallic Nanoparticles

Figure 6.2: Formation of Silver, Gold and Bimetallic (Gold-Silver) Nanoparticles by the effect of *Aspergillus niger* NCIM 616

After 8 hours in orbital shaker, culture with gold nanoparticles showed lavender colour, silver nanoparticles showed yellow colour and bimetallic nanoparticles showed red colour. On maintaining the media and the cell mass for 1 week, no colour change was observed in all cases. It confirms the cell mass to stable in nature.

By using *Trichoderma reesei* NCIM 992

The visual changes observed after the bioconversion of metal salts is depicted in Fig. 6.3 and it was noticed that the color varied for silver nitrate supplemented broth, gold chloride supplemented broth and for the combination of both metal salts.

Silver nanoparticles

Gold nanoparticles

Gold-Silver Bimetallic Nanoparticles

Figure 6.3: Formation of Silver, Gold and Bimetallic (Gold-Silver) Nanoparticles by the effect of *Trichoderma reesei* NCIM 992

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

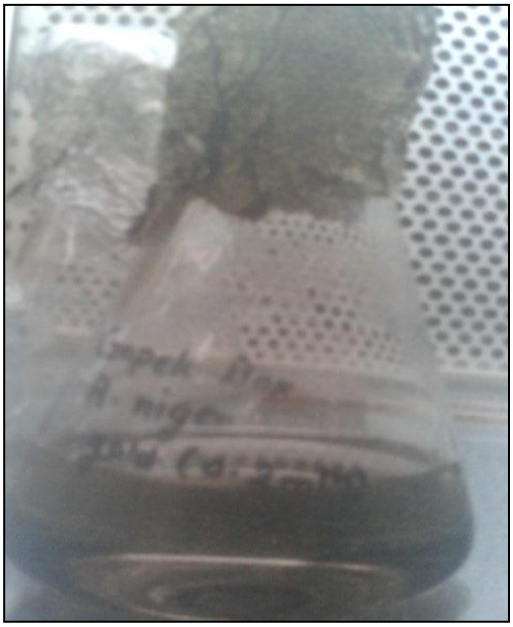
color). After two days, the biomass in the blank was white while it turned to yellowish color for silver nitrate supplemented broth. This indicated the formation of silver nanoparticles. Also, gold nanoparticles showed ruby-red color and the bimetallic nanoparticles showed dark purple color. The morphology 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 colour 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 [Sankar S.S. et. al. 2004, Shedbalkar U. et. al. 2014]. ICP-MS of the residual salts revealed that more than 75% of the metal salts were converted to nanoparticles as shown in Table 6.1.

Table 6.1: ICP-MS of the residual Bimetallic (Gold and Silver) salt

Metal Salt	**Original Salt Concentration**	**Residual Salt Concentration**	**Percentage of Bioreduction of Metal Salt**
Gold-Silver Bimetallic Salt	30 ppm & 70 ppm	7.0 and 16.5	77 and 76.5

Extracellular Production of Nanoparticles

Silver nanoparticles



Gold nanoparticles

Bimetallic nanoparticles

Figure 6.4: Extracellular production of Silver, Gold & Bimetallic (Gold-Silver) Nanoparticles

Intracellular Production of Nanoparticles

Blank **Silver NP** **Bimetallic NP** **Gold NP**

Figure 6.5: Intracellular production of Silver, Gold & Bimetallic (Gold-Silver) Nanoparticles

6.4 UV-Visible Spectroscopy of Bimetallic (Gold-Silver) Nanoparticles

Results obtained from *Aspergillus niger* NCIM 616

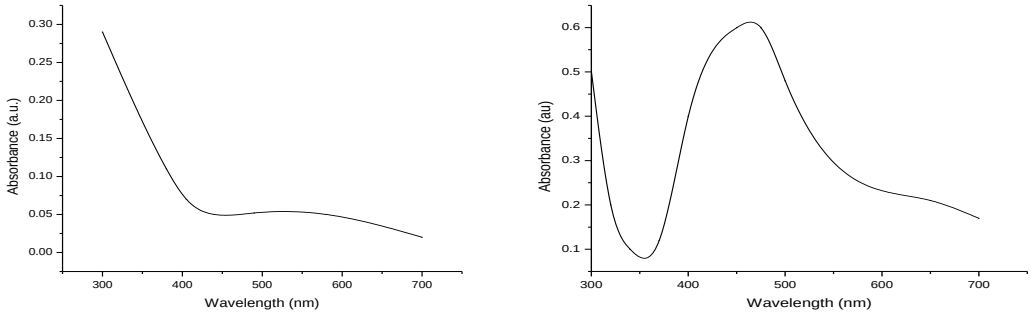
UV-Vis spectroscopy analysis was used to check the formation and stability of biosynthesized NPs in aqueous solutions; metallic nanoparticles display characteristic optical absorption spectra in the UV-Vis region. The spectra showed an intense peak at 540 nm [Magnusson *et al.* (1999)]. The mold culture exposed to (0.2 mm) Gold Chloride, Silver Nitrate and combination of both for 18-24 hours were tested for UV-Vis Spectroscopy. No characteristic peaks were obtained for the mold media concluding it be Intracellular Nanoparticles production.

The pale yellow cell filtrate from *Aspergillus niger* NCIM 616 . The solution turns purple after addition of $HAuCl_4$ solution to the stirred cell filtrate. Considering that the $HAuCl_4$ solution and the cell filtrate are both light in color, the change in color to the characteristic purple of gold nanoparticles clearly indicates their formation in the reaction solution [Link *et al.* (1999), Mukherjee *et al.* (2002), Kalimuthu *et al.* (2009)]. Visual observation of the biosynthesis process shows that it occurs rapidly. The color of the reaction solution changes to purple without further increase in intensity within 1 min. Compared with the previously reported times from several hours to several days for the extracellular biosynthesis of gold nanoparticles [Husseiny *et al.* (2007), Ahmad *et al.* (2003b)], the time required in this work is significantly shorter.

UV–Vis absorption spectroscopy was also used to measure the biosynthesis of gold nanoparticles. The UV–Vis spectrum illustrated in Fig. 6.6 shows no evidence of an absorption peak in the region of 300–700 nm for the cell filtrate from *Aspergillus niger* NCIM 616. After the $HAuCl_4$ solution has reacted with the cell filtrate, a well-defined absorption peak at 545 nm appears in Fig. 5.7.3 shows that corresponds to the wavelength of the surface plasmon resonance of gold nanoparticles [Mukherjee *et al.* (2002), Mulvaney

(1996)]. Various reports have established that the resonance peak of gold nanoparticles appears around this region, but the exact position depends on a number of factors [Link *et al.* (2000), Nair *et al.* (2002)]. The absorption peak is sharp, which indicates little aggregation of the nanoparticles in solution.

But after further sonication, the samples of the culture were incubated with metal salts. Analyzed the UV-Vis spectrum which gives specific peaks at specific wavelengths showing nanoparticle formation. As the reduction of gold and silver salts was an intracellular in case of reduction by fungal strain. We went for UV-Vis spectroscopy analysis of the synthesized nanoparticles.



Blank media (No peak) Bimetallic Nanoparticles (Peak at 480 nm)

Figure 6.6: UV-visible spectrophotometric characteristic peak of Blank and Bimetallic (Gold-Silver) Nanoparticles produced by *Aspergillus niger* NCIM 616

From figure no. 6.6 it is clear that, mold strain is acting as a reducing agent in converting Silver, Gold and Bimetallic salts into their respective nanoparticles which is emphasized by the peak at 435 nm, 525 nm & 475 nm respectively.

Results obtained from *Trichoderma reesei* NCIM 992

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. 6.7**.

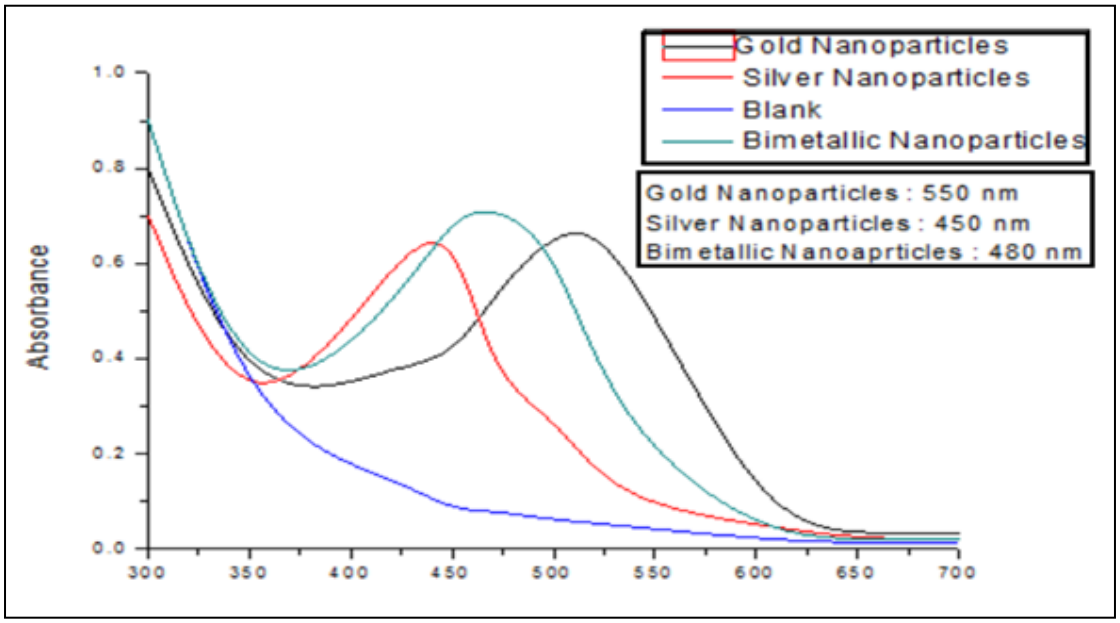


Figure 6.7: UV-Visible spectrophotometric characteristic peaks of Gold, Silver and Bimetallic (Gold-Silver) Nanoparticles produced by *Trichoderma reesei* NCIM 992

6.5 Characterization of Bimetallic (Gold-Silver) Nanoparticles

6.5.1 X-ray Diffraction of Bimetallic (Gold-Silver) Nanoparticles

XRD studies 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. 5.8**.1 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* [Shawle *et al.* (2008)]. 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.*, [Shawle *et al.* (2008), Basavraja *et al.* (2008), Shankar *et al.* (2003), Shankar *et al.* (2004)]. 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, β1/2 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.

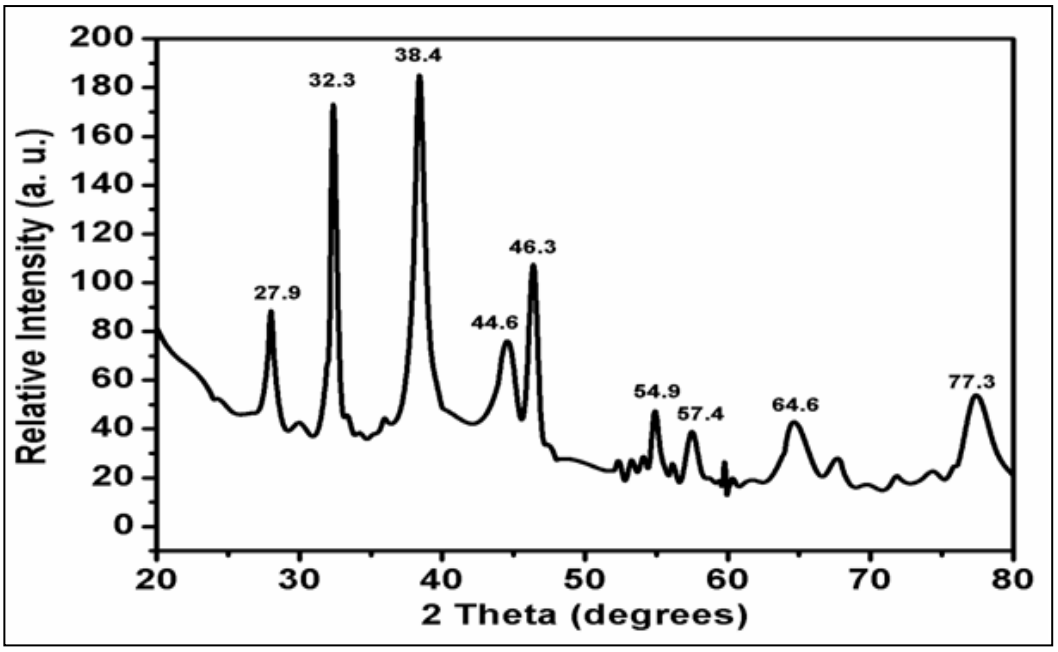


Figure 6.8**:** XRD Spectra of mold biomass treated with Gold and Silver salts

6.5.2 FTIR studies of Bimetallic (Gold-Silver) Nanoparticles

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 sp3 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.

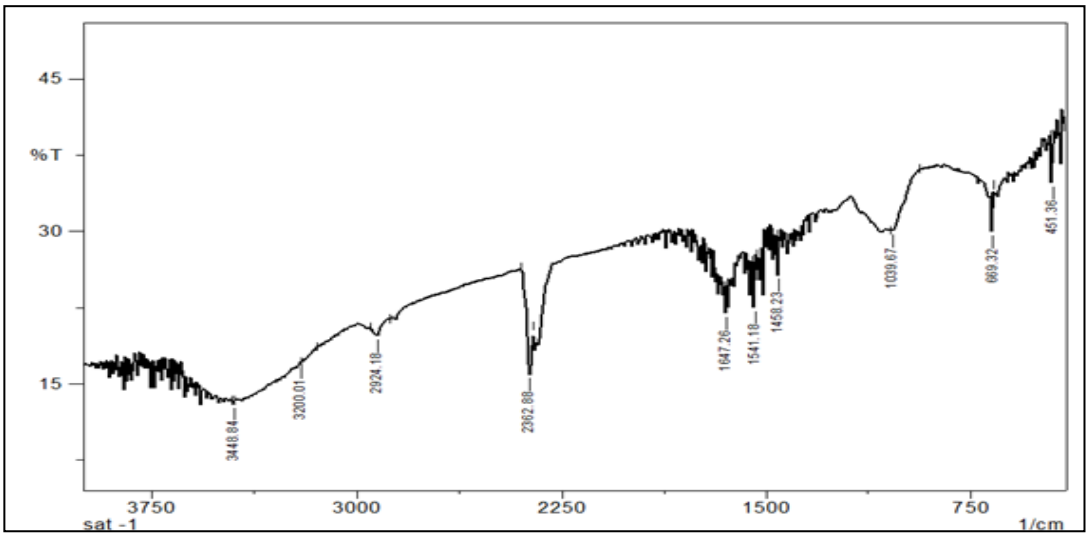


Figure 6.9: FTIR Spectra of Mold biomass

This was further confirmed by band at 3421 cm^{-1}. Similarly, Honery *et al.*, and Sandt *et al.*, 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.

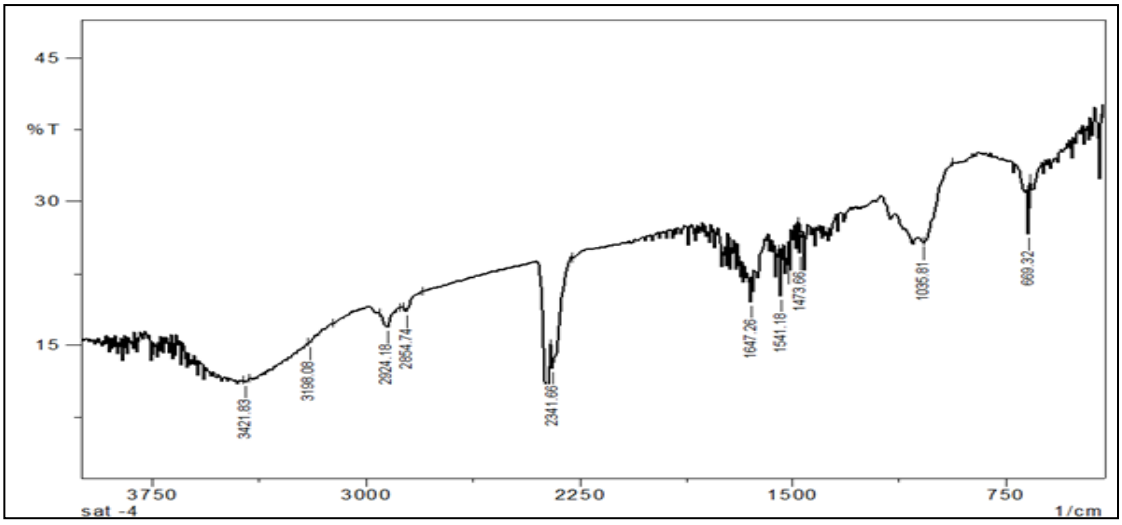


Figure 6.10: FTIR spectra of Mold biomass treated with Gold and Silver salts

6.5.3 Particle Size Analysis of Bimetallic (Gold-Silver) Nanoparticles

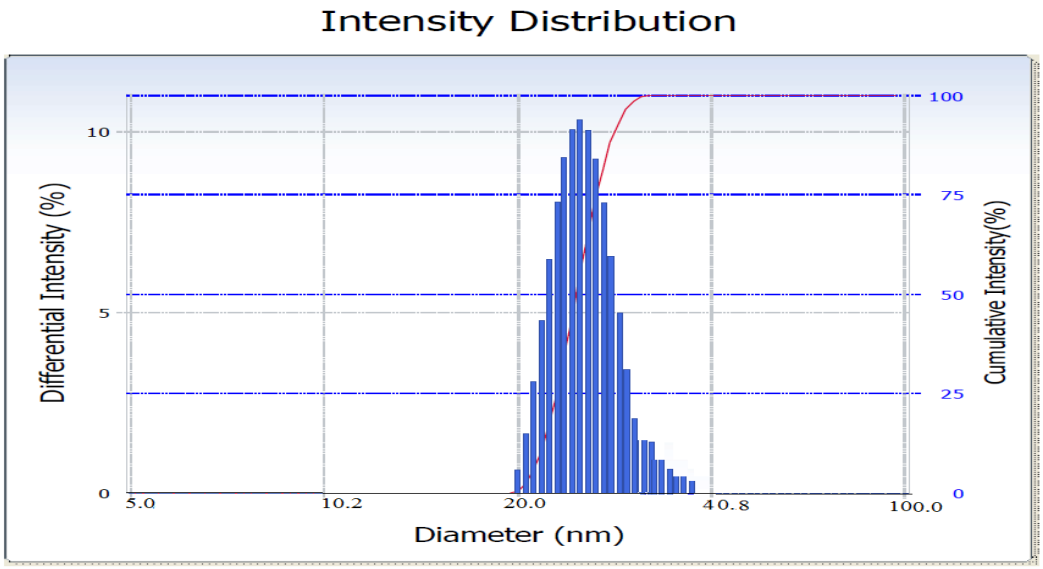


Figure 6.11: Particle Size of Bimetallic (Gold-Silver) Nanoparticles

Size range of Bimetallic Nanoparticle 20-40 nm

6.5.4 SEM-EDX of Bimetallic (Gold-Silver) Nanoparticles

SEM is done to characterize the structural properties of the produced gold nanoparticles. Since the gold nanoparticles are good conductors, they can be observed without any prior carbon coating. Gold nanoparticles accumulated on the mycelium intracellular.

Nanoparticles were identified from shiny particle on the surface of mold cells. The shiny particle observed to be irregular shaped with no definite morphology. The particle sizes of nanoparticles were identified by XRD [Biswarup *et al.* (2007)].

Figure 6.12, represents the SEM & EDX images of the mold biomass samples & after treating with Bimetallic solutions.

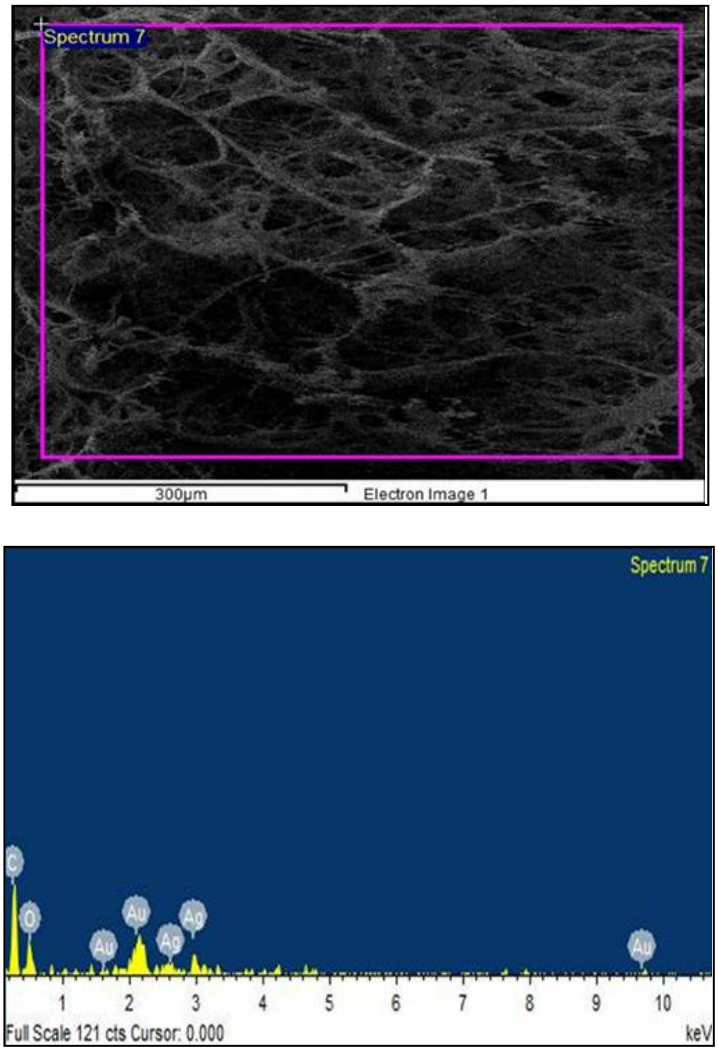


Figure 6.12: SEM & EDX images of Bimetallic Nanoparticles

The SEM instrument equipped with EDX studies were carried out for the composition analysis. EDS spectrum of mold biomass samples before and after treating with Gold-Silver salt solutions are displayed in Fig. 6.12.

EDX spectrum shows strong carbon and oxygen peaks, which could be attributed to the bio-molecules that were bound to the nanoparticles. Carbon and Oxygen were acting as stabilizing agents. It was clear that except Carbon and Oxygen, no other peaks were observed to the measurable amounts.

Four peaks of Gold and Silver in the EDX spectrum appeared around 1.5 – 3 keV indicates the existence of Gold and Silver atoms. Thus Gold and Silver atoms were identified in the mold biomass by SEM-EDX. EDX image of the mold biomass treated with gold and silver salts showing the gold and silver peaks along with carbon and oxygen peaks. 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. 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 mold biomass [Westsson *et al.* (2014)].

6.5.5 TEM Analysis of Bimetallic (Gold-Silver) Nanoparticles From *Aspergellus niger* NCIM 616

TEM is used for the morphological examination of nanoparticles.

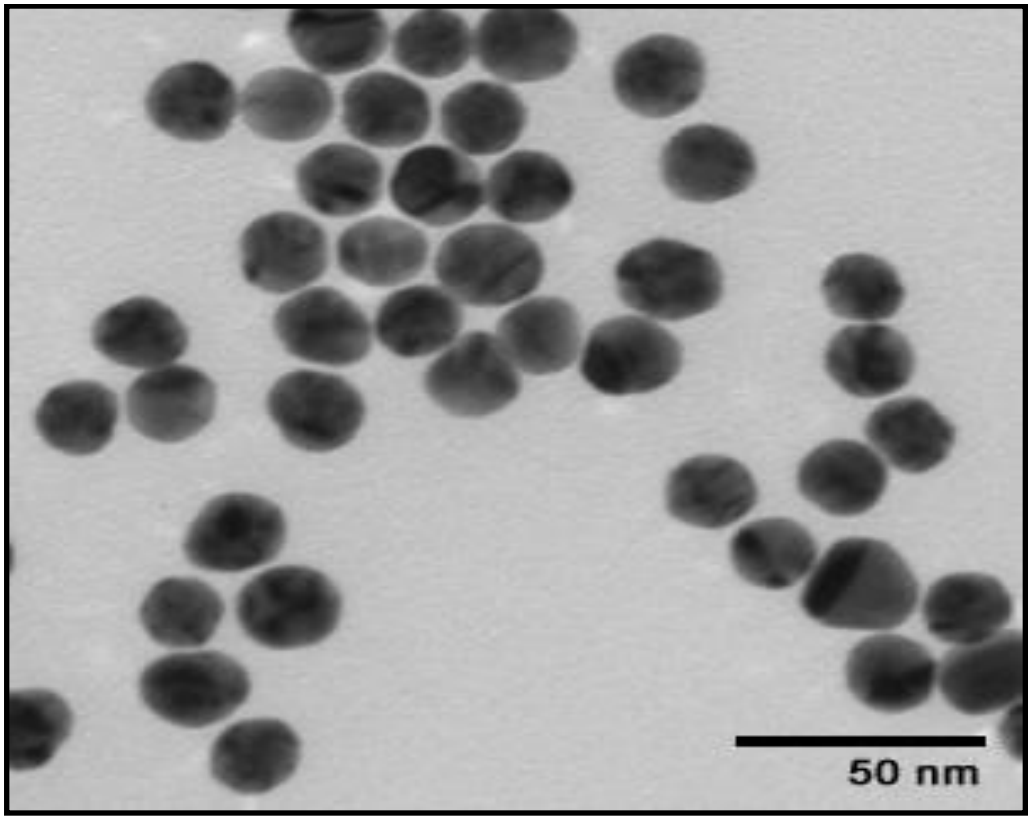


Figure 6.13: TEM micrograph of Bimetallic (Gold-Silver) Nanoparticles

TEM image showed that the nanoparticles formed were characterized by uniform distribution with spherical shape.

Bimetallic silver-gold nanoparticles formed 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.