Biogenesis of Metal Nanoparticles

Dissertation thesis

Submitted in partial fulfillment of the requirement
For the award of the degree of Masters of Science in
Biotechnology

By

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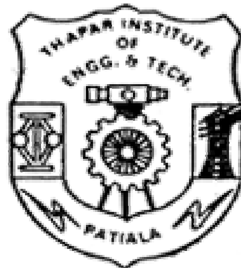

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DEPARTMENT OF BIOTECHNOLOGY & ENVIRONMENTAL SCIENCES
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I hereby declare that the work presented in the dissertation entitled "biogenesis of metal nanoparticles", submitted in partial fulfillment of the requirement for the award of degree of Masters of Science in Biotechnology to Thapar Institute of Engineering and Technology (Deemed University), Patiala, is an authentic record of my dissertation work during a period of five months from January 2005 to May 2005, under the supervision of Dr. N. Tejo Prakash, Assistant Professor, Department of Biotechnology and Environment Sciences. The matter embodied in the dissertation has not formed the basis for award of any degree or diploma in this or any other university or institute.

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This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge.

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Certificate

This is to certify that the thesis entitled "biogenesis of metal nanoparticles" submitted by Jesse S. Samuel in partial fulfillment of the requirements for the award of Degree of Masters of Science in Biotechnology to Thapar Institute of Engineering and Technology (Deemed University), Patiala, is a record of the students own work carried out by him under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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Nanotechnology can best be considered as a description of activities at the level of atoms and molecules that have applications in the real world. A nanometer is a billionth of a meter, that is, about 1/80,000 of the diameter of a human hair, or 10 times the diameter of a hydrogen atom. The field of nanotechnology has generated great enthusiasm in recent years because of its expected impact on various fields of sciences. For the advancement of nanotechnology, development of reliable procedures for the synthesis of nanoparticles of controlled size and chemical composition is imperative.

Many chemical routes are known to use toxic chemicals for the synthesis of the nanoparticles. The need-of-the-hour, however is to evolve procedures for nanoparticles synthesis through environmentally benign routes. Researchers in this field, therefore, have been eagerly looking at biological systems as alternative ecofriendly systems.

The present work was carried out to examine the formation of gold nanoparticles by the fungi viz. *Cladosporium resinae, Aspergillus niger* and *Paecilomyces variotii.*

The work culminated into the following results:

- XRD analysis confirming the presence of elemental gold in the biomass of test fungi.
- Light scattering (Nanosight™ technology) analysis confirming the formation of nano-sized particles extracellularly.

The field of nanotechnology has opened up new worlds of possibility and has spawned a proliferation of new terminology. The two fundamentally different approaches to nanotechnology are graphically termed 'top down' and 'bottom up'. 'Top-down' refers to making nanoscale structures by machining and etching techniques, whereas 'bottom-up', or molecular nanotechnology, applies to building organic and inorganic structures atom-by-atom, or molecule-by-molecule. Top-down or bottom-up is a measure of the level of advancement of nanotechnology. Nanotechnology, as applied today, is still in the main at what may be considered the more primitive 'top-down' stage. A breakthrough that may signal the beginning of the 'bottom-up' stage of nanotechnology has been the discovery of elemental and molecular structures. These may open the door to huge applications for medicine and information technology (Seeman and Belcher; 2002).

Another feature of nanotechnology is that it is an area of research and development that is truly multidisciplinary. Research at the nanoscale is unified by the need to share knowledge on tools and techniques, as well as information on the physics affecting atomic and molecular interactions in this new realm. Materials scientists, mechanical and electronic engineers and medical researchers are now forming teams with biologists, physicists and chemists.

New applications of nanomaterials are emerging rapidly. The synthesis of nanoparticles is a cornerstone of nanotechnology. New methods to study synthesis of nanoparticles are an area of active research. The methods currently being used encompass chemical routes. A big impediment to encouragement of these methods is that the byproducts associated with metal production have become a great concern with respect to environmental pollution, additionally; some of these processes are expensive. In addition, the synthetic procedures involve conditions such as high temperature, pressure and environmental inertness, which are cost intensive (Rao *et al.,* 2003).

Alternative metal recovery/removal methods are being considered which are based on metal sequestering and or metal uptake from solution by biological systems. Nature however, is no stranger to nanotechnology; living organisms from bacteria to beetles rely on nano-sized protein-based machines that do everything from whipping flagella to flexing muscles. The molecular machinery of nature out performs anything that mankind knows how to construct with conventional manufacturing technology by many orders of magnitude (Lowe, 2000). Biological systems have a unique ability to control the structure, phase, orientation and nano-structural topography of inorganic crystals.

It is well known that inactivated biological systems interact with metal ions; the connection between the two is more in depth. As is well known that many elements in trace concentration are essential to plant growth and propagation; however these very elements become toxic to the plants at higher concentrations (Gardea-Torresdey *et al.,* 2002). It has been shown that many plants as well as bacteria can actively uptake and reduce metal ions from soil and solutions. A well-known example of reduction and production of nanoparticles is the magneto-tactic bacteria that can synthesize magnetic nanoparticles (Schuller and Frankel, 1999) which have an enormous number of applications (Safarik and Safarikova, 2002). Another example is the production of gold nanoparticles using inactivated Alfalfa biomass (Gardea-Torresdey *et al.,* 2002). The possibility of using Lactic acid bacteria in the whey of buttermilk has shown the production of gold-silver composite materials when challenged to a mixture the ions of the two metals (Nair and Pradeep, 2003). These are some examples that show the biotechnological solutions to material-science. Although many biotechnological applications such as remediation of toxic metals employ microorganisms such as bacteria and yeast, it is only relatively recently that material science has been viewing these as possible eco friendly nano-factories (Ahmad *et al.,* 2002a).

Microbes affect the redistribution of metal by oxidation, reduction or biosorption. Microbes may solublize the metals as in the case of uranium, or reduce them, as in the case of iron and manganese. Microbial biomass can retain relatively high quantities of metal by biosorption (passive mode) or by bioaccumulation (actively by viable cells) (Volesky, 1995). It has been recently shown that several types of

inactivated biomasses and living organisms have the ability to remove high concentrations of Au^{3+} from solution by converting it to Au^0 (Gardea-Torresdey *et al.,* 1999).

Fungi, due to their tolerance and bioaccumulation ability of metals, are taking the centre-stage of studies on biological metal nanoparticle generation (Sastry *et al.,* 2003). A few advantages of using fungal mediated green approach for the synthesis of nanoparticles are as follow:

- Economic viability.
- Ease in scale up as in thin solid substrate fermentation method, thus making it possible to easily obtain biomass for processing.
- Large-scale secretion of extracellular enzymes (fungi are extremely efficient producers and secretors of extracellular enzymes).
- Ease in handling biomass.

Keeping these points in view, the present study was focused to examine the potential of fungal species, which had been isolated earlier from bio-contaminated fuel at Defense Materials Stores, Research and Development (DMSRDE), Kanpur had been found to be resistant to metals, to generate metal nanoparticles.

Nanotechnology is an upcoming field the benefits of which are believed to revolutionize various other fields like computers, pharmaceuticals etc. The synthesis of nanoparticles of different chemical composition, controlled size is an important area of research in nanotechnology. Nanoparticles of metals and semiconductors have an immense use in various other branches of sciences. There are various chemical methods (Murray *et al.,* 2002) and physical methods (Ayyub *et al.,* 2001) to synthesize nanoparticles, but these routes for synthesis of particles/crystallites require tedious and environmentally challenging techniques. The growing needs to develop clean, non-toxic and eco-friendly procedures for synthesis of nanoparticles has resulted in researchers seriously looking at biological systems for inspiration.

Ever increasing pressure to develop environmentally benign technique for nanoparticle synthesis has lead to a renewed interest in biotransformation as a route to growth of nanoscale structures. Biological systems have a unique ability to control the structure, phase and nanostructural topography of the inorganic crystals (Cui and Gao, 2003). It is well known that microbes such as bacteria (Beveridge and Murray, 1980; Brierley, 1990), Yeast (Huang *et al.,* 1990), fungi (Frilis and Myers 1986; Volesky 1995) and algae (Sakaguchi *et al.,* 1979; Darnall *et al.,* 1986) are able to adsorb and accumulate metals and can be used in the reduction of environmental pollution and also for the recovery of metals from waste. Amongst these microorganisms, only a few groups have been confirmed to selectively reduce certain metal ions (Tobin *et al.,* 1987; Kamilo *et al.,* 1991; Klaus *et al.,* 1999, Sharma *et al.,* 2000; Mukherjee *et al.,* 2001; Nair and Pradeep 2002; Oremland *et al.,* 2004). The potential of microbes to reduce metals has lead to another new dimension of ‘Quantum Dots’ or Semiconductor bimetallic nanoparticles with immense use in the semiconductor devices (Dameron *et al.,* 1989).

Biogenesis of metal nanoparticles

Bacteria

Magnetotactic bacteria are a group of gram-negative prokaryotes that are diverse with respect to morphology and habitat. They have the ability to synthesize fine (50-100nm) intracellular membrane bound ferromagnetic particles composed of magnetite (Fe_3O_4) or greigite (Fe_3S_4) which are covered with an intracellular phospholipids membrane; the structures thus formed are called magnetosomes (Schuller and Frankel, 1999). Klaus and co-workers (1999) reported the generation of Ag crystals of very well defined shapes such as pyramidal and hexagonal in *Pseudomonas stutzeri* AG259. This bacterium was also found to generate AG-nanoparticles of upto 200nm size. TEM, quantitative energy dispersive X ray diffraction established that the presence of three different shaped crystals. These Ag-nanoparticles were found embedded in the organic matrix of the bacterial cell.

Sharma and co-workers (2000) isolated a highly cadmium-resistant *Klebsiella planticola* strain *Cd-1*, from reducing salt marsh sediments. The strain was found to grow in up to 15 mM $CdCl_2$ under a wide range of NaCl concentrations and at pH ranging from acidic or neutral. In growth medium amended with thiosulfate, it precipitated significant amounts of cadmium sulfide (CdS), as confirmed by x-ray absorption spectroscopy. In comparison with various other strains tested, *Cd-1* was found to be superior for precipitating CdS in cultures containing thiosulfate.

Nair and Pradeep (2002), reported the generation Ag, Au and Ag-Au alloy crystals of submicron dimensions upon exposure to the precursor ions by *Lactobacillus* strains, common in buttermilk. Crystal growth was observed to occur by the coalescence of clusters, and number of crystals was found within the bacterial contour. Coalescence appeared to be a route by which surface area of the crystal is reduced so that it can be effectively protected to avoid biological damage.

Oremland and co-workers (2004) reported the structural and spectral features of selenium nanospheres produced by Se-respiring bacteria. They examined three selenate and selenite respiring bacteria for the occurrence of elemental selenium.

When grown in the presence of selenium oxyanions as the electron acceptor, all three organisms formed stable, uniform extracellular nanoparticles of diameter ~300 nm of elemental selenium having monoclinic crystalline structure. Formation of intracellular Se-nanoparticles was also observed. After harvesting and cleaning of the cellular debris, a large difference in the optical properties of the nanoparticle formed by the three bacteria was observed. The spectral property in turn differed substantially from that of amorphous Se^0 formed by the chemical oxidation of H_2Se and that of vitreous (black) Se^0 formed chemically by reduction of selenite with ascorbate. The microbial synthesis Se^0 nanoparticles result in unique, complex, compact nano-structured arrangement of Se atoms. These arrangements probably reflect a diversity of enzyme involved in the assimilatory reductions that are subtly different in different microbes

Actinomycetes

Ahmad and co-workers (2003a) reported the Intracellular biogenesis of gold nanoparticles. The organism used was *Rhodococcus sp* which is an alkalotolerant Actinomycete. The gold nanoparticles obtained showed a good monodispersity with size ranging from 5-15 nm. The particles were found on the cell wall as well as on the cell membrane but in a larger amount in the former. This observation was probably due to reduction of the gold ions by enzymes present in the cell wall and cell membranes. The metal ions were not toxic to the cells and they remained viable even after the reduction of gold ions.

Ahmad and co-workers (2003 b) have also reported the extracellular generation of gold nanoparticles by chemical reaction of the biomass with chloroaurate ions. Formation of a high concentration of gold nanoparticles of an average size of 8nm was observed. The exact mechanism leading to the reduction of the metal ions in the organism has not yet been elucidated. As a first step in this direction, this group analyzed the proteins released into water by the organism in terms of number of proteins secreted and their molecular weights. Preliminary gel-electrophoresis analysis indicated the presence of four different proteins ranging from 10-80 KDa. It is believed that one or more copy of these proteins may be enzymes that reduce the chloroaurate ions and cap the gold nanoparticles formed

by the reduction process. It is also possible that different proteins affect capping and stabilizing of the gold nanoparticles.

Yeast

The biosynthesis of quantum crystallites in yeast have been reported in *Candida glabarata* and *Schizosaccharomyces pombe* cultured in the presents of cadmium salt (Dameron *et al.,* 1989). Short chelating peptides of general structure [(γ-glu-cys) $_n$ –gly] control the nucleation and growth of CdS crystallites (a well known semiconductor material) to peptide capped intracellular particles of diameter 20Å. These quantum CdS crystallites are more monodisperse than the ones synthesized chemically. *C.glabarata* and *S.pombe* respond to Cd salts by synthesizing γ-glutamyl peptide. Cytoplasmic cadmium ions were sequestered within organic complexes contained in these γ-glu peptides. Cadmium binds to these peptides forming Cd-γ-glu peptide complex. In both cases, X-ray pattern of lyophilized material showed a single sharp peak, indicating a dense aggregate of homogeneous particles.

Kowshik and co- workers (2002) reported the intracellular synthesis of cadmium sulfide nanoparticles by *S. pombe* strain when challenged with 1mM cadmium solution. The CdS nanoparticles exhibited an absorbance maximum at 305nm. X-ray scattering data showed that the nanoparticles had Wurtzite ($Cd_{16}S_{20}$) type hexagonal lattice structure and most of the nanoparticles were in the size range of 1-1.5nm. The nanoparticles were used in the fabrication of hetero-junction with poly-(p-phenylenevinylene). The diode exhibited ~75mA/cm^2 current at 10 V when forward biased and a breakdown at ~15 V in the reverse biased mode. These characteristics are considered ideal for a diode. This particular study was the first of its kind in the application of biogenic nanoparticles in optoelectronic electronic devices.

Extracellular synthesis of silver nanoparticles was observed in silver tolerant *yeast strains* MKY3 when challenged with 1mM soluble silver in the log phase of growth (Kowshik *et al.,* 2003). It was shown that silver was not reduced when the supernatant of MKY3 culture (grown in the absence of silver) was exposed to an

environment of silver ions. It was reported that although the exact mechanism leading to reduction of silver ions to elemental silver is yet to be elucidated, it is possible that certain biochemical reducing agents are secreted by yeast cells in response to silver stress. TEM and X-ray diffraction analysis confirmed that metallic silver nanoparticles of size 2-5nm were being formed. Extracellular synthesis of nanoparticles could be highly advantageous from the point of view of synthesis in large quantities and easier downstream processing as compared to the intracellular synthesis.

Higher Plants

Sankar and co-workers (2002) reported use of geranium leaves (*Pelargonium graveolens*) and its endophytic fungus in the extra-cellular synthesis of gold nanoparticles. Sterilized geranium leaves and an endophytic fungus (*Colletotrichum sp.*) growing in the leaves were separately exposed to aqueous chloroaurate ions. In both cases, rapid reduction of the metal ions was observed resulting in the formation of stable gold nanoparticles of variable size. In the case of gold nanoparticles synthesized using geranium leaves, the reducing and capping agents appear to be terpenoids while they are identified to be polypeptides/enzymes in the case of *Colletotrichum sp*. The biogenic gold nanoparticles synthesized using the fungus were essentially spherical in shape while the particles grown using the leaves exhibited a variety of shapes that included rods, flat sheets and triangles.

Gardea-Torresdey and co-workers (2002) reported the formation of gold nanoparticles inside live Alfalfa plants. Alfalfa plants were grown in $AuCl_4$ rich environment. The seeds were soaked in 3% formaldehyde to avoid contamination by fungus and thoroughly washed thrice with deionized water. The seeds were then transferred to nutrient medium to allow plant growth to take place. Gold (III) from potassium tetracholoaurate was added in different concentrations in different jars containing the seeds. Four replicates of each treatment were prepared at the optimum pH of 5.8. The plant growth conditions were maintained at 12-hour photoperiod (25^0C) and dark period (18^0C). The Alfalfa plant was harvested after 2 weeks of growth, washed thoroughly, frozen in liquid nitrogen and then placed in a

freeze-dry system for 2 days. The absorption of Au metal by the plant was confirmed by X-ray absorption studies (XAS) and TEM. Atomic resolution analysis confirmed the nucleation and growth of Au nanoparticles inside the Alfalfa plant. TEM images also showed defects such as twins in the crystal structure and in some cases icosahedral nanoparticles were also observed. X-ray EDS studies confirmed that the nanoparticles formed were that of pure gold.

Armendariz and co-workers (2004) studied Oat (*Avena sativa*) biomass as an alternative to recover Au (III) ions from aqueous solutions and for its capacity to reduce Au^{3+} to Au^0 forming Au nanoparticles. To study the binding trend of Au^{3+} to oat and the possible formation of Au nanoparticles, the biomass and a solution of Au^{3+} were reacted for a period of 1 hr at pH values ranging from 2 to 6.The results demonstrated that Au^{3+} ions were bound to oat biomass in a pH-dependent manner, with the highest adsorption (about 80%) at pH 3. HRTEM studies showed that oat biomass reacted with Au^{3+} ions formed Au nanoparticles of FCC (face centered cube), tetrahedral, decahedral, hexagonal, icosahedral multi-twined, irregular and rod shape. These studies also showed that the pH of the reaction influenced the nanoparticle size. The smaller nanoparticles and the higher occurrence of these were observed at pH values of 3 and 4, whereas the larger nanoparticles were observed at pH 2.

Fungi – as model systems for nanoparticles biosynthesis

From the days of discovery of penicillin to the present day applications for synthesis of metabolites, the uses of fungi have been known for a long time. Since many metals play an important role in key metabolic reactions catalyzed by enzymes, fungi are capable of sequestering and accumulating them. These features are probably due to either active metal intake for metabolic purpose and/ or passive metal uptake and biosorption.

For large-scale biosorption of metals, fungi possess unique advantages over bacteria:

1. Most fungi have a very high wall-binding capacity as well as intracellular metal uptake capacities (Volesky and Holan 1995).

2. They are easy to culture on a large scale by solid substrate fermentation, thus making a large amount of biomass available for processing.
3. Fungi can grow over the surface of inorganic substrate during culture. This leads to the metal being distributed in a more efficient way as a catalyst.
4. Fungi produce large amount of enzymes per unit biomass.

The use of specific enzymes such as reductases secreted by fungi opens up exciting possibilities of designing a rational biosynthesis strategy for metal nanoparticles of different chemical composition.

Mukherjee and co-workers (2001a) demonstrated that the fungus *Verticillium* sp. When subjected with an aqueous solution of Chloroaurate ($AuCl_4^-$) resulted in the reduction and consequent intracellular formation of gold nanoparticles of good monodispersity. The average size of the nanoparticles was around 17 nm. A careful examination of the TEM image revealed extremely small particles of gold organized on the walls of the cells. Larger particles of gold were seen within the cells and were considerably smaller in number. The number of gold nanoparticles was observed to be higher on the cytoplasmic membrane than on the cell wall. Negligible of the metal ions was observed in solution. X-ray diffraction analysis confirmed the synthesis of gold nanoparticles by the fungus.

Mukherjee and co-workers (2001b) also reported the synthesis of silver nanoparticles by using *Verticillium* sp. On subjecting the fungal biomass to aqueous silver ions solution, formation of nanoparticles of dimension 25 ± 12 nm was observed. TEM analysis of thin sections of the fungal cell revealed the presence of silver nanoparticles on the cell wall surface, possibly due to reduction of the metal ions by the enzymes present on the cell membrane. The metal ions were found to be non-toxic to the fungus and it continued to grow after the synthesis of silver nanoparticles.

Mukherjee and co-workers (2002) demonstrated the capability of *Fusarium oxysporum* to synthesize gold nanoparticle by treatment of the fungal biomass with aqueous solution containing $AuCl_4^-$ ions. The formation of colloidal gold particles was characterized visually by the appearance purple colour in the

aqueous solution. When this solution was filtered and the fungal biomass separated, it was observed that the colour was absent in the biomass and present only in the solution, thereby showing the phenomena of extracellular reduction. TEM confirmed the morphology and the size range of 20-40nm of the nanoparticles.

Ahmad and co-workers (2002a) reported their discovery that fungi *Fusarium oxysporum* may be used to synthesize CdS and other metal Sulfide nanoparticles extracellularly by a purely enzymatic process. *F. oxysporum* when exposed to aqueous solution of Cd^{2+} and SO_4^{2+} ions lead to the formation of very stable CdS nanoparticles in solution.

Ahmad and co-workers (2002b) reported the use of fungi in intracellular production of metal nanoparticles. As part of the investigations, they observed that aqueous silver ions when exposed to the fungus *Fusarium oxysporum* get reduced in solution, thereby leading to the formation of an extremely stable silver hydrosol. The silver nanoparticles are in the range of 5-15 nm in dimensions and are stabilized in solution by proteins secreted by the fungus. It is believed that the reduction of the metal ions occurs by an enzymatic process, thus creating the possibility of developing a rational, fungal-based method for the synthesis of nanomaterials over a range of chemical compositions, which is currently not possible by other microbe-based methods.

Chen and co-workers (2003) reported the biosynthesis of silver nanoparticles through pretreatment of *Phoma sp.*3.2883 with silver nitrate. The fungal mycelia were freeze-dried and then kept on a shaker in a silver nitrate solution. It was found that silver production as high as up to 13.4 mg of was produced per gram of the dry weight of the mycelia as analyzed by atomic absorption spectroscopy. Transmission electron microscopy analysis revealed silver particles adsorbed on the mycelial walls. The size of the nanoparticles formed was found to be 71.06 ± 3.46 nm. Further examination of the particles via X-ray photoelectron spectroscopy confirmed the presence silver particles. It was concluded by them that pre-treatment of freezing the mycelia of *Phoma sp.*3.2883 has a potential to be used in silver nanoparticle production.

Although, extensive studies have been carried out on the biogenesis of nanoparticles using variety of organisms ranging from bacteria to higher plants, very limited studies have been reported using fungi for nanoparticles biogenesis. As on date only three research groups have explicit reports indicating the nanoparticles synthesis using three different fungal systems viz. *Verticillium, Phoma* and *Fusarium,* in addition to *Aspergillus* and *Acremonium* examined by the present group (Guneet kaur *et al*., 2004).

Keeping the lacunae in view, the present study attempted to examine the biogenesis of nanoparticles using *Aspergillus niger, Paecilomyces variotii* and *Cladosporium resinae* which were observed to be metal tolerant. The study focused on their potential to bio-reduce chloroauric acid extracellularly and/or intracellularly to generate gold nanoparticles.

Test fungi

1. *Cladosporium resinae*
2. *Aspergillus niger*
3. *Paecilomyces variotii*

Chemicals

1. Tetrachloroauric acid ($AuCl_4^-$)
2. Potato dextrose agar
3. Potato dextrose broth
4. Citric acid 1- hydrate
5. Sodium citrate 2-hydrate

Reagents

Citrate buffer (0.5 M) (S.D. fine chemicals)

26.27gm of citric acid-1-hydrate was dissolved in 250ml of distilled water in a 500ml volumetric flask. 36.76gm sodium citrate-2-hydrate was further added and pH maintained at 5-6.

Chloroauric acid solution ($HAuCl_4$ 2%W/V)
(S.D. fine chemicals)

For making different concentrations of Chloroauric acid solution:

Molecular weight of $HAuCl_4.3H_2O$ = 1+ 197+ 35.5*4 = 340 + 54 =394

394 gms = 197 Au

Given = 2% solution

394 gm in 1000ml = 1M

39.4 gm in 100ml = 1 M

1 gm in 100ml = $^1/_{39.4}$ M

2 gm in 100ml = $[\{(^1/_{39.4}) \times 2\} \times 1000] \approx 50$ mM

Therefore to obtain different concentrations we use the formula $M_1V_1=M_2V_2$

Media preparation

To prepare 1 liter of potato dextrose agar medium (HiMedia), 39gm of PDA was dissolved in water and the volume made up to 1 liter.

Similarly 24gms of potato dextrose broth (HiMedia) was dissolved in water and the volume made up to 1 liter.

These were the autoclaved at 121^0C at 15-psi pressure for 15 minutes.

Washing and exposure to Chloroauric acid.

The master cultures of the fungi were maintained on PDA plates and were sub-cultured from time to time. From these subcultures, a small amount of fungal biomass was cut out using a borer and inoculated into the autoclaved Potato dextrose broth in sterile conditions (laminar air flow). These PDB bottles were kept on a shaker at 150 rpm for the first 2 hours and then at 120 rpm at 28^0C for 3 days.

On obtaining sufficient growth, the fungal beads thus formed were thoroughly washed 6 times at 6 – 8 hourly intervals (while keeping them under shaking conditions between the washing sessions). This step was carried out to facilitate removal of any adhered material/media components to the fungal beads.

The washed beads were then subjected to different concentrations of chloroauric acid (20µM, 40µM, 60µM, 80µM, 100µM, 200µM, 300µM, 400µM, 500µM, and 1000µM). These were kept on a shaker for 48 hours. In the case of extracellular reduction observed (only *C.resinae*) at 40µM, 60µM, and 80µM, gold on being reduced from $Au^{3+} \rightarrow Au^0$ in solution turns the colour of the solution deep pink. In the case of intracellular/surface reduction at 300µM, 400µM, 500µM, and 1000µM the presence of purple-pink colour of the fungal beads was taken and indication of the intracellular reduction by the fungal biomass.

Viability check

The fungal beads were checked for viability after observing reduction of gold. The few beads from each flask were picked and placed in separate PDA plates under

sterile conditions (laminar flow). These plates were placed in kept in an incubator at 28^{0}C for 2 days. These plates were checked for any signs of fungal growth from the beads.

Sample preparation for X ray diffraction

Fungal beads exposed to Chloroauric acid were washed by vigorously shaking them in citrate buffer (0.5M) for 1 minute. These were then filtered through coarse filter paper, oven-dried at 70^{0}C for 3 days. Dried samples were then powdered in a ball-mill (Giegerflex). The finely powdered samples were then analyzed by an X-ray diffractometer (Rigaku D Max IIIC) with Cu-Kα radiation at 40 Kv and 30 mA current.

Sample preparation for examination of extracellular reduction

The aqueous solution with fungal biomass was initially filtered with a coarse filter paper. The biomass free filtrate was further filtered with 0.45µm filter and 0.22µm membrane filter (Whatmann) to free the solution of any cellular debris. The filtered samples were sealed in culture bottles and sent to UK to be analyzed using Halo™ nanoparticle tracking system. The methodology of the particle tracking system is based on the light scattering by the particles due to Brownian motion in solution. The samples were re-filtered and diluted for better analysis.

Biological systems have a unique ability to be self-organized and synthesize molecules that have highly selective properties. These properties make them a prospective tool that can be used to synthesize nanoscale sensors and nano-devices (Cui and Gao, 2003). Many biological systems are able to create an interface with these materials to use them. This project "biogenesis of nanoparticles" was focused on examining the potential of three test fungi which were found to be tolerant to metal, to generate metal nanoparticles.

Viability of organisms to chloroauric acid exposure

On subjecting the three test fungi (*A. niger, C. resini, P. variotii*) to different concentrations of $AuCl_4^-$ ions, visual observations of the biotransformation indicated the formation of nanoparticles either extracellularly or intracellularly, which resulted in the solution or biomass turning pink-purple in colour. This colour was very distinct as compared to the control, which was white in colour. On examining the viability of the organisms exposed to different concentrations, it was observed that the all the test organisms were tolerant upto 1mM (**Figure 1**). These observations similar to earlier studies by Mukherjee and co workers (2001; 2002) wherein the extra/intracellular generation of nanoparticles was observed at concentration upto 0.1mM $HAuCl_4$ in the case of *Verticillium sp.* and 1mM in the case of *Fusarium sp.*

Extracellular generation of nanoparticles

The extracellular reduction was observed only in the case of *C. resinae*. In case of extracellular reduction, the solution showed a deep pink colour (Figure 2). The samples indicating extracellular reduction of gold were analyzed using Halo™ nanoparticle tracking system (Nanosight, UK). The NanoSight™ technology is based on a metallized optical element illuminated by laser beam at the surface of which deeply sub-micron (nanoscale) particles in suspension can be directly visualized, counted and analyzed in real time using optical microscope. The

amount of light scattered by a particle (I_{scatt}) varies strongly (sixth power) as a function of its size (r, radius). Large particles scatter significantly more light than small particles, $I_{scatt} \alpha r^6$. Furthermore small particles move rapidly under Brownian motion appearing to 'jump' distances significantly larger than their apparent 'size'. This is particularly characteristic of particles below 100nm and increases in smaller particles. Larger particles move much more slowly and accordingly can be easily distinguished from smaller ones through their slower Brownian motion and brighter appearance. The system uniquely allows particles as small as 15nm diameter to be individually detected and analyzed both in terms of their light scattering characteristics and Brownian motion using the Halo 2.1 Nanoparticle Tracking Analysis. **Figures 3(a) and 4(a)** show the particle size distribution in the filtered and unfiltered samples and **figure 3(b) and 4(b)** show the particles light scattering intensities in both samples. The following table shows the size distribution of the particles in the samples as analyzed by the Halo 2.1 system.

	Size Distribution of particles (%)	
Size in nanometers	**Unfiltered sample**	**Filtered sample**
0-250	13.400	18.080
250-500	15.500	26.300
500-750	18.140	18.490

The results as analyzed by the above system also indicated that the suspension contained aggregation of particles indicating grouping of particles in a biomass matrix.

Various research groups have reported the visual observation of colour change in solution after the biotransformation. Mukherjee and co-workers (2002) reported that the appearance of the purple colour clearly indicates the formation of gold nano-particles in the reaction mixture during the studies carried out on *Fusarium oxysporum*. The biomass had a pale yellow colour before reaction with the gold ions, which changed to dark purple on completion of the reaction. The appearance of the purple colour clearly indicated the formation of gold nanoparticles in the

reaction mixture. The characteristic pink-purple colour of colloidal gold solutions is due to excitation of surface plasmon vibrations in the nanoparticles and provides a convenient spectroscopic signature of their formation. The researchers reported that upon filtration, the biomass was still pale yellow but that the aqueous solution contained the gold nanoparticles. This indicates that the reduction of the $AuCl_4^-$ ions takes place intracellularly. The researchers suggested that most probably, the reduction of the $AuCl_4^-$ ions occurs due to reductases released by the fungus into solution. The FTIR analysis of the particles generated extracellularly also indicated coating of lower molecular weight peptides around the particles facilitating monodispersity. TEM analysis of the particles generated by *F. oxysporum* showed size range of 20 ± 40 nm. Majority of these particles were found in aggregates as observed in the present study also. Even though there is large-scale association of the particles, individual, discrete gold nanoparticles could be clearly noted in the present studies.

Extracellular reduction of gold using fungal systems has been reported only in *Fusarium* sp. as on date. There are no other reports indicated the formation of gold nanoparticles in solution mediated through fungi.

Studies carried out on extracellular biogenesis of gold nanoparticles using *Thermomonospora* sp. (actinomycetes) by Sastry and co-workers (2003) resulted in generation of gold particles in solution ranging from 7-12 nm (avg. 8 nm) as indicated by TEM analysis. The particles were essentially spherical in nature. The uniqueness of this observation by the researchers was the formation of gold particles at extreme conditions of pH 9 – 11 and temperature above 50^0C.

Extracellular reduction of gold using higher plants such as *Pelargonium graveolens* [Geranium] (Shankar *et al.,* 2003) and *Avena sativa* [Oat] (Armendariz *et al*., 2004) have indicated that the extracellular biogenesis of gold nanoparticles was carried out by non-viable biomass only.

Intracellular/Surface formation of nanoparticles

Incase of the intracellular/surface reduction, the formation of purple colour was observed with the three test fungi examined (*A.niger, C.resinae, P.variotii*). This colour was distinctly different from the control, which was white/pale yellow. **Figures i to viii** show the X-ray diffraction peaks of the biomass after proper sample processing. The presence of distinct peaks corresponds to elemental gold

(powder diffraction data- refer **appendix**). Gold crystals are FCC (111, 200, 220) in nature. The "2θ" and "d" values at the three major peaks obtained for the test are given in **Table 1**. An estimated mean size of the crystallites obtained in the present study was calculated using the Debye-Scherrer equation; this gave a value of approximately 17 nm (**Table 1**). This indicates that the crystallites formed are nano-sized.

Various research groups have reported the visual observation of colour change in biomass after the biotransformation of chloroauric acid. Mukherjee and co-workers (2001) reported that after the biotransformation, aqueous medium in which chloroauric acid was added along with the fungal biomass remained colorless; however, the colour of the fungal biomass turned purple indicating the presence of gold nanoparticles in the biomass. Further evidence of intracellular generation of gold nanoparticles was provided by X-ray diffraction analysis of *Verticillium* biofilm deposited on Si Substrate. The presence of intense peaks corresponding to 111, 200, 220 Bragg reflections for gold agree with those reported for gold nanocrystals (Leff *et al*., 1996). TEM analysis of these particles indicated the size to be approximately 20 nm. Calculations using Debye-Scherrer e.g. with XRD data indicated the particle size to be approximately 25nm. In the present study, the particle size analysis using XRD data indicated the particle size to be 17 nm. Similar observations were also noted in the present study with prominent Bragg's peaks in fungal biomass exposed to all the concentrations. Notably the XRD peaks became more prominent with little or no interference of other elemental components, as the concentration of the gold increased from 500μM to 1mM (**Figures i to viii**).

Shankar and co-workers (2004) during their work on *Geranium sp.* and *Colletotrichum sp.* reported a number of prominent Bragg reflections (XRD) in both cases that could be indexed based on the fcc structure of gold. The reports indicated that the reflections appeared to be broader for the gold nanoparticles synthesized using the endophytic fungus showing that the particles are of smaller dimensions relative to those synthesized using the geranium leaf broth. Gold nanoparticles grown using the endophytic fungus *Colletotrichum* sp. showed little morphology variation/control and are similar in that respect to gold nanoparticles grown using other fungi and actinomycetes. In the case of gold nanoparticles

synthesized using the *Colletotrichum sp*., the particles were predominantly spherical and aggregated into larger irregular structures of approximately 50nm with no well-defined morphology.

In addition to the studies carried out on fungal systems, observations on intracellular generation of gold nanoparticles by *Rhodococcus* sp. (Ahmad *et al.,* 2003) also indicated microbial mechanisms that facilitate nanoparticle biogenesis. During these studies gold nanoparticles were obtained in the biomass with size range from 9-16 nm, as observed by TEM, which agreed with the particle size determination (12nm) using XRD data.

Studies carried out on formation and growth of Au nanoparticles inside live Alfalfa plants (Gardea-Torresday *et al*., 2002) indicated bioreduction of potassium tetrachloroaurate by the plants and accumulation of gold nanoparticles in the plant biomass. TEM analysis of the particles indicated 2-20 nm size.

It is important to note that there are limited studies examining the metal nanoparticles generation using microbial systems especially fungi. The present study contributes to the possibility of using metal tolerant fungi in biogenesis of metal nanoparticles and confirms the nanoparticles presence using material analysis techniques.

In conclusion, the study carried out on biogenesis of metal nanoparticles presents observation on intra- and extra-cellular generation of gold particles and confirmation of their presence using XRD and light scattering methods. The use of fungi as a system for nano-material biosynthesis has come in vogue in recent past as an effective alternative to chemical synthesis. The ability to synthesis gold nanoparticles rapidly with morphology control by eco-friendly biological methods is exciting and represents an important advance in making them viable alternatives to the more popular chemical methods. Especially, extracellular synthesis offers the advantage of obtaining significant quantities in relatively pure state and can easily be processed by filtering the cells and isolating the particles through cell-free filtrate. The study also exemplifies the application of metal-tolerant fungi for the metal biotransformations.

Figure1.Viability check showing fungi viable at various concentrations of chloroauric acid up to 1mM. The bead kept at 2mM concentration was non-viable.

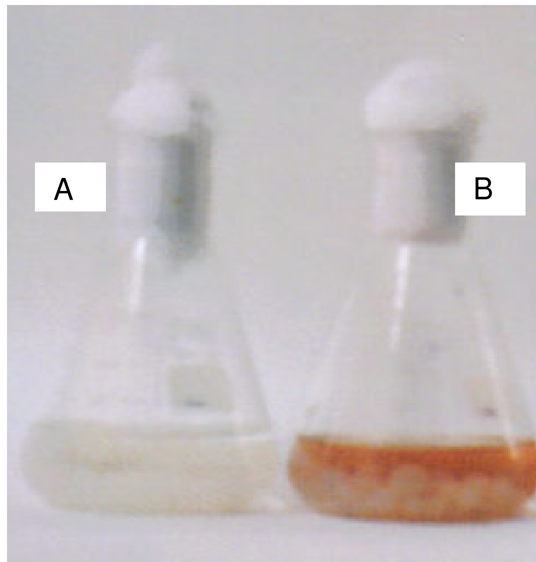

Figure 2. Indication of extracellular reduction of gold in solution Cladosporium resinae 40µm $HAuCl_4$ (B) in comparison to solution of control without $HAuCl_4$ (A)

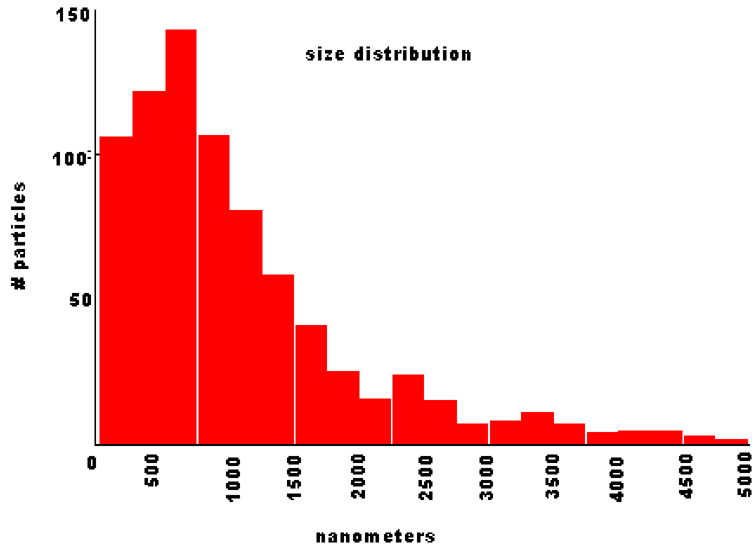

Figure 3(a) Particle size distribution in unfiltered cell free suspension as indicated by light scattering nanoparticle tracking system.

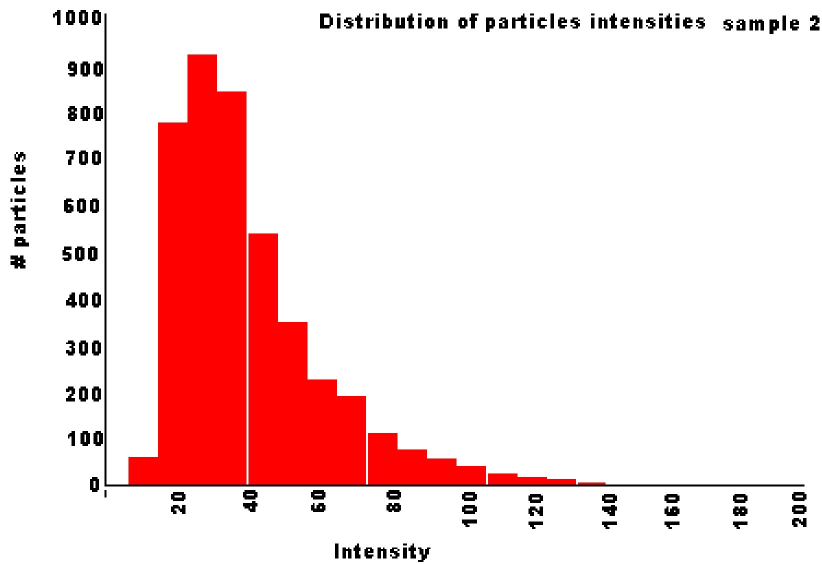

FIGURE 3(b) – Light scattering intensities of particles in unfiltered sample.

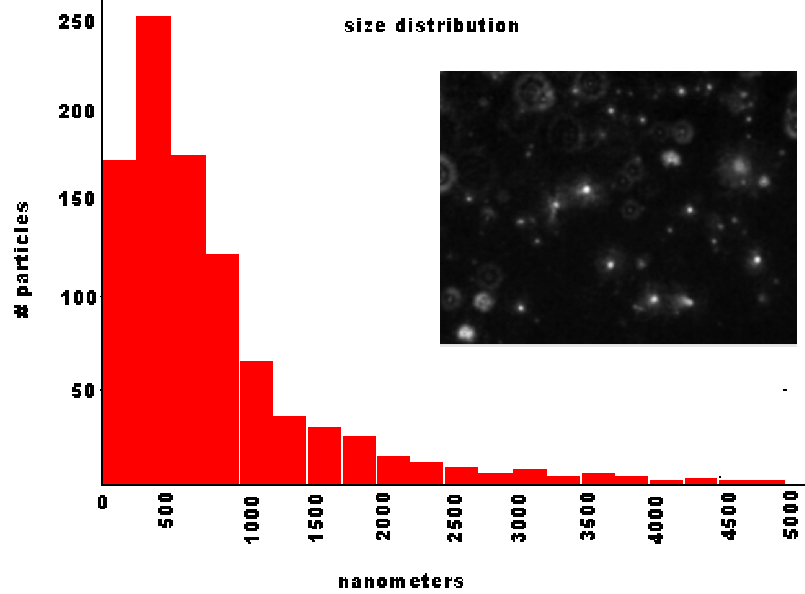

Figure 4(a) – Particle size distribution in filtered cell free suspension as indicated by light scattering nanoparticle tracking system. Image showing the scattering of light by particles (inset).

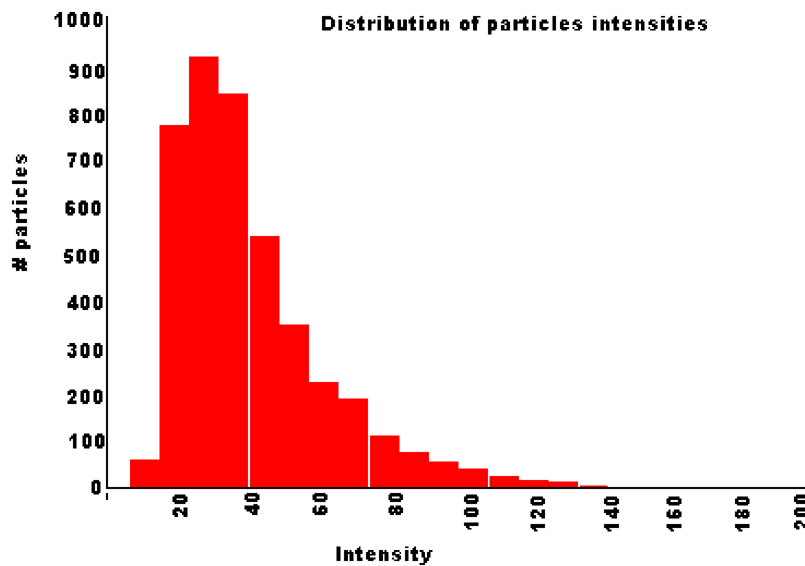

Figure 4(b) – Light scattering intensities of particles in filtered cell free suspension.

Table 1 – XRD data and size of particles (at $^{I}/_{I_o}$ = 100)

Organism	$HAuCl_4$ conc.	2θ	d	Particle size nm
Cladosporium resinae	1mM	38.120	2.359	18.94
		44.320	2.042	
		64.620	1.441	
	500µM	38.080	2.361	15.74
		44.200	2.047	
		44.380	2.040	
	400µM	38.280	2.349	14.21
		44.080	2.053	
		44.400	2.039	
	300µM	38.160	2.356	16.71
		44.440	2.037	
		64.720	1.439	
Paecilomyces variotii	1mM	38.300	2.348	18.95
		44.520	2.033	
		64.800	1.438	
	500µM	38.300	2.348	13.53
		44.460	2.036	
		64.800	1.348	
Aspergillus niger	1mM	38.320	2.347	16.72
		44.500	2.034	
		64.740	1.439	
	500µM	38.180	2.355	17.76
		44.320	2.042	

Calculation of particle size from XRD data

An estimate of the particle size can be determined from the x-ray diffraction data by using the Debye-Scherrer equation.

Debye-Scherrer equation = (K *λ) / (w * Cosθ)

Where K = constant = 0.91

λ = Wavelength of Cu radiation

W = width at half peak height

θ = Bragg angle

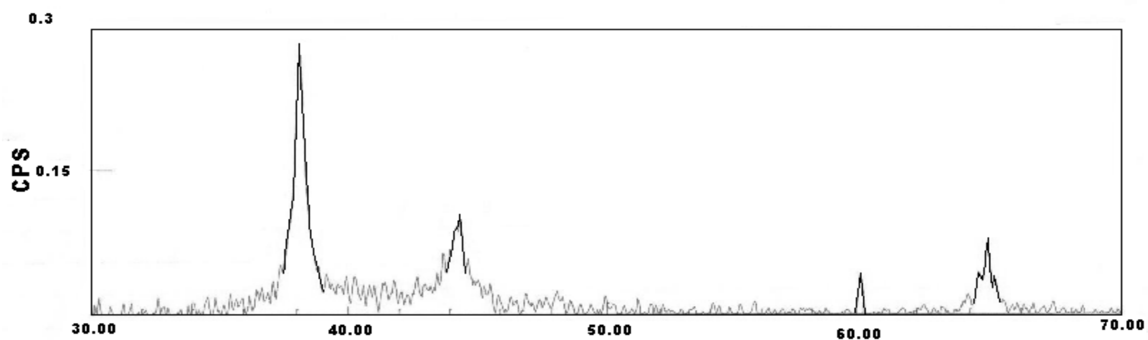

Figure i → XRD of *C.resinae* biomass exposed to 0.3mM $HAuCl_4$

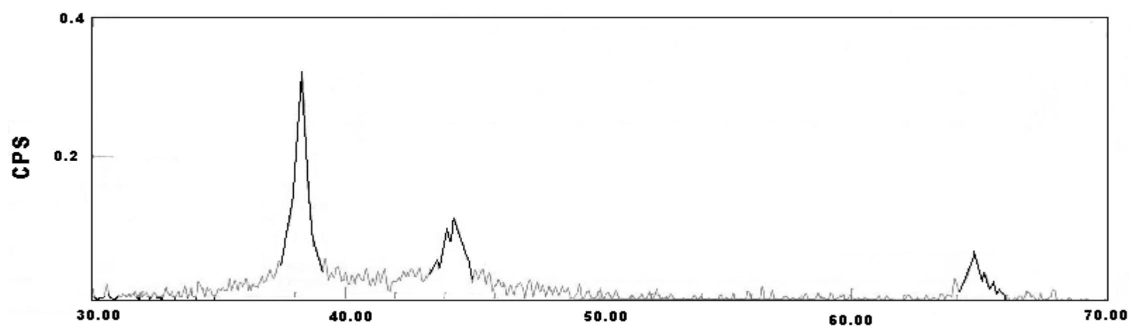

Figure ii→ XRD of *C.resinae* biomass exposed to 0.4mM $HAuCl_4$

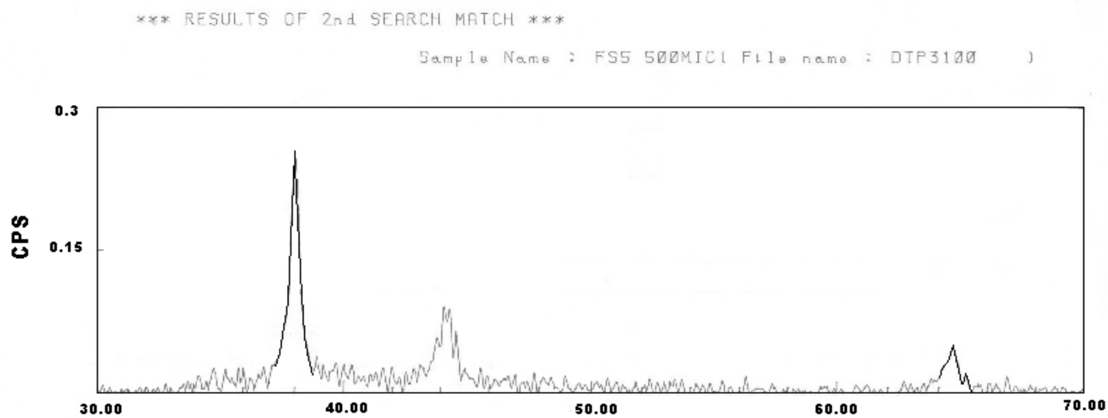

Figure iii→ XRD of *C.resinae* biomass exposed to 0.5mM HAuCl4

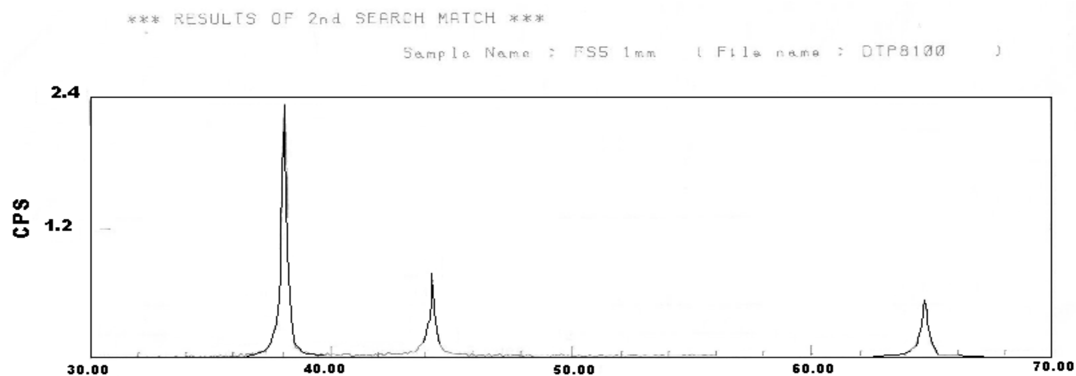

Figure iv → XRD of *C.resinae* biomass exposed to 1mM HAuCl4

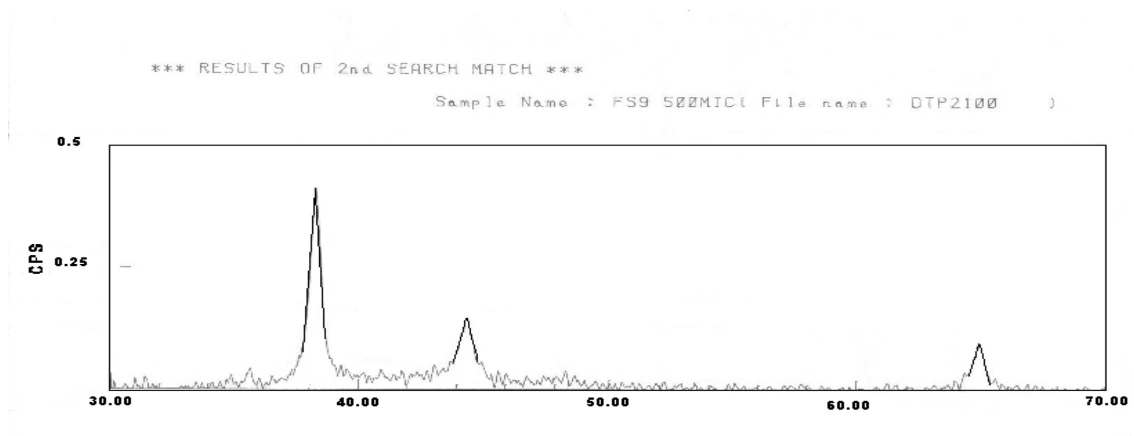

Figure v → XRD of *P.varioti*i biomass exposed to 0.5mM HAuCl4

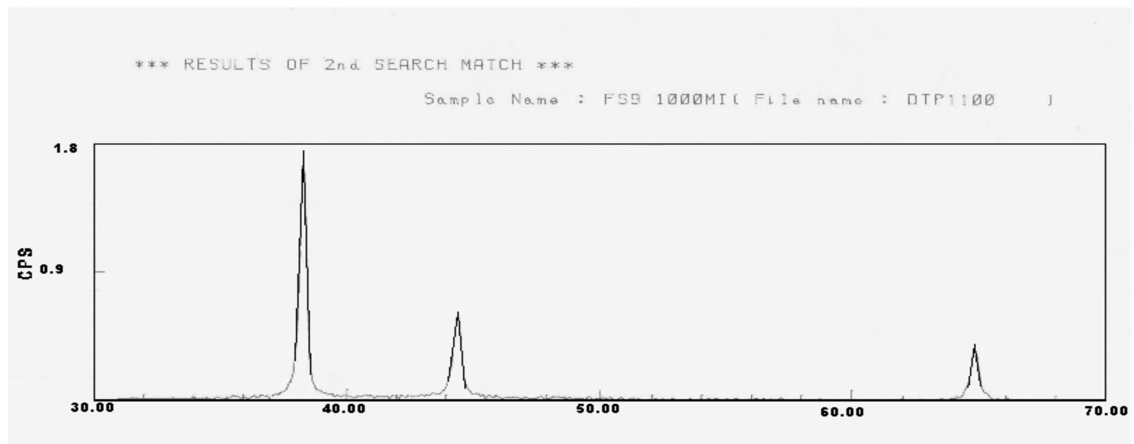

Figure vi → XRD of *P.variotii* biomass exposed to 1mM HAuCl4

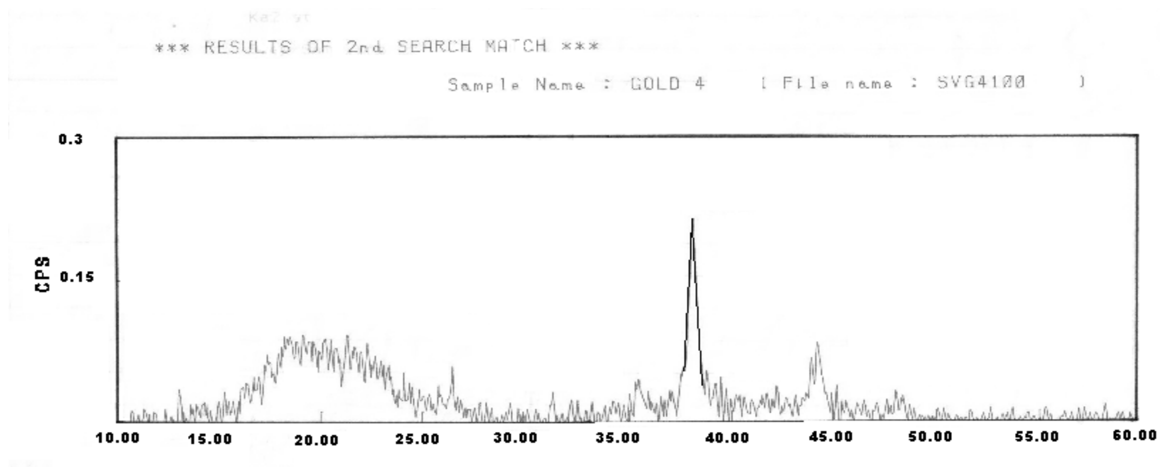

Figure vii → XRD of *A.niger* biomass exposed to 0.5mM HAuCl4

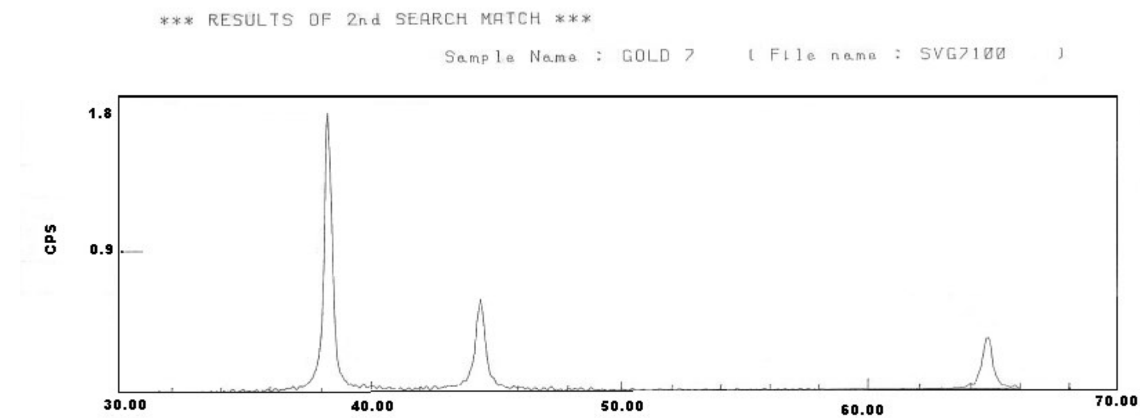

Figure viii → XRD of *A.niger* biomass exposed to 1mM HAuCl4

APPENDIX

APPENDIX

Powder diffraction pattern of Au

04-0784 Au Gold
Gold, syn
Rad.: CuKal[lambda]: 1.54056 Filter: Ni Beta
cut off: Int.: Diffract.
Ref: Swanson, Tatge, Natl. Bur. Stand. (U.S.), Circ. 539, 1, 33
Sys.: Cubic
1: 4.0786 b:
[alpha]: [beta]:
Ref: Ibid.
Dx: 19.283 Dm: 19.300
[epsilon][alpha]: [eta][omega][beta]: 0.366
Ref: Winchell, Elements of Optical
Mineralogy, 17

04-0784 Au Gold
Gold, syn
Rad.: CuKal[lambda]: 1.54056 d-sp:
cut off: I/Icor.:
Ref: Swanson, Tatge, Natl. Bur.
Stand. (U.S.), Circ. 539, 1, 33

Sys.: Cubic S.G.: Fm3m (225)
1: 4.0786 c:
[alpha]: [gamma]:
Ref: Ibid.

Dx: 19.283 SS/FOM:[F.sub.g] = 129 (.0078, 9)
[epsilon][alpha]: [epsilon][gamma]:
Ref: Winchell, Elements of Optical
Mineralogy, 17

04-0784
Au d(A)

Gold
2.3550

2.0390

1.4420
Gold, syn
1.2300
Rad.: CuKal[lambda]: 1.54056
1.1770
cut off:
1.0190
Ref: Swanson, Tatge, Natl. Bur.
0.9358
Stand. (U.S.), Circ. 539, 1, 33
0.9120

0.8325
Sys.: Cubic
1: 4.0786 A: C:
[alpha]: Z: 4 mp: 1061.6 – 1063.2
Ref: Ibid.

Color:
Yellow metallic Pattern taken at 26 C. Sample purified at NBS, Gaithersburg, Maryland, USA and is about 99.997% Au. CAS #: 7440-57-5. Spectrographic analysis (%): Si 0.001, Ca 0.001, Ag 0.001(?). Opaque mineral optical data on specimen from unspecified locality: RR2Re=71.6,
Disp.=16, VHN100-53-58, Color values=.384, .391, 72.7, Ref.: IMA Commission on Ore Microscopy QDF. Cu type. Gold SuperGroup, 1C-disordered Group. PSC: cF4. Optical data reference: Winchell, Elements of Optical Mineralogy, 17. Structural reference: Winchell, Elements of Optical Mineralogy, 17. Mwt: 196.97. Volume[CD]: 67.85.

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