3.1 Designs for the Synthesis of Nanoparticles

Different approaches designed for the synthesis of nanoparticles. There are two strategies for the synthesis of metal nanoparticles: a bottom-up (self-assembly) and a top-down [Marchiol (2012)].

Top-down approach: Bottom-up strategy refers to the construction of a structure atom-by-atom, molecule-by-molecule, or by self organization. Deals with physical methods such as thermal decomposition, diffusion, irradiation, arc discharge etc. It could be to process bulk materials by milling, laser ablation, lithography/itching etc. In the top-down approach, a suitable starting material is reduced in size using physical or chemical means. Cutting, grinding and etching are typical fabrication techniques, which have been developed to work on the nano scale [Singh *et al.* (2011)].

Bottom-up approach: Basically deals with Chemical and Biological methods which involve seeded growth method, polyol synthesis method, electrochemical synthesis, chemical reduction and biological entities for fabrication of nanoparticles [Reddy (2006)]. Bottom-up strategy refers to the construction of a structure atom-by-atom, molecule-by-molecule, or by self organization. Mycosynthesis of nanoparticles is a kind of bottom-up approach (Fig. 3.1), whereby the main reaction occurring involves reduction/ oxidation of substrates, giving rise to colloidal structures [Moghaddam (2010)]. Mold enzymes or metabolites with antioxidant or reducing properties are usually responsible for reduction of metal compounds into their respective nanoparticles [Zhang *et al.* (2011)]. Mycoreduced metal atoms undergo nucleation with subsequent growth, leading to the generation of nanostructures. The production of functional nanometer sized objects and semiconductor QDs are good example of bottom-up approach [Saravanan *et al.* (2008)]. An advantage of the bottom-up approach is the better possibilities to obtain nanostructures (nanorods,

nanocubes, nanotubes, nanowires and nanosheets etc.) with minor defects and more homogeneous chemical compositions [Thakkar *et al.* (2010)]. This is because the bottom-up approach is driven mainly by the reduction of Gibbs free energy, so that such synthesized nanostructures/nanomaterials are in a state closer to a thermodynamic equilibrium state [Behari (2010)].

Bottom-up strategy for the mycogenic synthesis of nanoparticles start with one or more precursor molecules, which undergo certain processes that result in well-organized assemblage of atoms and molecules using fungus biomass. Examples of bottom-up approaches include systems that self-assemble and triggered by minor alterations in a chemical or physical condition such as change in pH, temperature, incubation, presence of specific enzyme, media, concentration and stabilising agent etc.

The nanoparticles can be synthesized by Physical, Chemical and Biological methods. Physical and Chemical methods involve the use of strong chemical reducing agents such as Sodium borohydride and weak reducing agents such as Sodium citrate, alcohols, use of gamma and UV rays etc. studies has reported that the Biological methods depict an inexpensive and eco-friendly method for the synthesis of Nanoparticles [Rai *et al.* (2011)]. To date, biosynthesis of nanoparticles has been demonstrated by the use of biological agents such as bacteria, fungi, yeast, actinomycetes and plants. Synthesis of nanoparticles using microbes or plants is a new and emerging eco-friendly science [Huang *et al.* (2007)].

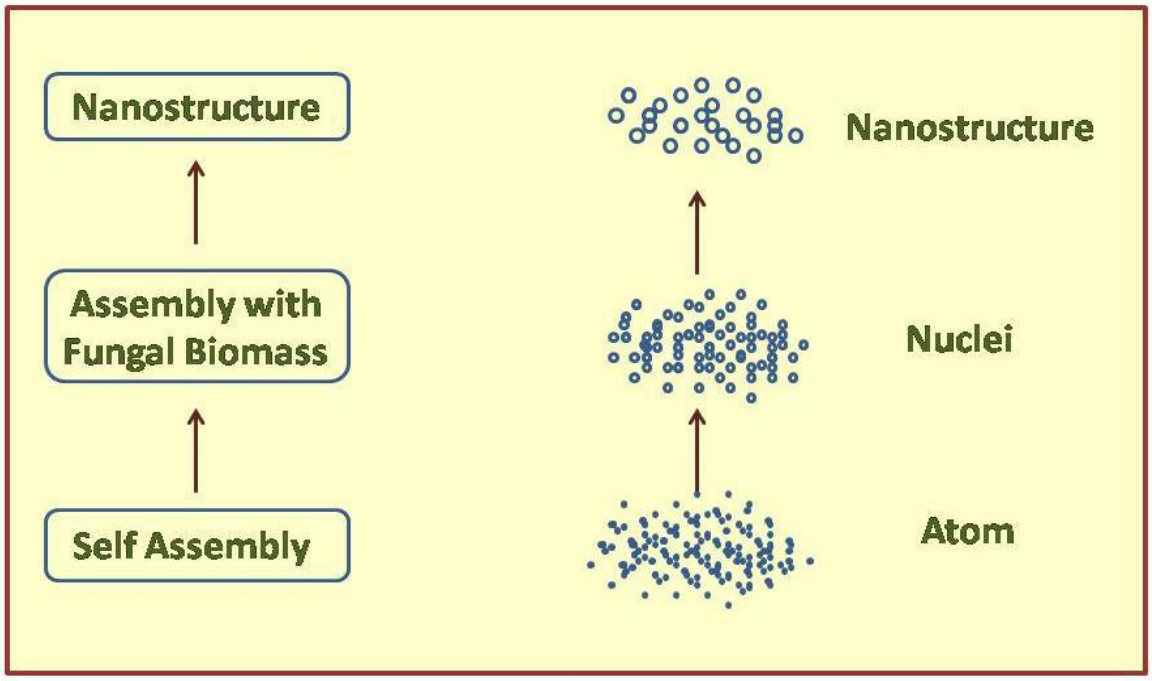

Figure 3.1: Bottom-up strategy for the mycogenic synthesis of Nanoparticles

3.2 Mechanism for the Synthesis of Nanoparticles

At present the mechanism for nanoparticles(s) fabrication by mold is not clear, according to speculation by various researchers, the hypothetical representation for the mycosynthesis of nanoparticles is depicted in Fig. 4.2. The ability of microorganisms to grow in the presence of high metal concentrations might result from specific mechanisms of resistance and their adaptability in extreme conditions. Efflux systems, alteration of solubility, and toxicity by changes in the redox state of the metal ions, extracellular complexation or precipitation of metals, and the lack of specific metal transport systems are some of the mechanisms possessed by microorganisms [Dhillon *et al.* (2012)]. Thus, it might be speculated that in the mycogenic synthesis of metal nanoparticles, the mold mycelium is exposed to the metal salt solution that prompts the mold to produce enzymes and metabolites for its own survival. In this process the toxic metal ions are reduced to the nontoxic metallic nanoparticles through the catalytic effect of the extracellular enzymes and metabolites of the fungus. It includes Intracellular & Extracellular Synthesis.

Intracellular Synthesis

In case of **mold**, nanoparticles are intracellularly synthesized by treating the fungal mycelium with metal salt solution and further incubation for 24 h [Mukherjee *et al.* (2001)]. Dried mycelium of fungi is also used for synthesis of nanoparticles. In this method, the mold mycelium is harvested by centrifugation and subsequently freeze-dried, and this freeze dried mycelium is immersed in metal salt solution and kept on a shaker [Chen *et al.* (2003)].

The mold cell surface when comes in contact with metal ions interacts electrostatically and traps the ions. Next, the enzymes present in the cell wall bio-reduce the metal ions. There will be an electrostatic interaction between metal clusters mold cell which lead to the formation of nano-clusters. Finally, aggregation of particles and synthesis of nanoparticles take place [Rai *et al.* (2011)].

The synthesis of nanoparticles using **bacteri**a and **actinomycetes** usually involves the intracellular synthesis method. In which the bacterial cell filtrate is treated with metal salt solution and kept in a shaker in dark at ambient temperature and pressure conditions [Ahmad *et al.* (2003a), Ahmad *et al.* (2003b), Mouxing *et al.* (2006), Nair *et al.* (2002)]. The negatively charged cell wall of the microorganisms interacts electrostatically with the positively charged metal ions. The enzymes present within the cell wall bio-reduce the metal ions to nanoparticles, and the small sized nanoparticles get diffused of through the cell wall. The mechanism of synthesis of nanoparticles includes trapping, bio-reduction and synthesis.

Extracellular Synthesis

Extracellular Synthesis of nanoparticles using mold. The mold culture is centrifuged at 8,000g [Das *et al.* (2009), Kalishwarlal *et al.* (2008), Ogi *et al.* (2010)]. However, the filtrate of the mycelium is treated with metal salt solution and incubated for 24 h [Basavaraja *et al.* (2007), Fong *et al.* (2006), Shaligram *et al.* (2009)].

The mechanism of extracellular synthesis of nanoparticles using microbes is basically found to be Nitrate reductase mediated synthesis. The enzyme Nitrate reductase secreted by the fungi helps in the bio-reduction of metal ions and synthesis of nanoparticles [Gade *et al.* (2008), He *et al.* (2007), Ingle *et al.* (2008), Kumar *et al.* (2007a), Kumar *et al.* (2007b)]. Duran *et al.* (2005) conducted the nitrate reductase assay test through the reaction of nitrite with 2,3-diaminophthalene.

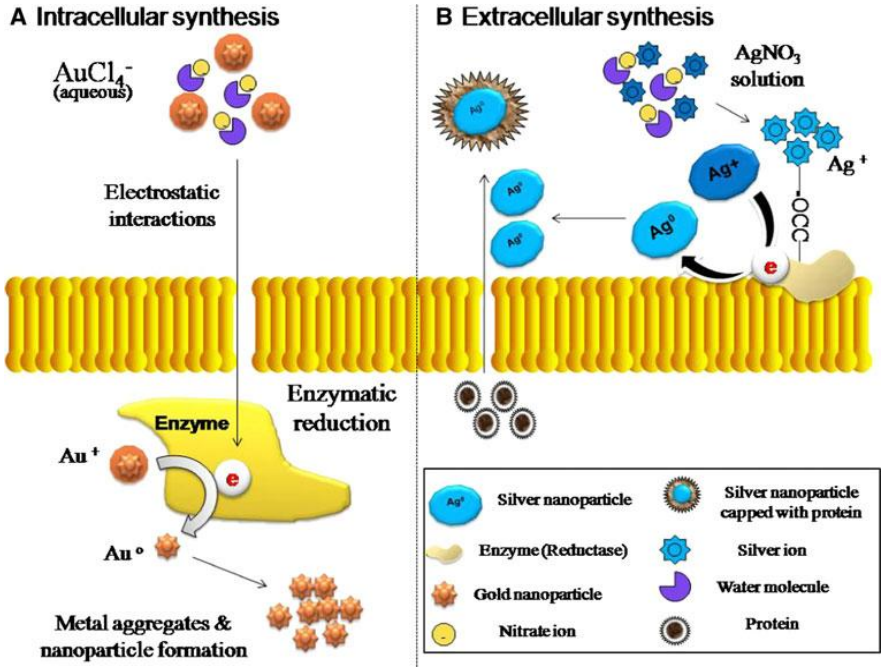

Figure 3.2: Mechanism of intracellular and extracellular synthesis of Gold (Au) and Silver (Ag) Nanoparticles through Mold [Kashyap *et al.* (2012)]

Putative mechanisms of nanoparticle synthesis using mold biosystems. During intracellular synthesis (a), metal ions (e.g. gold) initially bind on the mold cell surface through electrostatic interaction. The adsorbed metal ions are then reduced by positively charged groups in enzymes present in the cell wall, leading to their aggregations and the formation of nanoparticles. In case of extracellular synthesis (b), the exposure of metal ions to fungus resulted in the release of reductase enzyme (such as nitrate reductase) and subsequent formation of highly stable nanoparticles (e.g. silver) in solution.

Further, Duran *et al.* (2005) conducted nitrate reductase assay to explore the possible role of reductase activity or electron shuttle quinones or both in the extracellular mycosynthesis of nanoparticles. They concluded that the enzyme reductase is responsible for the reduction of Ag^{+} ions and the subsequent extracellular formation of silver nanoparticles. The role of nitrate reductase in the synthesis of nanoparticles was also studied by Kumar *et al.* (2007). The enzyme a-NADPH-dependent nitrate reductase was isolated from *F. oxysporum* and used for in vitro synthesis of silver nanoparticles. The spectra of reaction mixture showed strong surface Plasmon resonance at 413 nm which intensified with time, while the absence of absorption band at 413 nm in the absence of enzyme clearly depicted that the reduction of silver

involves enzymatic reduction of nitrate to nitrite. Thus, indicating that the synthesis of silver requires the reduction of NADPH to $NADP^{+}$ and the hydroxyquinoline probably acts as an electron shuttle transferring the electron generated during the reduction of nitrate to Ag^{+} ions converting them to Ag^{0} (Fig. 4.2). In accordance with the above studies, Ingle *et al.* (2008) conducted the nitrate reductase test using commercially available nitrate reductase discs to clarify the above assumptions. The colour of the disc turned reddish from white when challenged with fungal filtrate signifying the presence of nitrate reductase. Briefly, it can be concluded that the electrostatic interaction and specific enzyme(s) of fungi

(e.g. NADPH dependent reductase enzyme and hydroxyquinoline, phytochelatin etc.) are major factors in the mycogenic synthesis of nanoparticles, even though the real mechanism of biosynthesis of nanoparticles is still unclear.

3.3 Methodology used for the Synthesis of Nanoparticles

There are a large number of physical, chemical, biological and hybrid methods available to synthesize different types of nanoparticles. The physical and chemical methods involve the use of strong chemical reducing agents such as sodium borohydride and week reducing agents such as sodium citrate, alcohols, use of gamma and UV rays, etc. Some chemical methods cannot avoid the use of toxic chemicals in the synthesis process [Azim *et al.* (2009), Tapan *et al.* (2009)]. Usually the chemical methods followed are carried out under very high temperatures to form AuNPs and also result in environmental pollution due to toxicity of the reagents (reducing agents like acetylene) used. Therefore, there is an urgent need to develop a green process of nanoparticle synthesis; biological methods of nanoparticle synthesis using either microorganisms or plant extracts have offered a reliable, eco-friendly alternative to chemical and physical methods. [Wang *et al.* (2007), Rai *et al.* (2011)]. Studies have reported that the biological methods depict expensive and ecofriendly method for synthesis of nanoparticles [Rai *et al.* (2011)].

Physical method includes Mechanical which includes high energy ball milling, melt mixing etc. and Vapour process which includes physical vapour deposition, Laser ablation, Sputter deposition, electric arc deposition, ion implementation.

Chemical method includes colloidal, sol-gel, L-B films, inverse micelles etc.

Biological method includes using DNA, proteins (enzymes), plants and microorganisms.

Hybrid method includes Electrochemical, Chemical vapour deposition, Particle arresting in glass or zeolites or polymers, Micro emulsion.

Biological Method

Use of microorganisms like fungi, yeasts (eukaryotes) or bacteria and actinomycetes are employed for the production of silver and gold nanoparticles. Plant Extract or Enzymes are also used for the biosynthesis of nanoparticles [Ahmad *et al.* (2002), Ahmad *et al.* (2003b), Mukherjee *et al.* (2008), Rai *et al.* (2008), Shaligram *et al.* (2009)]. Further the biosynthesis of nanoparticles are done by the use of proteins & template like DNA, membranes, viruses and diatoms [Kulkarni (2009)].

Biosynthesis of nanoparticles using microorganisms are widely employed in the industrial biotechnology field. One of the important area is Microbial Fuel Cell (MFC). In a MFC, microorganisms are separated from a terminal electron acceptor at the cathode so that the only means for respiration is to transfer electrons to the anode. The electrons flow to the cathode as a result of the electrochemical potential between the respiratory enzyme and the electron acceptor at the cathode. Electron transfer from the anode to the cathode must be matched by an equal number of protons moving between these electrodes so that electro neutrality is preserved [Rabaey *et al.* (2005)]. The nano-decoration of electrode with nanoparticles is one effective way to enhance power output of MFCs. Improved power generation of MFC with gold nanoparticles was modified by carbon paper [Alatraktchi *et al.*, (2014)].

Molds (2.0 - 10.0 µm) are eukaryotic; multicellular (septate / non septate) with many distinctive structural features; cultivated in our laboratory under specified conditions, which is reproduced by asexual & sexual

processes. They are fungi that grow in the form of multicellular filaments called hyphae. A connected network of these tubular branching hyphae called mycelium is considered a single organism. The hyphae are generally transparent, so the mycelium appears like very fine, fluffy white threads over the surface. They are considered to be microbes and do not form a specific taxonomic or phylogenetic grouping, but can be found in the divisions Zygomycota and Ascomycota. In the past, most molds were classified within the Deuteromycota. They multiply through reproducing very large number of small spores, which may contains a single nucleus or be multinucleate. Mold spores can be asexual (the product of mitosis) or sexual (the product of meiosis); many species can produce both types. Mold spores may remain airborne indefinitely, may cliche to clothing or fur or may be able to survive extremes of temperature and pressure. It is responsible for decomposition (deterioration) of many materials; useful for industrial production of many bio-chemicals, including penicillin; cause diseases of humans, other animals and plants. It includes *Acremonium, Aspergillus, Cladosporium, Fusarium, Mucor, Penicillium, Phoma, Rhizopus, Stachybotrys, Trichoderma* and *Alternaria species.* Yeasts (5-10 µm) which are eukaryotic; unicellular are cultivated much like that of bacteria. It involves in the production of alcoholic beverages and also used as food supplement. It includes *Saccharomyces, Candida, Kluyveromyces, Eremothesium* and *Candida* species [Pelczar *et al.* (2008)].

3.4 Superiority of Biological Methods

Green synthesis gives advantage over chemical and physical methods in terms of its cost effectiveness, environment friendly and can be easily scaled up to large scale industrial level. Chemicals which were used for nanoparticle synthesis were mostly toxic and harmful to ecosystem. The facile synthesis of silver nanoparticles by the combination of chemical

agents (Sodium dodecyl sulphate, m-hydroxy benzaldehyde, ammonia etc.) studied by S. Sarkar *et al.*(2007). Though it was very fast facile synthesis however there are some toxic effects of such nanoparticles was observed [Kumar *et al.* (2008), Hiramatsu *et al.* (2004), Rebello *et al.* (2013)]. Hence, microbial synthesis of nanoparticles was considered better as compared to other methods.

In Biological methods, the shape and size of nanoparticles can be controlled by modulating pH and temperature of the reaction mixture. Gericke and Pinches (2006b) have obtained different shape morphologies (Triangular, Spherical, Hexagonal and Rod shaped) by modulating the pH of the reaction mixture to 3, 5, 7 and 9. Rai *et al.* (2009) demonstrated that the amount of nanoparticles synthesized at 35 °C is more than at 65 °C. The biological systems secrete a large amount of enzymes, which are capable of hydrolyzing the metals and thus bring about enzymatic reduction of metal ions. In case of fungi, the enzyme nitrate reductase is found to be responsible for nanoparticle synthesis [Ahmad *et al.* (2003), He *et al.* (2007)]. Hence biological method for the synthesis of nanoparticles are environmental friendly approach and also considered as an economically viable technique.

3.5 Chemicals and Instruments

Chemicals

1) Dextrose	S. D. fine chemicals
2) Agar	Merck
3) Yeast extract	S. D. fine chemicals

Instruments

1) Balance	Contech
2) Laminar flow hood	Narang Scientific
3) Shaker-incubator	Orbitex
4) Refrigerator	Godrej
5) Autoclave	Narang Scientific
6) Centrifuge	Remi

Figure 3.3: Instruments used in study of Nanoparticles (A) Shaker incubator (B) Autoclave (C) Laminar Air Flow (LAF) & (D) Centrifuge

3.6 Mold Selected for Nanoparticle Synthesis

Potato Dextrose Agar (PDA) media sterilised at 15 psi at 121°C for 15 minutes

Inoculation of mold into the medium & incubated at 28°C for 3 days

Revived and sub-culturing of master plates

Store master plates at 4°C in refrigerator till further use

3.7 Media Composition for Sub-culturing of Mold Culture

Ingredients	Gram/Litre (g/L)
Potato	200
Dextrose/Glucose	20
Yeast Extract	1
Agar	30

pH: 5.5±0.2

Potato Dextrose Agar (PDA): Composition and Methods

200 g peeled potato, cut into small pieces

500 ml Distilled Water added into potato

Boiled for 2 hours

Filter out with muslin cloth, took extract

20g glucose, 1g yeast extract

30g agar added

Volume make up to 1L

To prepare PDA media, 200 g of peeled potatoes were chopped & boiled for 30 minutes in 1000 ml of double distilled water. Churn it properly by using glass rod. Sieved it through a muslin cloth. Added 20 g of dextrose. Make up the volume to 1000 ml using Distilled water. Put the beaker in a boiling water bath, added 30 g of agar in fraction & continuous stirring. Transfer the PDA media in the various sub culturing glassware like test-tubes, Petridis plates etc. Put tight cotton plugs over the test-tubes. Cover the test-tubes and Petridis plates with brown paper. They were autoclaved at 121°C for 15 minutes. After autoclaving, put test-tubes inclined at around 45° to make slants.

Revival of strain was done to revive the culture from the freeze-dried state so that the microorganisms can be grown till it achieves the lag phase. Strains of microorganisms were revived to ensure the survivability. Revival of mold strain was done on Czapek Dox Broth media. Mold growth was observed between 4-5 days of growth.

3.8 Media Composition for Growth Characterisation

Czapek Dox Broth: Composition and Methods

Czapek Dox Broth media: Composition

Sucrose: 30g

Sodium Nitrate: 3g

Dipotassium Hydrogen Phosphate: 1g

Magnesium Sulphate: 0.5g

Potassium Chloride: 0.5g

Ferrous Sulphate: 0.01g

Distilled Water: 1L

pH: 5.5±0.2

100 ml media prepared in 250 ml

Erlenmeyer flask

Sterilised at 15 psi at 121°C for 15 min.

Inoculated with mold strain

Culture allowed to grow in a orbital shaker

incubator at 28°C and 200 rpm

At regular time intervals, fixed sample volumes taken out

Follow the same protocol with $AgNO_3$,

$HAuCl_4$ and $AgNO_3$.$HAuCl_4$ supplementation

3.9 Media Composition of Bacterial Culture for Antimicrobial Assay

Lysogeny Broth (LB) Agar: Composition and Methods

500 ml Distilled Water

10g Tryptone + 5g Yeast + 10g NaCl

15g agar added

Volume make up to 1L

pH: 7.2±0.2

3.10 Cyclic Voltammetry Study

Cyclic Voltammetry Study of Mold Strains:

Study in normal Czapek Dox media:

i. Mold strains were inoculated in 100 ml each of sterile Czapek Dox media and incubated at 28°C and 200 rpm.
ii. After 2 days of incubation, mycelium biomass was separated from cell filtrate.
iii. Three samples were prepared – cell mass only, cell mass and broth, cell filtrate.
iv. Cyclic Voltammetry (CV) was performed between -1V to 1 V at 0.01 V/s scan rate and 0.001 V sample interval.

Study in Nanaoparticles supplemented media:-

i. Mold strains were inoculated in 100 ml each of sterile Czapek Dox media and incubated at 28°C and 200 rpm.
ii. After 2 days of incubation, mycelium biomass was separated from cell filtrate.
iii. In three different flasks containing distilled water, 4-5 g of mycelium biomass was added and tested with 0.1 mM $HAuCl_4$, $AgNO_3$ and Bimetallic (Gold-Silver) in each three flasks.
iv. The culture was incubated at 28°C and 200 rpm for 24 hours.
v. After incubation, three samples were prepared – Gold Nanoparticles cell mass, Gold Nanoparticles cell mass and broth, Cell filtrate.
vi. Cyclic voltammetry (CV) was performed between -1V to 1 V at 0.01 V/s scan rate and 0.001 V sample interval.

Standard Reduction Potential (E^0) of Gold salt [Au(III)/Au(0)] is +1.50 and Silver salt [Ag(I)/Ag(0)] is +0.799. As lesser the E^0, more stronger is the reducing agent**.**

Measurement of Reduction Potentials

Cyclic Voltammetry is the most widely used technique for acquiring qualitative information about electrochemical reactions. It offers a rapid location of redox potentials of the electroactive species. The fact that certain bacteria can direct electrons to their outer surface, or transfer electrons from their outer surface to their inner compartments, make it possible to measure the rate of electron flux from a living organism. Microbial electron transfer to electrodes combines a series of enzymatic reactions such as oxidation of acetate via th TCA cycle to yield NADH and reduce menaquinone), with a pathway of reversible electrochemical reactions that allow electrons to pass to the cell surface.

While catalytic Voltammetry is obviously related to situations such as continuous electricity production in fuel cells and produces easily measurable large signals, it represents the interplay between diffusion of donor, irreversible oxidations and electron transfer. Voltammetry in the absence of electron donor can reveal the potentials of exposed redox proteins, and can often be used to estimate interfacial rate constant or long range diffusion parameters [Andrienko (2008)].

Cyclic Voltammetry study was conducted to analyze the reducing capacities of the mold strain [Marsili *et al.* (2008)]. Its experimental setup consists three-electrode electrochemical cell (CH1222)

- Ag/AgCl electrode cell as the reference electrode (CH1111)
- Platinum wire as counter electrode (CH1115)
- Glassy carbon electrode is used as working electrode (CH1104)

After 5 days of growth, media samples were collected, filtered and used for cyclic Voltammetry analysis. Cyclic Voltammetry (CV) was performed between -1V to 1 V at 0.01 V/s scan rate and 0.001V sample interval. Cultivating mold strains in Czapek Dox media. The study in gold chloride supplemented media was conducted with 0.1 mM

$HAuCl_4$ at 28°C & 220 rpm in shaker incubator. In the two stage cultural study, after 3 days of growth, the cultures were supplemented with fresh media and with 0.1 mM $HAuCl_4$. CV analysis of the filtered media was done after three mere days of incubation anaerobically. The redox potential of the mediators was measured as the average of the electrode potential (Ep) value found from the forward and the reverse scale of CV. Since, the measurements were carried out in the reference to standard Ag‖AgCl electrode, the values were converted to standard electrode potential in reference to Normal Hydrogen Electrode (NHE).

Precedure:

i) Sterile media taken as control.

ii) Electrochemical cell filled with sample solution (20 ml).

iii) Silent period of 1000 second used to stabilize cell and contents.

iv) Cyclic Voltammetry (CV) performed between -1V to 1V at 1 mV/s scan rate.

v) Differential graph of I vs. V obtained to determine peak range.

3.11 Nanoparticle Production

Synthesis of Gold, Silver, Bimetallic (Gold-Silver) Nanoparticles from Mold Strain

100 ml CDB prepared, sterilised at 15 psi at 121°C for 15 minutes

Inoculation of mold into the medium & **incubated** at 28°C at 200 rpm for 3 days

After incubation, separate cell biomass by **filtration** and washed thrice with DW.

Wet biomass **suspended** into a Erlenmeyer flask containing 100 ml of each Silver Nitrate/Gold Chloride/Bimetallic solutions

Culture was incubated at 28°C at 200 rpm for 1 day.

For control, Mold biomass suspended in Distilled Water, incubated at 28°C at 200 rpm for 1 day

3.12 Nanoparticles Characterization Techniques

1. **UV-Visible Spectroscopy Analysis**

- The culture flasks were removed from orbital shaker after 24 hour of incubation.
- Take Control which does not contain any metal ions as a **Blank**.
- **2 ml** of sample was withdrawn and absorbance was measured at a resolution of 1nm using UV-Visible Spectrophotometer (UV-Vis) at a **wavelength range of 300-700 nm**.
- For measurement of UV-Vis absorbance, Shimadzu UV- 1800 was used.
- As the **particle sizes** of Gold Nanoparticles varies between **5 – 100 nm**, the value of **peak wavelength** changes between **515 – 572 nm**.
- As the **particle size** of Silver Nanoparticles varies between **5–100 nm**, the value of peak **wavelength** changes between **393 – 462 nm**.

2. **X-Ray Diffraction (XRD) analysis:**

   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°.

3. **Scanning Electron Microscope-Energy Despersed X-Ray (SEM-EDX) Analysis**

- Gold/Silver/Bimetallic Nanoparticles solutions were centrifuged at 10,000 rpm for 15 minutes.
- Pellet was collected and placed at -54°C followed by **lyophilisation**.
- The lyophilised mass was **powdered** using mortar and pestle.

The powdered samples were placed on pin stubs and examined by Scanning Electron Microscopy (SEM) on ZEISS: EVO18 equipped with an Energy Dispersive Spectrometer (EDS) on OXFORD INSTRUMENTS.

Applications:- Scanning Electron Microscopy has been applied to the surface studies of metals, ceramics, polymers, composites and biological materials for both topography as well as compositional analysis. An extension (or sometime conjugation to SEM) of this technique is Electron Probe Micro Analysis (EPMA), where the emission of X-rays, from the sample surface, is studied upon exposure to a beam of high energy electrons. Depending on the type of detectors used this method is classified in to two as: Energy Dispersive Spectrometry (EDS) and Wavelength Dispersive Spectrometry (WDS). This technique is used extensively in the analysis of metallic and ceramic inclusions, inclusions in polymeric materials, diffusion profiles in electronic components.

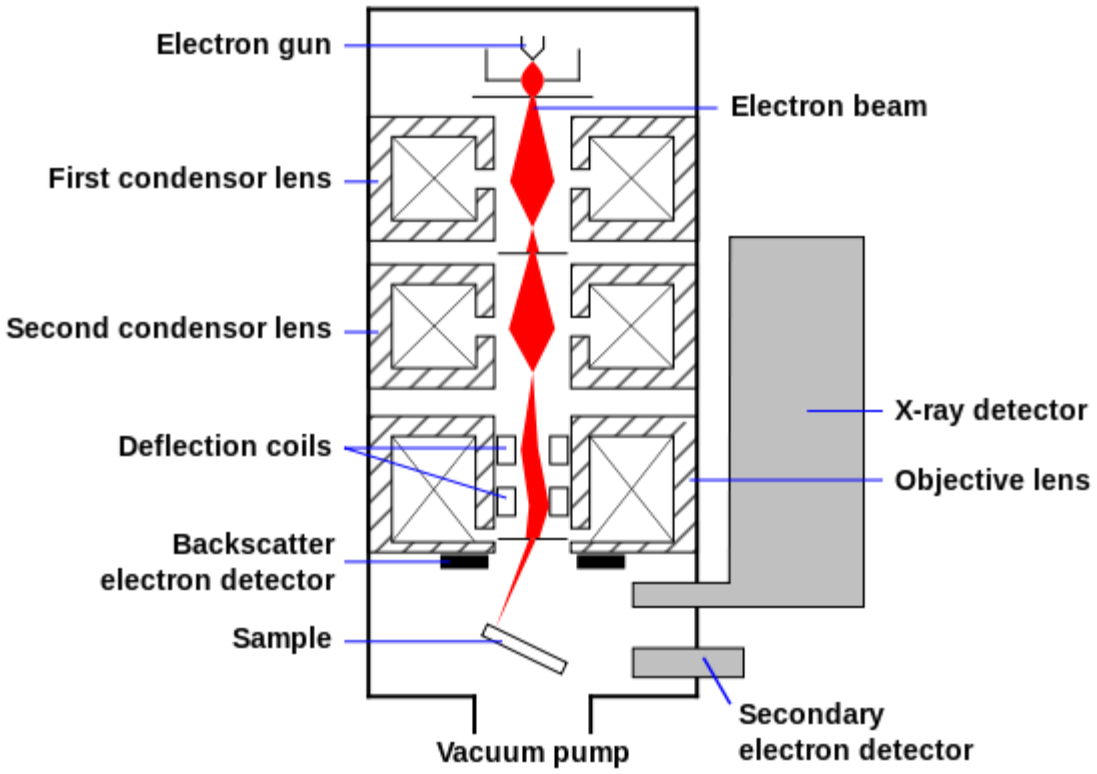

Fig. 3.4 Schematic diagram of Scanning Electron Micrograph (SEM)

Disadvantage:- The instrumentation is complicated and needs high vacuum for the optimum performance.

As the metal particles are good conductors, they can be observed without any prior carbon coating at a magnification of 1000× in a voltage of 10kV. Fig.1 (a) and (b) are the SEM image of *A. fumigatus* biomass after the addition of the chloroauric acid at 2 µm and 500 nm respectively. It was identified from SEM images that the fungal mycelia loaded with glittering particle. This depicts that the glittering particles on the mycelia should be gold nanoparticles accumulated on the mycelia intracellularly. The gold nanoparticles loaded in the mycelia were found to be in the size range of 85.1-210 nm.

4. Transmission Electron Microscopy (TEM) Analysis

- Gold/Silver/Bimetallic Nanoparticle solution were centrifuged at 10,000 rpm for 15 minutes.
- Pellets was collected and placed at -54°C followed by lyophilisation to preserve.
- Lyophilised mass was powdered using mortar and pestle.
- Powdered sample dissolved in **ethanol. Ultra-sonicate** the sample **for dispersion**.
- **10µl** of dispersed sample was coated on **cupper grid** under vacuum and examined by Transmission Electron Microscopy (TEM) on ZEISS: EVO18 equipped with an Energy Dispersive Spectrometer (EDS) on OXFORD INSTRUMENTS.