CHAPTER 2

MATERIAL AND METHODS

2.1 CHEMICALS

Chemicals for media preparation and other general chemicals were procured from different sources such as Hi-Media (Mumbai), SRL (Chennai), Merck (Mumbai) and Qualigens Fine Chemicals (Mumbai). All the chemicals used were of analytical grade. The consumables like filters, membranes, were purchased from the following companies and stored according to the manufacturer’s recommendation. All the reagents were prepared using deionised (Millipore) or glass distilled water.

2.2 CULTURE MEDIUM

Bold’s Basal Medium

Major stock solutions

\[ \begin{align*}
\text{NaNO}_3 & : 25\text{g/L} \\
\text{CaCl}_2.2\text{H}_2\text{O} & : 2.5\text{g/L} \\
\text{MgSO}_4.7\text{H}_2\text{O} & : 7.5\text{g/L} \\
\text{K}_2\text{HPO}_4 & : 7.5\text{g/L} \\
\text{KH}_2\text{PO}_4 & : 17.5\text{g/L} \\
\text{NaCl} & : 2.5\text{g/L}
\end{align*} \]
Minor stock solutions

\[ \text{H}_3\text{BO}_3 : 11.42\text{g/L} \]

Alkaline EDTA solution

\begin{align*}
\text{EDTA} & : 50\text{g/L} \\
\text{KOH} & : 31\text{g/L}
\end{align*}

Acidified Iron solution

\begin{align*}
\text{FeSO}_4.7\text{H}_2\text{O} & : 4.98\text{g/L} \\
\text{H}_2\text{SO}_4 & : 1.0 \text{ ml}
\end{align*}

Trace Metals solution

\begin{align*}
\text{ZnSO}_4.7\text{H}_2\text{O} & : 8.82\text{g/L} \\
\text{MnCl}_2.4\text{H}_2\text{O} & : 1.44\text{g/L} \\
\text{MoO}_3 & : 0.71\text{g/L} \\
\text{CuSO}_4.5\text{H}_2\text{O} & : 1.57\text{g/L} \\
\text{Co(NO}_3)_2.6\text{H}_2\text{O} & : 0.49\text{g/L}
\end{align*}

The final pH of the medium was adjusted to 6.6.

2.3 ALGAL CULTURE

The microalga *Chlamydomonas reinhardtii* was obtained from the Culture Collection Center, CAS in Botany, University of Madras, India. Initially 1ml of starter culture was inoculated into 9 ml of Bolds Basal medium (BBM) and maintained at 25°C with a 12 hour photoperiod for seven days. This was the seed culture, subsequently, this was inoculated into 40 ml of BBM and the culture was thus grown to required volume.
2.4 SYNTHESIS OF NANOPARTICLES

2.4.1 Synthesis of Gold Nanoparticles

The microalga was grown as a suspension in a 1L Erlenmeyer flask containing 225 ml of Bold's basal medium and this was treated as the source culture. Four such flasks were prepared for each experiment. The culture (~1g wet weight) was pelleted at 12,557 g at 4°C. The supernatant was discarded and the pellet was washed repeatedly (3 times) with sterile distilled water. The cell pellet was sonicated for twenty minutes at 60% amplitude with a 10 second on and 50 second off cycle. Following sonication, the cell extract was centrifuged at 8,720 g for 10 minutes and the supernatant was collected for further experiments. To initiate the experiments, 40 ml of sterile water was taken, to which 5ml of cell extract was added. The mixture was heated to 37°C with vigorous stirring for ten minutes, following which 5ml of chloroauric acid solution (final concentration 1mM) was added. The experiments were conducted at 37°C and the reaction was completed within two and a half hours. At the end of the reaction the gold nanoparticles were separated by centrifugation at 5,581 g, they were washed repeatedly with sterile water to remove other proteins or organic matter that may bind to the nanoparticles. Control experiments without the addition of chloroauric acid were also performed.

2.4.2 Synthesis of Zinc Sulphide Nanoparticles

*Chlamydomonas reinhardtii* was cultured in 225 ml bolds basal medium in a 1L Erlenmeyer flask at 25°C with a twelve hour photoperiod for a week. The cells were pelleted at 12,557 g for twenty minutes and at 4°C. The cell free extract that was obtained was used to synthesize zinc sulphide nanoparticles. 25 ml of cell extract was taken and made up to 100 ml using deionized water; to this zinc sulphate was added to obtain a final
concentration of 1mM. The reaction was allowed to proceed for 24 hours. Following the completion of the reaction the zinc sulphide nanoparticles were separated by centrifuging at 8,720 g; the pellet was washed repeatedly with de-ionized water to remove other organic matter that may be bound to the nanoparticles. Control experiments of cell extract without zinc sulphate was performed.

2.4.3 Synthesis of Cadmium sulphide nanoparticles

The microalgal culture was prepared by inoculating 25 ml of *Chlamydomonas reinhardtii* into 225 ml of bolds basal medium prepared in a 1L Erlenmeyer flask. The culture was maintained at 25°C with a twelve hour photoperiod for a period of seven days after which the cells were harvested. The cell free extract was obtained by centrifuging the culture at 12,557 g for a period of twenty minutes at 4°C. 25 ml of the extract was made upto to 100 ml with de-ionized water; this was placed in a water bath at 65°C. Cadmium chloride and sodium sulphide were added to the extract to obtain a final concentration of 1mM for both. The reaction was completed in twenty minutes and following the completion of the reaction the cadmium sulphide nanoparticles were separated by centrifuging at 8,720 g. The pellet was washed repeatedly with de-ionized water to remove other organic matter that may be bound to the nanoparticles. Control experiments involving the use of cell extract alone were performed.

2.4.4 Synthesis of Zinc Oxide Nanoparticles

The preparation of the cell extract was as described above. 25 ml of cell free extract was taken and made upto 100 ml using de-ionized water. To this zinc acetate was added to obtain a final concentration of 1mM, the pH of the reaction was adjusted to 8 and the reaction was conducted in a water bath set at 80°C and monitored for the formation of white floccules that
precipitated. The reaction was completed within an hour. Following the completion of the reaction, the zinc oxide nanoparticles were pelleted by centrifuging at 8,720 g and washing the pellet repeatedly with de-ionized water. The pellet was then dried and used in further experiments.

2.5 CHARACTERIZATION OF NANOPARTICLES

2.5.1 UV-Visible Spectroscopy

UV-visible spectroscopy is a useful tool to determine the formation of nanoparticles as well as their stability. The reduction of nanoparticles was monitored by periodic sampling (1mL) and measuring the UV-visible spectrum of the solution in a quartz cuvette using a UV-visible spectrophotometer (Perkin Elmer Lambda 35) at a resolution of 1nm and scanning speed of 930 nm/minute. The samples were suitably diluted with deionized water to avoid errors due to high optical density of the solution.

2.5.2 High Resolution Transmission Electron Microscopy (HR-TEM) Analysis

HR-TEM micrographs of the nanoparticles were obtained using a FEI Tecnai G2, model T-30 S-Twin operating at 200 kV. The samples were drop casted on a carbon coated copper grid and allowed to dry at room temperature.

2.5.3 High Resolution Scanning Electron Microscopy (HR-SEM) and Energy Dispersive X-ray Spectroscopy (EDX) Analysis

The surface morphology of the nanoparticles was characterized using HR-SEM (FEI Quanta FEG 200) equipped with energy dispersive X-Ray spectrometer (EDX). Energy Dispersive analysis of X-rays (EDX) is a
chemical microanalysis technique. The samples were drop casted on a carbon tape with an accelerating voltage of 30 kV.

2.5.4 Powder X-Ray Diffraction Analysis

Powder X-ray diffraction is an efficient technique used to determine the structure of materials. The crystalline nature of the nanoparticles and their crystal type can be identified by this method. Following repeated washes with sterile water, the nanoparticles were dried and used for powder X-ray diffraction. The diffraction data was recorded on Rigaku MiniFlex II diffractometer, with 2θ values ranging from 20° to 90° and Cu Kα (1.5406 Å) source running at 30 kV and 15 mA.

2.5.5 Dynamic Light Scattering (DLS) and Zeta Potential Measurements

The particle size and zeta potential was analyzed using Malvern Zetasizer Nano ZS system. Dynamic light scattering is a technique that has been used to measure particle size in liquid media while the zeta potential provides information about the electrophoretic mobility of nanoparticles and their stability in solution. Prior to analysis the nanoparticles were filtered using either a 0.2 μm or 0.4 μm filter. The particles were analyzed within a range of 0.1 nm to 1000 nm under the following conditions, water refractive index-1.33 and at a temperature of 25°C.

2.5.6 Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared spectroscopy is a useful tool in identifying compounds wherein the appearance or non-appearance of specific vibrational frequencies is indicative of the structure of the molecule. The nanoparticles were lyophilized and mixed with potassium bromide (KBr) in a
ratio of 1:100; a tablet was formed with the aid of a bench press. The functional groups were determined using FT-IR spectroscopy (BRUKER RFS-27, Stand alone FT Raman Spectrophotometer). Scanning was performed between 4000 cm\(^{-1}\) to 400 cm\(^{-1}\) at a resolution of 2 cm\(^{-1}\) in the transmittance mode.

2.5.7 **Fluorescence Spectroscopy Analysis**

Fluorescence spectroscopy or photoluminescence (PL) spectroscopy is a popular tool in analyzing fluorescence spectra and determining the electronic and vibrational states in a molecule. Dilute solutions of the nanoparticles were prepared and the emission spectra were measured on Jobin Yvon Fluorolog 3-11 spectrophotometer and Perkin Elmer Enspire.

2.6 **CHARACTERIZATION OF PROTEIN CAPPED GOLD NANOPARTICLES AND CATALYSIS EXPERIMENTS**

2.6.1 **Partial Purification of Proteins in Cell Extract**

The proteins in the sonicated cell extract were partially purified by slowly adding solid ammonium sulphate at a final concentration of 60\% (w/v). The mixture was gently stirred at 4°C overnight. The resulting precipitate was centrifuged at 12,557 g for 15 minutes at 4°C. The pellet was resuspended in buffer (10mM Tris buffer at pH 7) and was dialysed in a 12 kDa cutoff dialysis bag that had been pretreated at 4°C. The buffer was changed 2-3 times over a 24 hour period; following dialysis the sample was collected and analyzed using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).
2.6.2 Protein Estimation

Protein concentration was estimated by using the Lowry method (Lowry et al 1951). It is one of the most widely used methods to estimate protein concentrations in biological samples. It is based on the reactivity of peptide bonds with copper [II] ions under alkaline conditions and the subsequent reduction of Folin Ciocalteau reagent. A series of samples containing 20 µg to 100 µg of BSA in 100 µl volume was mixed with 2 ml of alkaline copper sulphate reagent and incubated for ten minutes. Following which 0.2 ml of Folin Ciocalteau reagent was added and further incubated for thirty minutes. Absorbance was measured at 600 nm. Standard graph of amount of protein versus absorbance was plotted (Appendix 2).

2.6.3 Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) (Laemmli 1970)

SDS polyacrylamide gel electrophoresis was performed using the discontinuous gel system. The stacking and separating gels were prepared using 4% (w/v) and 12% (w/v) acrylamide respectively. The gel was loaded in duplicate with 40 µg of the ammonium sulphate precipitated protein and was run at 20°C under a constant voltage of 60 V. The gel was run for a period of about 4 hours until the dye front reached the end of the gel. Following which the gel was stained using Coomassie brilliant blue dye (CBB R-250) for about 4 hours. It was then destained and protein bands were visualized in a gel imaging system.

Separating Gel (12%) : 10 ml

- Distilled water : 3.4 ml
- 1.5M Tris-HCl (pH 8.8) : 2.5 ml
- 30% Acrylamide-bisacrylamide : 4.0 ml
10% SDS : 50 μl
10% APS : 45 μl
TEMED : 5 μl

Stacking Gel (4%) : 5 ml
Distilled Water : 3.025 ml
1 M Tris-HCl (pH 6.8) : 1.25 ml
30% Acrylamide-bisacrylamide : 670 μl
10% SDS : 25 μl
10% APS : 25 μl
TEMED : 5 μl

Sample Buffer (4X)
Tris-HCl (pH 6.8) : 0.25 M
Bromophenol blue : 0.02%
SDS : 8%
2- Mercaptoethanol : 10%
Glycerol : 30%

Running Gel Buffer (pH 8.3)
Glycine : 14.14 g
Tris : 3.03 g
SDS : 0.1%
2.6.4 Catalysis of 4-nitrophenol by Gold Nanoparticles

The reduction of 4-nitrophenol (4-NP) by sodium borohydride (NaBH₄) using gold nanoparticles as nanocatalysts was studied. A 3 mM solution of 4-nitrophenol and 0.25 M solution of sodium borohydride was prepared. The reaction was initiated by adding 1 ml of 4-nitrophenol to 22 ml of water followed by the addition of 1 ml of freshly prepared sodium borohydride solution. The mixture was stirred vigorously for ten minutes to obtain a bright yellow coloured solution. To this solution 1 ml of gold nanoparticle solution was added and the reaction was monitored using UV-visible spectroscopy till the solution turned colourless. The rate constant of the reduction reaction of 4-NP was determined by measuring the change in absorbance of the peak initially observed at 400 nm for the nitrophenolate ion, as a function of time.

The effect of temperature on catalysis was studied by varying the temperature from 298 to 313 K and studying its effect rate of the reaction. Similarly the role of catalyst concentration on 4-nitrophenol degradation was analysed by varying the amount of catalyst used and studying the reaction.