Chapter IV

Bacterial synthesis of Metal Sulfide nanoparticles

This chapter describes the biosynthesis of metal sulfide nanoparticles using bacteria. Semiconductor sulfide nanoparticles like CdS, ZnS, PbS and Ag$_2$S are synthesized by two different bacterial species namely *Pseudomonas aeruginosa* and *Actinobacter* spp. Biogenic CdS, ZnS and PbS nanoparticles exhibited size quantization effect and hence can be considered as quantum dots. Moreover, bacterially synthesized CdS, ZnS and PbS quantum dots show excellent fluorescent properties. This chapter also describes the biosynthesis of magnetic iron sulfide–greigite (Fe$_3$S$_4$) nanoparticles by *Actinobacter* spp. The molecular mechanism responsible for the synthesis of metal sulfide is studied in detail and two genes underlying the process of sulfate reduction in *P.aeruginosa* were identified, cloned and successfully overexpressed in *E.coli* cells. Exposure of *E. coli* cells overexpressing sulfate reductase genes to cadmium, zinc and lead sulfate solution resulted in the formation of respective metal sulfides. Greigite nanoparticles synthesized using *Actinobacter* spp. exhibited superparamagnetic behavior at low temperatures. One of the genes, known as *cys H* encoding sulfate reductase had been identified in *Actinobacter* spp. Formation of all sulfide nanoparticles proceeds via sulfate reduction by an assimilatory sulfate reduction pathway operating in *P.aeruginosa* and *Actinobacter* spp.


*Ph.D. Thesis* Atul Bharde University of Pune
4.1 Introduction

The reliance of future technologies on developing scalable and economic methods for the fabrication of one-dimensional (1D) systems has spurred intense and rapid progress in the area of materials synthesis. Semiconductor nanocrystals constitute an important class of nanomaterials due to their unique size dependent chemical and physical properties that render them applicable in the emerging field of nanoelectronics [1]. Therefore developing reliable protocols for the synthesis of nanometer scale semiconductor particles is a problem of great importance. Among nanocrystalline semiconductors, metal sulfides are significant due to their optical, electronic and magnetic properties. Sulfide nanoparticles like CdS, ZnS and PbS are tremendously explored and find applications as fluorescent biological labels [2], optoelectronics such as nonlinear optics, flat panel displays, light emitting diodes, transistor components [3], photocatalysts [4], sensors [5], photoelectric and thermoelectric materials [6], photoimaging and photodetection [7] etc. The interesting properties of semiconductor sulfide nanocrystals are because of electronic quantum confinement and the large number of exposed atoms on the surface [8]. Quantum confinement in luminescent semiconductors enables tuning of the absorption and emission via particle size and shape [9]. Transition metal sulfides are useful as dry lubricants, catalysts and solar cells [10].

There are few reports on the synthesis of nanoparticles of transition metal sulfides like iron and nickel which show magnetic properties [11]. Iron sulfide with structural formulae Fe₃S₄ and Fe₇S₈ are magnetic minerals and called as greigite and pyrrhotite respectively. These magnetic sulfides behave as a soft magnet and are mostly studied from geomagnetic field paleoclimatic magnetism point of view [12].

Numerous protocols have been designed for the synthesis of metal sulfide nanocrystallites over a range of composition, size and shapes [13]. However most of the methods employ non – polar organic solvents or caustic chemicals. Recently, biological methods for the synthesis of metal sulfide nanoparticles are gaining importance since they occur in aqueous medium under ambient experimental conditions of temperature and pressure. Biological methods comprise use of microorganisms, small molecules of biological origin, biological templates and small peptides for the synthesis of various metal sulfide nanoparticles. Also biological molecules have been used for capping of
sulfide nanoparticles. Living organisms can exert tight control on the synthesis of materials [14]. Therefore, most of the work till date is centered on the use of micro-organisms for the synthesis of sulfide nanoparticles.

Dameron and co-workers have described the intracellular synthesis of quantum semiconductor crystallites of CdS using two different yeast species, Candida glabrata and Schizosaccharomyces pombe respectively. Short chelating peptides called as phytochelatins with the general structure \((\gamma – \text{Glu} – \text{Cys})_n – \text{Gly}\) controls the nucleation and growth of CdS nanocrystallites, where \(n\) varies from 2 – 6 [15]. Recently synthesis of nanocrystalline CdS and subsequent fabrication of diodes have been shown to occur in the yeast S. pombe [16]. Another yeast species, Torulopsis sp. has been shown to synthesize semiconductor nanocrystalline PbS [17]. Though CdS and PbS nanocrystallites synthesized by yeasts are in the quantum confinement regime, they are synthesized intracellularly [16-17]. To understand the biochemical mechanism of CdS and PbS nanocrystallite synthesis, it is argued that yeast cells upon exposure to Cd or Pb salts synthesize metal chelating peptide to nullify the stress generated by metal ions. On addition of the metal ions, a metal ion – \(\gamma\) glutamyl complex is initially formed and this is accompanied by an increase in the intracellular sulfide levels. Later this sulfide complexes with Cd or Pb to form CdS or PbS nanocrystals, which accumulate in vacuoles present inside the yeast cells [18]. A previous report from this laboratory describes the biological synthesis of CdS nanoparticles using plant pathogenic fungi Fusarium oxysporum [19]. Unlike previous reports, CdS nanoparticles were shown to be synthesized extracellularly and sulfate reductases from the fungus was speculated to be responsible for biosynthesis of CdS nanoparticles. However the process of CdS biosynthesis is considerably slower in comparison to yeasts.

Prokaryotic organisms like bacteria have long been explored for the synthesis of inorganic sulfides. Sulphate reducing bacteria use inorganic sulfates for cellular respiration and generate energy for growth and metabolism. In doing so, inorganic sulfates acts as terminal electron acceptor and are converted into respective sulfide [20]. In nature biofilms of sulfur reducing bacteria form cubic ZnS (sphalerite) from very dilute natural solutions [21]. A bacterial species Klebsiella planticola Cd-1 has been shown to synthesize CdS nanoparticles in high quantity under anaerobic conditions [22].
An enzyme thiosulfate reductase produced by bacterial cells has been shown to be responsible for the synthesis of CdS. Also biosynthesis of CdS has been shown to occur by *Klebsiella pneumoniae* [23]. In this case an enzyme cysteine desulfhydrase converts cysteine into H$_2$S, which in turn reacts with Cd$^+$ ions to form CdS. Recently Belcher and co-workers have shown biosynthesis of nanocrystalline CdS using *E. coli* [24]. However sulphur source used in this study was exogenous and bacterial cells merely act as scavenger for CdS nanoparticles that precipitate intracellularly.

Many biological molecules have been used either for synthesis or capping of CdS nanoparticles. Glutathione, cysteine and thiolates able to form high-affinity metal ligand clusters, and have been shown to promote the formation of CdS and ZnS nanocrystals [25]. Further control over nanocrystal synthesis has been gained by using fatty acids, which have been found to promote the formation of CdSe, CdS and CdTe nanocrystals [26]. Further biological approaches to semiconductor nanocrystal synthesis have been extended to intact biological particles. Viral scaffolds have been used as template for the nucleation and assembly of CdS and PbS crystalline nanowires [27]. Peptides capable of nucleating nanocrystal growth have been identified by combinatorial screens and displayed on the surface of M13 bacteriophage. This genetically engineered phage promoted the synthesis of CdS and ZnS single crystalline nanowires [28]. CdS superlattices were also shown to be directed by using self assembled S – layers from *Bacillus stearothermophilus* NRS2004/3 [29].

Additionally a strain of *Pseudomonas stutzeri* isolated from a silver mine produced nanocrystalline silver sulfide together with silver nanoparticles [30]. Bacterial synthesis of iron sulfide with crystallographic phase Fe$_3$S$_4$ (greigite) has been shown to occur in magnetotactic bacteria [31] and sulfate reducing bacteria [32]. Iron sulfide nanoparticles synthesized by magnetotactic bacteria are typically in between 35 – 120 nm and this size range is within the permanent single magnetic domain size. Like magnetite, greigite nanocrystals are synthesized in magnetosomes by magnetotactic bacteria [31]. Along with greigite iron sulfide magnetosome houses cubic and tetragonal FeS – which is thought to be precursors of Fe$_3$S$_4$. However greigite nanocrystals found in magnetotactic bacteria are synthesized under natural habitat only and no strain of magnetotactic bacteria has been shown to synthesize greigite in pure cultures in the laboratory. However, strict
anaerobic conditions are required for the synthesis of magnetosomes of greigite in natural habitats. Due to the stringent growth requirements and difficulties associated in cultivation of magnetotactic bacteria that harbors greigite magnetosomes, biomolecular mechanism of greigite synthesis is not studied in detail.

Though ample work has been done to understand the basic biomolecular mechanism responsible for the synthesis of inorganic materials, very few reports describe the genetic analysis of material formation in living microorganisms. Here we have shown the biosynthesis of metal sulfide nanoparticles using bacterial species *Pseudomonas aeruginosa* and *Actinobacter* spp.

**Outline of the present work**

Bacterial synthesis of technologically important metal sulfides like semiconductor quantum dots and magnetic iron sulfide has been illustrated in this chapter. Unlike previous reports, where metal sulfide nanoparticles produced by microorganisms are either intracellular, unstable or require anaerobic conditions, we demonstrate here extracellular, aerobic synthesis of metal sulfide nanoparticles, which are found to be stable for weeks in aqueous solution. Furthermore a detailed biochemical and genetic analysis has been described in this chapter which, we believe is responsible for the formation of metal sulfide nanoparticles or controls the crucial biochemical steps responsible for the process. This chapter also describes identification and cloning of two genes responsible for the synthesis of quantum dot semiconductor crystallites like CdS, ZnS and PbS by *P. aeruginosa*. In addition, overexpression of these two genes in *E. coli* and isolation and purification of overexpressed proteins from *E. coli* has been demonstrated. Second part of this chapter describes the biosynthesis of silver sulfide and iron sulfide using *Actinobacter* spp. Biosynthesis of silver sulfide and iron sulfide is found to be precursor dependent and essentially occurs by reduction of sulfate, which is exogenously provided as a precursor. All the semiconductor nanoparticles synthesized by *P. aeruginosa* are well within the limits of quantum confinement and hence can be considered as quantum dots. These semiconductor nanoparticles show strong fluorescence signal. Iron sulfide nanoparticles synthesized by *Actinobacter* spp. are composed of greigite (Fe$_3$S$_4$) and pyrite (FeS$_2$) and exhibit superparamagnetic behavior.
4.2. Part I: Biosynthesis of CdS, ZnS and PbS semiconductor nanocrystallites using
*Pseudomonas aeruginosa*

This part of the chapter describes the biosynthesis and characterization of group II-VI semiconductor sulfide nanoparticles like CdS, ZnS and PbS using a gram negative aerobic bacterium *Pseudomonas aeruginosa*. Further, the biomolecular mechanism for the synthesis of these sulfide nanoparticles has been discussed.

### 4.2.1 Experimental details

A Gram negative bacterium *Pseudomonas aeruginosa* was isolated from Arabian sea, Goa, India. For cultivation of bacteria in 100 ml batch, the bacterial seed culture was grown overnight in small volume (~2 ml) of Luria broth (LB) medium. This seed culture was then inoculated in 100 ml of LB medium in 500 ml Erlenmeyer flask for propagation. For the biosynthesis of CdS, ZnS and PbS nanoparticles bacterial cells were freshly grown in 100 ml of LB medium in 500 ml Erlenmeyer flask and incubated on a rotary shaker (200 rpm) at 37 °C for ~ 36 h. The bacterial biomass was then harvested from the growth medium by centrifugation at 7000 rpm (5533 X g). The supernatant was discarded and the cell pellet was washed twice with saline (0.85 % NaCl, W/V) and finally with deionised water. This is followed by mixing of bacterial biomass with 100 ml of sterilized 10⁻³ M solution of CdSO₄, and ZnSO₄ each in separate Erlenmeyer flask. For synthesis of PbS nanoparticles, bacterial biomass was mixed with 100 ml sterilized 10⁻³ M solution of PbCl₂ and Ca(SO₄)₂. In this case PbCl₂ acts as a source of lead ions while Ca(SO₄)₂ provides sulphate. PbSO₄ is very sparingly soluble in water and hence can not be used for the synthesis of PbS nanoparticles. All the flasks were incubated on the rotary shaker at 37 °C for 48 h. Next the biomass was separated from each flask by centrifugation at 7000 rpm and the supernatant was collected for further characterization. The supernatants containing sulfide nanoparticles were analyzed by TEM, UV – vis spectroscopy, fluorimetry, and XRD. For the genetic analysis of sulfate reductase genes, genomic DNA of *P. aeruginosa* was isolated and genes for phosphoadenosyl phosphosulfate reductase (PAPS) and sulfite reductase (SIR) were PCR amplified by template specific primers. Primer sequences for PAPS and SIR are

**attB1 forward:** 5′-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC CTG -3′ **attB2 reverse:** 5′−GTG CCG CGC GGC AGC ATG CTG CCC TTT GCT ACC ATT CCC G -3′ for

---

*Ph.D. Thesis*  
Atul Bharde  
*University of Pune*
PAPS reductase and \textit{attB1} 5'–GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC-3' \textit{attB2} reverse: 3' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC-5' respectively. The amplified genes were cloned and overexpressed in \textit{E. coli} BL21-AI™ (F', gal, Cm') cells using Gateway® Technology according to manufacturer’s instructions [33]. Initially PCR products i.e. amplified \textit{cysH} and \textit{cysI} genes were separately cloned in pDONR™221 plasmid, known as an entry vector. Addition of 5'-terminal \textit{attB} sequences to PCR primers allows synthesis of a PCR product that is an efficient substrate for recombination with a donor vector in the presence of BP CLONASE Enzyme Mix. This reaction produces an Entry Clone of the PCR product [33]. LR recombination reactions were performed between entry clones and a Gateway® destination vector pDEST™17. Next, the expression clones were transformed in \textit{E. coli} BL21-AI cells and induced with L- arabinose for the expression of recombinant proteins [33]. For overexpression and assay of PAPS and SIR enzymes, \textit{E. coli} BL21-AI cells were grown in the presence of ampicillin (100 μg/ μl) together with 10⁻³ M CdSO₄, ZnSO₄ and PbSO₄ respectively. The clones were induced in the presence of L – arabinose for the expression of recombinant proteins. Control experiments were performed without changing the above mentioned procedure except that non-transformed \textit{E. coli} BL-21 AI cells without pDEST™17 plasmid containing \textit{cysH} and \textit{cysI} genes for PAPS and SIR respectively were used. Overexpressed proteins were analysed by 10 % SDS-PAGE and visualized by staining the gels with coomassie blue R 250 stain.

\subsection*{4.2.2 Biosynthesis of CdS nanoparticles}

\subsubsection*{4.2.2.1 TEM analysis}

Representative TEM images of CdS nanoparticles synthesized using \textit{P. aeruginosa} are shown in Figure 4.1. A dense population of CdS nanoparticles was found to be evenly distributed on the grid area as observed by low magnification images (Figure 4.1 A- C). The size of CdS nanoparticles appears to be nearly uniform. Careful analysis of high magnification image indicates that CdS nanoparticles observed in TEM are clusters of smaller nanoparticles that are loosely assembled together (Figure 4.1 E). These clustered nanoparticles are spherical in shape with size between 4.5 – 6.5 nm. Particle size distribution analysis performed on 100 particles in different micrographs indicated
the mean diameter of CdS nanoclusters to be 5.5 nm with a small standard deviation value of 0.59 (inset Figure 4.1E).

![Figure 4.1 TEM images of CdS nanoparticles synthesized using P. aeruginosa. Low magnification images (A – C) show the presence of very high number of CdS nanoparticles. High magnification image (E) shows the loose assembly of smaller particles into bigger clusters. Inset in image E shows particles size distribution analysis. Image F shows a HRTEM image of CdS nanoparticles. The d values correspond to (100) and (111) crystal planes.](image)

Particles smaller than 5 nm were difficult to resolve with the microscope used in this study. A representative HRTEM analysis performed on CdS nanoparticles is shown in the Figure 4.1F. Clearly resolved lattice fringes of CdS nanoparticles are observed in the image indicating the crystalline nature of CdS nanoparticles. The interplanar spacing of 3.33 Å (0.33 nm) and 2.79 Å was assigned to (100) and (111) planes of cubic CdS system respectively. [34].

4.2.2.2 UV – vis spectroscopic analysis

Figure 4.2 shows the UV-vis spectroscopic profile of CdS nanoparticle formation using *P. aeruginosa*. CdS is a direct band gap semiconductor with bang gap energy value of 2.4 eV in the bulk form [35]. Bulk CdS shows a featureless absorption edge around 518 nm. UV-vis spectra recorded as a function of time of reaction between CdSO₄ and *P. aeruginosa* is shown in Figure 4.2A. UV-spectroscopic analysis reveals the presence of small absorption peak with absorption maxima around 370 nm, and corresponds to
excitonic transitions in CdS nanocrystallites [36a]. Presence of such a sharp absorption peak rather than absorption hump or shoulder indicates the narrow size distribution of nanocrystallites, since large size distribution usually results in wide distribution of the band gaps yielding a broad and featureless absorption edge [36b]. The absorption spectra shown in Figure 4.2A show a large blue shift in comparison with bulk CdS. The absorption of CdS nanoparticles increases with time indicating that CdSO₄ is gradually converted to CdS. After around 32 h of reaction all CdSO₄ molecules are reduced to CdS by the bacterium. UV-vis spectroscopic analysis suggests the size of CdS nanocrystallites to be in between 2.5 – 3 nm [36a]. This observation is in well agreement with the TEM analysis that shows small clusters of CdS nanoparticles 5 – 6 nm in diameter composed of smaller particles 2.5 to 3 nm in diameter.

Figure 4.2 (A) Time dependent UV-vis spectroscopic analysis of CdS nanoparticle formation using P.aeruginosa. Curves 1 to 9 represent excitonic absorption of CdS nanoparticles obtained after every 4 h during the course of reaction. The reaction was carried out for 32 h. (B) Band gap energy curve of CdS nanoparticles obtained by UV-vis spectroscopic analysis performed on CdS nanoparticles after 32 h of reaction. Tangent drawn to the absorption edge (dotted line) corresponds to the band gap energy of CdS nanoparticles.

A fundamental property of semiconductors is the band gap; the energy separation between the filled valence band and the empty conduction band. As mentioned above bulk CdS has band gap energy of 2.4 eV. All semiconductors show size quantization effects as the size of semiconductor material is reduced [1b, 13a]. Figure 4.2B corresponds to the absorption threshold or band gap energy analysis after 32 h of reaction. The band gap energy value obtained is approximately 3.2 eV, which is
considerably higher than the band gap energy of bulk CdS. The increase in band gap energy is due to the reduction in size of CdS and could be a consequence of size quantization effect.

4.2.2.3 Photoluminescence (fluorescence) spectroscopic analysis

CdS nanocrystallites frequently display excellent photoluminescence generally in the visible region of the electromagnetic spectrum depending on the size of CdS nanocrystallites. Figure 4.3 shows the photoluminescence (PL) spectra obtained from the aqueous solution of CdS nanoparticles synthesized using *P. aeruginosa* after 32 h of reaction. The PL spectra were obtained by exciting the aqueous solution of CdS nanoparticles at 300, 320 and 350 nm respectively. PL spectra obtained from excitation at 320 nm and 350 nm are shown in Figure 4.3A.

![Figure 4.3](image)

**Figure 4.3** (A) PL spectrum obtained from aqueous solution of biogenic CdS nanoparticles synthesized using *P. aeruginosa* excited at 320 nm (solid curve) and 350 nm (dotted curve). Inset in (A) shows a photograph of biogenic CdS nanoparticle solution after irradiation with UV light of 365 nm showing blue – green light emission. (B) Same spectrum in terms of the energy scale corresponding to emitted light.

The PL spectrum manifests a strong, sharp and uniform sized curve with emission maxima centered at 485 nm. For all emission spectra, the peak height varies in magnitude and all the spectra have same general features. Since not much difference is observed in the PL spectra obtained from the aqueous solution of CdS nanoparticles exited at various wavelengths, only emission spectra obtained at excitation wavelength of 320 nm (solid curve) and 350 nm (dotted curve) are shown in the Figure 4.3A. The large blue shift
observed in the PL band can be attributed to the small size of nanoparticles. The inset in Figure 4.3A shows a photograph of aqueous solution of CdS nanoparticles from which PL measurement was carried on. A strong blue – green emission from the solution is observed when excited at wavelength of 365 nm while, no florescence was obtained from the aqueous solution of $10^{-3}$ M CdSO$_4$.

Figure 4.3B shows the PL spectrum obtained from aqueous solution of biogenic CdS nanoparticles in terms of energy. Bulk CdS shows a broad peak with very weak photoluminescence (PL) intensity with red emission and photon energy of 0.4 eV much below its absorption threshold. The PL of bulk CdS can be explained as the recombination of charged carriers trapped in the surface states. Therefore PL can be related to the size of CdS nanoparticles, and the PL emission blue-shifts with decreasing particle size [36a]. A sharp and strong peak at 2.5 eV corresponding to 485 nm is observed from biogenic CdS nanoparticles. The large blue shift in the energy of PL spectrum could be assigned to the emission from holes and electron traps frequently located at surface on the particles due to its small size as the density of electron traps increases on the surface due to the large surface area of the particle at smaller size. Therefore the observed blue shift in the PL spectrum can be attributed to the decrease in size of CdS nanoparticle [36b]. Line width of the PL spectra is narrow and regular indicating that no impurity is associated with CdS nanocrystallites.

**4.2.2.4 X-ray diffraction (XRD) analysis**

Figure 4.4 shows the XRD analysis performed on a solution cast film of biogenic CdS nanoparticles, synthesized by reaction between *P.aeruginosa* and $10^{-3}$ M CdSO$_4$ on glass substrate. A number of strong Bragg reflections are observed originating from the film surface. The diffraction pattern was indexed on the basis of wurtzite and cubic phase of CdS as observed in Figure 4.4A. CdS nanoparticles in particular size regime exhibit mixed phase structure [37]. The XRD peaks appear to be broadened, however the broadening observed in this case is not very prominent as reported previously [37]. This is most probably due to the fact that the XRD pattern was obtained from solution cast film on glass surface and not from the powder. There is a possibility of aggregation among the nanoparticles due to evaporation while the film was cast. However the broadening of XRD peak is clearly evident from the enlarged view of the spectrum.
Figure 4.4 (A) XRD pattern obtained from a solution cast film of aqueous CdS nanoparticles on glass surface. The Bragg reflections arising from the film are indexed with the respective crystal planes. (B) Enlarged view of the XRD pattern in the region of (100) and (111) reflections indicating the peak broadening due to the finite size of CdS nanoparticles.

Figure 4.4B shows the enlarged view of the XRD spectrum in 2θ value range in between 24 – 27 degrees. The broadening of XRD peaks in this region is considered since it consist of two most intense Bragg reflections corresponding to (100) and (111) planes. The crystallite size calculated by Debye – Scherrer formula is found to be 6 nm. The XRD pattern obtained from aqueous biogenic CdS is in well agreement with the HRTEM analysis indicating the highly crystalline nature of the nanoparticles. The respective crystal planes with their corresponding d values (in Å) are as follows; 3.36 (100), 2.82 (111), 2.46 (200), 2.48 (305), 1.93 (037), 1.79 (526), 1.73 (820) [34].

4.2.3 Biosynthesis of ZnS nanoparticles

4.2.3.1 TEM analysis

Figure 4.5 illustrates the TEM analysis performed on the aqueous reaction mixture of 10⁻³ M ZnSO₄ and P. aeruginosa after 28 h of reaction. A large population of ZnS nanoparticles was found to be evenly distributed on the surface of the grid as indicated by low magnification images (Figure 4.5 images A, B and C). Biogenic ZnS nanoparticles are nearly uniform in size with narrow size distribution. As observed with CdS, ZnS nanoparticles shown in TEM images could also be resulted by the loose aggregation of smaller particles. Particle size distribution analysis performed on 100 particles selected from different micrographs indicated the mean diameter of ZnS
nanoclusters to be 3.5 nm with a small standard deviation value of 0.5. (inset in Figure 4.5E).

![Image](image_url)

**Figure 4.5** TEM images of ZnS nanoparticles synthesized using *P. aeruginosa* (A-E). The mean size of ZnS nanocrystallites is 3.5 nm (inset, image E). Image F shows HRTEM analysis of ZnS nanoparticles. The $d$ values correspond to (100) and (101) crystal planes.

Careful analysis of high magnification TEM images reveals the presence of nanoparticles, predominantly with two different sizes. Most of the particles are up to 3.5 nm in diameter while others are in between 4 to 5 nm. HRTEM analysis performed on ZnS nanoparticles clearly indicates the presence of lattice fringes and highly crystalline nature of ZnS nanoparticles (Figure 4.5F). The interplanar spacing of 3.31 Å and 2.83 Å was assigned to (101) and (100) planes of wurtzite ZnS structure respectively. [38].

### 4.2.3.2 UV – vis spectroscopic analysis

Figure 4.6 shows the UV-vis spectral analysis as a function of time of the reaction mixture containing $10^{-3}$ M ZnSO$_4$ and *P. aeruginosa*. The progress of reaction, i.e. the formation of ZnS nanoparticles is depicted in Figure 4.6A. ZnS belongs to the family of II–VI semiconductor with direct band gap energy of 3.64 eV at 300 K in bulk form [36a]. Bulk ZnS shows a featureless absorption edge at around 380 nm in the UV-vis spectrum. Figure 4.6A shows time dependent analysis of the formation of ZnS nanocrystallites using *P. aeruginosa*. 
Figure 4.6 (A) Time dependent UV-vis spectroscopic analysis of the formation of ZnS nanoparticles. The reaction between $10^{-3} \text{ M ZnSO}_4$ and P. aeruginosa was carried out for 28 h. Curves 1 – 9 represent the spectra obtained after every 4 h during the progress of reaction. (B) Band gap energy curve of ZnS nanoparticles obtained by UV-vis spectroscopic analysis performed on ZnS nanoparticles after 28 h of reaction. Tangent drawn to the absorption edge (dotted line) corresponds to the band gap energy of ZnS nanoparticles.

Formation of ZnS nanoparticles starts immediately after 4 h of reaction as indicated by curve 1 in Figure 4.6A. An absorption hump is developed at 330 nm. This absorption at 330 nm is due to the excitonic transitions in ZnS nanocrystallites [36a]. The absorption hump increases in intensity with time and attains saturation after 28 h of reaction as indicated by curve 8 in Figure 4.6A. No appreciable rise in the absorption is observed further indicating completion of reaction (Curve 9 corresponding to the UV-vis spectrum after 32 h almost superimposes with curve 8, which corresponds to the UV-vis spectrum after 28 h of reaction). According to effective mass model, UV-vis spectrum corresponds to the nanoparticle with 3 – 4 nm diameter [36c]. Along with the absorption hump at 330 nm, another shoulder peak around 300 nm is observed in UV-vis spectra. Appearance of this absorption shoulder together with hump at 330 nm indicates the presence of nanocrystallites with two different sizes. This observation is well supported by TEM analysis which shows the presence of two types of particles corresponding to 2.5 to 3.5 nm of diameter and 4 to 5 nm of diameter respectively.

Figure 4.6B corresponds to the absorption threshold or band gap analysis of ZnS nanoparticles after 28 h of reaction. The band gap energy value obtained for ZnS nanocrystallites is approximately 3.75 eV, which is higher than the band gap energy of
bulk ZnS. The increase in band gap energy can be attributed to the reduction in size of ZnS and could be a consequence of a size quantization effect.

4.2.3.3 Photoluminescence spectroscopic analysis

Figure 4.7 shows the photoluminescence (PL) spectra obtained from the aqueous solution of ZnS nanoparticles synthesized using *P. aeruginosa* after 28 h of reaction. The PL spectra were obtained by exciting the aqueous solution of ZnS nanoparticles at 280, 320 nm respectively. As observed with the aqueous CdS nanoparticles synthesized using *P. aeruginosa*, aqueous solution of ZnS nanoparticles also exhibited a sharp emission peak and the wavelength of emitted fluorescence light showed intensity maxima at 440 nm.

![Photoluminescence spectra](image)

*Figure 4.7 (a) PL spectrum obtained from the aqueous ZnS nanocrystallites at room temperature. Curve 1 indicates the spectrum corresponding to the excitation wavelength of 325 nm while curve 2 corresponds to excitation at 280 nm. The inset shows the photograph of the aqueous solution of ZnS nanocrystallites excited with UV light.*

The curve 1 in Figure 4.7 shows a room temperature PL spectrum obtained from aqueous ZnS nanoparticles excited at 320 nm. The PL spectrum consists of a single, sharp emission peak at 440 nm that could be assigned to surface traps or band-edge emission corresponding to the surface states in the nanocrystallites. The presence of a
single peak further suggests that the nanocrystallites are composed of ZnS without detectable quantity of other impurities. The narrow and sharp emission spectrum suggests that the surface of nanocrystallites is fairly flat and regular. Also the lower line width of the emission band indicates regularity in the size of nanocrystallites that gives fluorescence at 440 nm.

The inset in Figure 4.7A shows the photograph of aqueous ZnS nanocrystallites after irradiation with UV light. The blue colored fluorescence emitted by the solution is in well agreement with the PL spectrum that shows the peak maxima, which corresponds to the wavelength of the blue light in the visible region of the electromagnetic spectrum. Curve 2 in Figure 4.7 corresponds to the PL spectrum obtained from the aqueous ZnS nanocrystallites excited at 280 nm. The spectrum shows a broad emission peak with the maximum intensity around 440 nm. The emission spectrum can be resolved in two separate components possibly indicating the presence of nanocrystallites with two different sizes. The low intensity portion of the spectrum with the maximum intensity centered at 390 nm corresponds to the smaller nanocrystallites 2.5 to 3.5 nm in diameter. On the other hand the high intensity component of the spectrum corresponds to the bigger nanocrystallites 4 to 5 nm in diameter. The intensity of the PL decreases with increasing the excitation energy, which means that the line width of the spectrum increases at higher energy of the absorption edge. This can be due to the simultaneous excitation of all nanocrystallites present in the solution resulting in the observed homogeneous profile of luminescence. However, the peak position in both the PL spectra is nearly independent on the excitation energy suggesting that the origin of the emitting state is similar for both spectra.

4.2.3.4 Energy dispersive X-ray analysis (EDAX)

The elemental analysis of nanoparticles can be very informative for the study of the structural or elemental composition. Figure 4.8 corresponds to the EDAX analysis performed on a single ZnS nanoparticle. The EDAX analysis shows the presence of Zn and S indicating that the nanoparticle is indeed ZnS.
Along with Zn and S, other elements like C, N and O are also present in the sample. The origin of these elements lies in the biological components, mostly proteins along with ZnS nanoparticles. The peak corresponding to copper arises from the copper grid on which the sample is prepared for the EDAX analysis.

4.2.3.5 X-ray diffraction (XRD) analysis

Figure 4.9 shows the XRD analysis performed on the solution cast film of the biologically synthesized ZnS nanoparticles by reaction between \( P. aeruginosa \) and \( 10^{-3} \) M \( \text{ZnSO}_4 \) on a glass substrate. A number of strong Bragg reflections are observed originating from the film surface. The XRD pattern was indexed on the basis of wurtzite structure of zinc sulfide, though one peak was corresponding to the sphalerite phase of ZnS (indicated by “*” in Figure 4.9A). The XRD pattern shows broadening of peaks indicating the finite size of the nanocrystallites. But the broadening of XRD peaks is not as prominent as observed previously. This could be due to the aggregation of nanoparticles while the film for XRD analysis was prepared by drop coating of the aqueous solution of ZnS nanocrystallites. The peak broadening was prominently observed in the magnified view of the XRD spectrum in the 20 region of 27 to 35...
degrees. The XRD peaks corresponding to (100), (102), (101) and (106) lattice planes are broadened considerably.

![XRD pattern](image)

**Figure 4.9** (A) XRD pattern obtained from the solution cast film of aqueous ZnS nanoparticles on glass surface. Bragg reflections arising from the film are indexed with the respective crystal planes. Symbol “*” indicates the XRD peak originating from the sphalerite phase of ZnS. (B) Magnified view of the XRD pattern in the region of (100) and (101) reflections indicating the peak broadening due to the finite size of ZnS nanoparticles.

The crystallite size calculated by Debye – Scherrer formula is found to be ~ 7 nm. The XRD pattern obtained from aqueous biogenic ZnS is well in agreement with the HRTEM analysis indicating the highly crystalline nature of these nanoparticles. The respective crystal planes with their corresponding $d$ values (in Å) are as follows for wurtzite: 3.31 (100), 3.25 (102), 2.92 (101), 2.32 (106), 1.63 (112), 1.41 (104) and for sphalerite: 0.914 (531) [38].

**4.2.4 Biosynthesis of PbS nanoparticles**

**4.2.4.1 TEM analysis**

Figure 4.10 illustrates the TEM analysis performed on biogenic PbS nanocrystallites synthesized using *P. aeruginosa*. Nearly spherical nanoparticles with fairly narrow size distribution are observed after 24 h of reaction. The low magnification TEM image shows (Figure 4.10E) small clusters of PbS nanocrystallites, which are composed of even smaller nanoparticles. These clusters of PbS nanoparticles are about 6 to 8 nm in the diameter. The smaller nanoparticles that form the bigger aggregates are about 2 nm in diameter, however, these small nanoparticles could not be resolved with the microscope used in the present study.
Figure 4.10 TEM images of PbS nanoparticles synthesized using P. aeruginosa (A-E). Insets in (B) and (C) show the particle size distribution analysis and SAED pattern obtained from PbS nanocrystallites respectively. The HRTEM image shows crystalline nature of PbS nanoparticles (F). The d values 3.4 Å and 3.07 Å corresponds to (111) and (200) crystal planes.

The particle size distribution analysis performed on the nanoparticles shown in the different images shows a mean diameter of PbS nanoparticles to be 6.5 nm with the standard deviation value of 0.28 (inset, Figure 4.10B). The SAED pattern obtained from PbS nanoparticles indicates the crystalline nature of the nanoparticles and the diffraction pattern was indexed on the basis of FCC structure of galena PbS (inset, Figure 4.10C). The crystalline nature of the nanoparticles was further confirmed by HRTEM analysis, which clearly indicates the presence of lattice fringes (Figure 4.10F). The interplanar spacing of 3.4 Å and 3.07 Å was assigned to (111) and (200) respective planes of galena structure of PbS [39].

4.2.4.2 UV-vis spectroscopic analysis

Figure 4.11 shows the UV-vis spectroscopic analysis performed on the aqueous solution of PbS nanocrystallites synthesized by P. aeruginosa. PbS belongs to the family of II–VI semiconductor with a small direct band gap energy value of 0.41 eV at 300 K in the bulk form [36a]. Bulk PbS shows the absorption in the infrared (IR) region of the electromagnetic spectrum with the absorption onset at ~3020 nm. Figure 4.11A shows UV-vis spectra of PbS nanocrystallites after 12 h and 24 h of the reaction. Two distinct
features are observed in the UV-vis spectrum obtained after 12 h of reaction, which could be assigned to the excitonic transitions in PbS nanocrystallite. A well defined absorption hump is developed at 350 nm that arises due to the presence of PbS nanoparticles. Further, the absorption hump at 350 nm is accompanied with another shoulder peak at higher wavelength region at 310 nm (grey curve, Figure 4.11A).

![Figure 4.11](image)

Figure 4.11 (A) The UV-vis spectroscopic analysis performed on the aqueous reaction mixture containing lead salt and *P. aeruginosa* biomass after 12 h (grey curve) and 24 h (black curve) of the incubation. (B) Absorption threshold or band gap energy curve of PbS nanoparticles obtained by the UV-vis spectroscopic analysis performed on PbS nanoparticles after 24 h of reaction. Tangent drawn to the absorption edge (dotted line) corresponds to band gap energy of PbS nanoparticles.

The remarkable blue shift in the absorption of PbS nanoparticles indicates very small size of nanocrystallites and can be attributed to the quantum confinement of the charge carriers in the nanoparticles [34]. After 24 h of reaction the absorption humps at 350 nm and 310 nm increases with the intensity and becomes sharper. Surprisingly, the absorption hump at 310 nm becomes more prominent and now appears as a sharp peak (black curve, Figure 4.11A). This feature is also observed previously in biologically synthesized PbS nanocrystallites [17]. Presence of two absorption peaks suggests that the overall population of PbS nanoparticles was composed of the particles with two distinct sizes. This observation was consistent with the TEM analysis.

Figure 4.11B corresponds to the absorption threshold or band gap energy analysis of PbS nanoparticles after 32 h of reaction. The band gap energy value obtained is approximately 3.05 eV, corresponding to the larger nanocrystallites among the overall
population, while the energy gap value calculated for the smaller nanocrystallites was found to be \( \sim 3.80 \text{ eV} \). These energy gap values for PbS nanocrystallites are considerably higher than the band gap energy of bulk PbS [36a].

4.2.4.3 Photoluminescence spectroscopic analysis

Figure 4.12 illustrates the PL spectrum obtained from the aqueous solution of PbS nanocrystallites synthesized after 24 h of reaction between lead ions and \( P.\text{aeruginosa} \). PL spectra were obtained by exciting the aqueous solution of nanocrystallites at 280 nm and 325 nm. The black curve in Figure 4.12 shows the room temperature PL spectrum obtained from the aqueous PbS nanoparticles excited at 330 nm. The PL spectrum consists of a single and sharp emission peak with the emission maxima centered at 460 nm.

![Figure 4.12: PL spectra obtained from the aqueous solution of biogenic PbS nanocrystallites excited at 280 nm (grey spectrum) and 330 nm (black spectrum). Both spectra were recorded at room temperature.](image)

The origin of this emission lies in the surface traps or band-edge emission corresponding to the surface states in the nanocrystallites. The presence of single peak
further signifies the purity of the nanocrystallites. The line width of the emission band is significantly lower and blue shifted in comparison with the previous reports [36a] indicating a regularity in the size of nanocrystallites that show emission peak at 460 nm. The grey spectrum in Figure 4.12 corresponds to the PL spectrum obtained from the aqueous PbS nanocrystallites excited at 280 nm. The spectrum shows a sharp emission peak with the maximum intensity around 460 nm as observed from the spectrum obtained by excitation at 330 nm (black spectrum in Figure 4.12). This emission spectrum clearly shows two separate emission maxima indicating the presence of nanocrystallites with two different sizes. The low intensity region of the spectrum with the maximum intensity centered at 360 nm corresponds to the smaller nanocrystallites. On the other hand the high intensity component of the spectrum corresponds to the bigger nanocrystallites. As observed with ZnS nanocrystallites, the intensity of the PL decreases with increasing the excitation energy, indicating that the line width of spectrum increases at higher energy of the absorption edge. This can be due to the simultaneous excitation of all nanocrystallites present in the solution giving rise to a homogeneous profile of luminescence. The peak position in both the PL spectra is almost independent of the excitation energy suggesting that the origin of the luminescence lies in PbS nanocrystallites.

4.2.4.4 X-ray diffraction (XRD) analysis

Figure 4.13 represents the XRD analysis performed on the drop cast film of aqueous PbS nanocrystallites on a glass substrate. The XRD spectrum consists of a number of Bragg reflections, most of which originate from PbS nanocrystallites. The XRD pattern was indexed on the basis of a mixed phase of lead sulfide consisting of FCC galena and cubic PbS. Almost half of the peaks could be indexed on the basis of cubic PbS, while other half belonged to galena (marked as “*” in the Figure 4.13A). The XRD pattern shows broadening of peaks indicating the small size of PbS nanocrystallites. The broadening of peaks is further evident in Figure 4.13B, that shows the magnified view of the XRD spectrum in the 2θ region of 29 to 32 degrees, which clearly indicates the peak broadening. The XRD peaks corresponding to (200) and (420) lattice planes are broadened considerably. The crystallite size of PbS calculated by Debye – Scherrer formula is found to be ~ 7 nm, which is larger in comparison to TEM analysis. The
broadening of XRD peak corresponding to (200) is considered for calculation of crystallite size by Debye – Scherrer formula, as it represents the most intense Bragg reflection arising from the PbS nanocrystallites.

\[ d_{-values} (\text{Å}) = \begin{align*}
3.38 & (111), 2.96 (200), 2.63 (420), 2.09 (220), \\
1.71 & (222), 1.48 (400), 1.14 (411), 0.85 (444) \end{align*}\]

4.2.5 Biomolecular mechanism of metal sulfide nanoparticle formation by *P. aeruginosa*

Biological synthesis of group II – VII sulfide nanoparticles semiconductors have been described previously using different microorganisms like yeast, fungi and bacteria. Though previous reports successfully describe the synthesis of CdS or PbS nanoparticles, the biological mechanism that lead to the formation of metal sulfide nanoparticles is unclear. It has been postulated that the yeast cells upon exposure to Cd or Pb ions synthesize metal chelating peptide to nullify the stress generated by metal ions. On addition of the metal ions, a metal ion – $\gamma$ glutamyl complex is initially formed and this is accompanied by an increase in the intracellular sulfide levels. Later Cd or Pb ions...
complex with sulfide to form CdS or PbS nanocrystals, which accumulate in vacuoles present inside the yeast cells. Bacteria like *Klebsiella planticola* and *K. pneumoniae* have been shown to synthesize CdS nanoparticles in high quantity under anaerobic conditions [22-23]. An enzyme thiosulfate reductase produced by *K. planticola* has been shown to be responsible for the synthesis of CdS nanoparticles, while cysteine desulphydrase secreted by *Klebsiella pneumoniae* converts cysteine into H₂S, which in turn reacts with Cd⁺ ions to form CdS. Cysteine desulphydrase gene has been successfully cloned and overexpressed in *E. coli* for the synthesis of CdS nanoparticles. However, synthesis of CdS was observed only after 48 h of the bacterial growth.

In this chapter we have described biological synthesis of semiconductor sulfide nanoparticles like CdS, ZnS and PbS using the bacterium *P.aeruginosa*. Unlike previous reports, synthesis of metal sulfide nanoparticles proceeds by reduction of the respective metal sulfate salts indicating that exogenous sulfur is utilized for the synthesis of CdS, ZnS and PbS nanocrystallites. This observation led to the hypothesis that bacterial sulfate reductases are responsible for the synthesis of sulfide nanocrystallites. In microorganisms, sulfate reductases are broadly categorized as the dissimilatory sulfate reductases (DSR) and assimilatory sulfate reductases (ASR). DSR are responsible for the anaerobic respiration in sulfate reducing bacteria [20], while ASR are responsible for the metabolism of sulfur containing amino acids like methionine and cysteine in aerobic bacteria. Sulfate ions are taken inside the bacterial cell by the action of sulfate permease. Once inside cell, the sulfate ions are acted upon by APS sulfurylase, which converts inorganic sulfate into adenosine phosphosulfate (APS). APS is further phosphorylated in 3′ phosphoadenosine 5′ phosphosulfate (PAPS) and this reaction is catalysed by APS kinase. PAPS is reduced to respective sulfite in the next step by the action of PAPS reductase, which generally called as sulfate reductase [40]. This step is followed by the action of sulfite reductase (SIR), converting sulfites into sulfide. The metal sulfides formed in the last step are effluxed in the surrounding medium by CZR transporter. The sulfate reduction pathway leading to synthesis of nanocrystalline semiconductor metal sulfides is illustrated in the following flow chart, which explains the uptake, reduction and efflux of metal sulfate ions by *P. aeruginosa*. 
The actual reduction of inorganic sulfate is a two step reaction. The first step is catalyzed by PAPS reductase in which, sulfate ions are reduced to sulfite ions. In the second step sulfite ions are converted into sulfide by the action of SIR. Therefore, keeping in mind the central role of these enzymes in sulfate reduction by assimilatory sulfate reduction pathway, genes encoding PAPS reductase (cys H) and SIR (cys I) in *P. aeruginosa* are identified, isolated and amplified using PCR (polymerase chain reaction). The amplified individual cysH and cysI genes could be successfully cloned and overexpressed in *E. coli* BL 21 AI™.
4.2.5.1 PCR amplification, sequencing and cloning of \textit{cysH} and \textit{cysI} genes and their overexpression in recombinant \textit{E.coli BL 21 AI}

Figure 4.14 shows PCR analysis performed on \textit{P.aeruginosa} genomic DNA for the amplification of \textit{cysH} and \textit{cysI} genes using sequence specific degenerative primers.

![Agarose gel electrophoresis image of cysH and cysI genes after PCR amplification using gene specific primers. Lane 1, 2 and M corresponds to amplified genes cysH, cysI and standard DNA size molecular weight marker respectively.](image)

The presence of two distinct bands at 0.9 kb and 1.2 kb on 0.8 % agarose gel indicates that gene specific primers are highly specific for \textit{cysH} and \textit{cysI} genes encoding enzymes PAPS reductase and SIR respectively. The molecular size of amplified DNA fragments is calculated to be 0.9 kb and 1.2 kb respectively, which show perfect match with the reported value of \textit{cysH} and \textit{cysI} genes. This result indicates the presence of functional \textit{cysH} and \textit{cysI} genes encoding two proteins, PAPS reductase and SIR, which catalyzes sulfate reduction in \textit{P.aeruginosa}. Lane 1 in Figure 4.14 corresponds to PCR amplified \textit{cysH} gene fragment with molecular size 0.9 kb, while lane 2 in Figure 4.14 represents PCR amplified \textit{cysI} gene fragment measuring molecular size of 1.2 kb. Lane M in Figure 4.14 is a standard molecular weight DNA marker. Further, 0.9 kb and 1.2 kb gene fragments were sequenced by eluting DNA from agarose gel on automated DNA
sequencer. The DNA sequences obtained were analyzed on BLAST program of NCBI server. GenBank database revealed that the amino acid sequences encoded by cysH and cysI gene fragment are most similar to that encoded by cysH and cysI homologues [GenBank accession no. CP 000 438 and AE 004091] indicating that amplified DNA fragments indeed belong to cysH and cysI genes.

To further illustrate the role of cysH and cysI genes in the formation of metal sulfide nanocrystallites such as CdS, ZnS and PbS, cys H and cysI genes were cloned and overexpressed separately as heterologous proteins in E.coli BL21 AI™ [33]. Initially two genes were separately cloned in plasmid vector pDONR™221 known as entry vector. This was followed by LR recombination reaction between entry vectors and destination vector pDEST™17 which was engineered in E.coli BL 21 AI™ cells by transformation. These transformed E.coli BL 21 AI™ cells were induced by L-arabinose in the presence of 100 μg/μl ampicillin. Overexpression of two proteins, PAPS reductase and SIR was analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the gels were visualized by staining with Coomassie blue R 250. Gateway® technology for cloning and overexpression of the gene has a limitation over the size of gene fragment that can be successfully overexpressed in E. coli BL 21 AI™ cells as a functional protein [33]. Usually proteins with molecular weight more than 20 kDa cannot be overexpressed as a functional protein because E.coli BL 21 AI™ senses it as non-functional by an unknown way. Therefore, the resulting overexpressed protein may not be folded in the appropriate conformation, which is destined for inclusion bodies as a non-functional protein. However, in spite of high molecular weights, PAPS reductase and SIR could be overexpressed functionally in E.coli BL 21 AI™ cells. A fraction of both proteins was found to be synthesized extracellularly as indicated by SDS-PAGE analysis of extracellular proteins. Activity of PAPS and SIR was analyzed by synthesis of metal sulfide nanocrystallites by recombinant E.coli BL 21 AI™ cells harboring cysH and cysI genes from P.aeruginosa as discussed in the following section. Figure 4.15 shows SDS-PAGE electropherograms of intracellular and extracellular proteins synthesized by E.coli BL 21 AI™ cells. Overexpression of PAPS reductase and SIR is evident by the analysis of intracellular and extracellular proteins on 10% SDS-polyacrylamide gel.
Figure 4.15A shows the electropherograms of intracellular proteins synthesized by *E.coli* BL 21 AI™ cells during the incubation of 4 h in LB medium at 37 °C.

![Image of electropherograms](image)

**Figure 4.15** (A) SDS-polyacrylamide gel electropherogram showing the intracellular protein profile obtained from *E.coli* BL 21 AI™ cells. Lanes 2 to 4 correspond to the intracellular-protein profile of *E.coli* BL 21 AI™ cells after 2, 3 and 4 h of induction respectively, while lane 1 corresponds to the protein profile obtained from uninduced cells. PAPS reductase and SIR are indicated by arrow facing to the left and right of the image. (B) SDS-PAGE electropherogram image of extracellular proteins synthesized by *E.coli* BL 21 AI™ cells during 4 h of induction. Lane 1 to 4 corresponds to protein profile obtained after 1, 2, 3 and 4 h of induction respectively. PAPS reductase and SIR are indicated by arrow facing to the left and right of the image. Central lane in both images corresponds to standard protein molecular weight marker with molecular weights (kDa) 212, 158, 116, 97.2, 66.4, 56.6, 42.7, 34.6, 27, 20 from top to bottom respectively.

Time dependent analysis of intracellular protein profile clearly indicates the synthesis of two proteins with molecular weights of 30 kDa and 65 kDa in an increasing amount during the incubation period of 4 h after induction (lane 2 to 4, Figure 4.15A). On the other hand, it is clearly observed that PAPS reductase and SIR are indistinguishable from other intracellular proteins synthesized by uninduced *E.coli* BL21 AI™ cells (lane 1, Figure 4.15). This result is obvious to consider the overexpression of PAPS reductase and SIR as intracellular proteins in recombinant *E.coli* BL21 AI™ cells. Also both overexpressed proteins remain functional after 4 h of induction.

Figure 4.15B shows the electropherograms of extracellular proteins synthesized by *E.coli* BL 21 AI™ cells during the incubation of 4 h in LB medium at 37 °C. Even, extracellular protein profile of recombinant *E.coli* BL 21 AI™ cells show similar trend observed with intracellular protein analysis. It is clearly observed that PAPS reductase...
and SIR with molecular weights of 30 kDa and 65 kDa are overexpressed with increasing incubation period up to 4 h of induction. Lane 1 to 4 in Figure 4.15B corresponds to the extracellular protein profile obtained from *E.coli* BL 21 AI™ cells after 1, 2, 3 and 4 h of induction respectively. It is clear that the amount of PAPS reductase and SIR increases in extracellular medium with time. Electropherogram also indicates that both proteins are stable even after 4 h of induction when secreted outside the cells in culture medium.

Figure 4.15 clearly shows the overexpression of PAPS reductase and SIR by recombinant *E.coli* BL 21 AI™ cells. Non transformed *E.coli* BL 21 AI™ cells do not show presence of any detectable proteins with molecular weights of 30 kDa and 65 kDa, indicating that presence of these proteins could be realized only after transformation with plasmid harboring *cysH* and *cys I* genes encoding PAPS reductase and SIR respectively.

### 4.2.5.2 Biosynthesis of CdS, ZnS and PbS using recombinant *E.coli* BL 21 AI™

The previous section describes PCR amplification, cloning and overexpression of PAPS reductase and SIR in *E.coli* BL 21 AI™ cells. Owing to the central role of PAPS reductase and SIR during sulfate reduction it has been hypothesized that metal sulfides are synthesized from metal sulfate by the sequential action of PAPS reductase and SIR. To analyze the functionality of PAPS reductase and SIR, growing recombinant *E.coli* BL 21 AI™ cells were exposed with \(10^{-3}\) M CdSO₄, ZnSO₄ and PbSO₄ for 12 h and the culture supernatant was analysed by TEM, UV-vis spectroscopy and photoluminescence spectroscopy for the presence of CdS, ZnS and PbS respectively. Since *cysH* and *cysI* could not be engineered in single plasmid owing to their large size, two different construct are used for transformation of *E.coli* BL 21 AI™ cells separately. For the synthesis of metal sulfides, different set of recombinant *E.coli* BL 21 AI™ cells separately growing and harboring *cysH* and *cysI* genes were mixed in equal numbers prior to addition of respective metal sulfates.

Figure 4.16 shows respective TEM, UV-vis spectroscopic, and PL spectroscopic analysis performed on CdS, ZnS and PbS nanocrystallites synthesized using equal population of recombinant *E.coli* BL 21 AI™ cells harboring *cysH* and *cysI* genes from *P.aeruginosa*. Figure 4.16A1 shows TEM image of CdS nanocrystals synthesized using recombinant *E.coli* BL 21 AI™ cells after exposure to \(10^{-3}\) M CdSO₄. Large population of
nanoparticles with average size of ~ 2 nm has been observed, which is smaller in comparison with CdS nanoparticles synthesized using \textit{P. aeruginosa}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4_16.png}
\caption{TEM, UV-vis and PL spectroscopic analysis of CdS, ZnS and PbS nanoparticles synthesized using recombinant \textit{E.coli} BL 21 AI\textsuperscript{TM} cells. Images A1, B1 and C1 are TEM micrographs obtained from CdS, ZnS and PbS nanoparticles respectively. A2, B2 and C2 correspond to respective UV-vis analysis of aqueous solutions of cdS, ZnS and PbS nanoparticles. Insets in A2, B2 and C2 are PL spectra obtained from the aqueous solution of CdS, ZnS and PbS nanoparticles respectively.}
\end{figure}

Corresponding UV-vis spectrum of aqueous solution of CdS nanocrystallites has been shown in Figure 4.16A2. Black curve in Figure 4.16A2 represents UV-vis absorption spectrum of the reaction mixture of 10\textsuperscript{-3} M CdSO\textsubscript{4} and recombinant \textit{E.coli} BL 21 AI\textsuperscript{TM} cells. Wild type \textit{E.coli} BL 21 AI\textsuperscript{TM} cells without pDEST\textsuperscript{TM}17 plasmid harboring cys\textit{H} and cys\textit{I} genes do not show formation of CdS nanoparticles as indicated by grey curve in Figure 4.16A2. The spectrum shows a well defined absorption peak with absorption maxima centered at 315 nm with large blue shift in comparison with bulk CdS, which can be ascribed to the size quantization effect in CdS nanocrystallites. Inset in Figure 4.16A2 represents the PL spectrum corresponding to the aqueous solution of CdS nanocrystallites synthesized using recombinant \textit{E.coli} BL 21 AI\textsuperscript{TM} cells after exciting at 300 nm and shows a well defined emission spectrum with intensity maxima centered at 460 nm.
Figure 4.16B1 shows the representative TEM image of ZnS nanocrystallites synthesized using recombinant *E. coli* BL 21 AI™ cells. Nanoparticles with the average size between 2-5 nm have been observed indicating the size distribution among nanoparticles. This feature is reflected in the UV-vis spectrum of ZnS nanoparticles, which shows a shoulder peak around 309 nm (Figure 4.16B2, black curve) instead of a sharp peak. Wild type *E. coli* BL 21 AI™ cells without engineered cysH and cysI genes do not show the formation of ZnS nanoparticles (Figure 4.16B2, grey curve). Inset in Figure 4.16B2 represents the PL spectrum obtained by exciting the aqueous solution of ZnS nanoparticles at 300 nm after 12 h of reaction between $10^{-3}$M ZnSO$_4$ and recombinant *E. coli* BL 21 AI™ cells and shows a sharp emission peak at 445 nm.

Figure 4.16C1 represents a TEM image of PbS nanoparticles synthesized using recombinant *E. coli* BL 21 AI™ cells and shows nanoparticles with average diameter of 5 nm. The corresponding UV-vis spectrum obtained from PbS nanoparticles shows clear absorption hump at 330 nm (Figure 4.16 C2, black curve), which is not observed in the UV-vis spectrum obtained from reaction between wild type *E. coli* BL 21 AI™ cells and precursor salt (Figure 4.16C2, grey curve). Inset in Figure 4.16C2 shows PL spectrum obtained by exciting the aqueous solution of PbS nanoparticles at 325 nm, which shows well defined emission peak with intensity maxima centered at 430 nm.

### 4.2.6 Discussion

This part of the chapter describes biological synthesis of metal sulfide semiconductor quantum dots using the bacteria *P. aeruginosa*. Biogenic nanocrystalline CdS, ZnS and PbS exhibited size quantization effect as indicated by their optical properties. Synthesis of all metal sulfide semiconductor nanocrystallites occurred under aerobic conditions and was found to be extracellular. Time dependent UV-vis spectroscopic analysis of the reaction indicated that metal sulfide nanoparticles are synthesized within 28 h of reaction. The average size of CdS, ZnS and PbS nanocrystallites was observed between 2-7 nm. The molecular mechanism of biological metal sulfite formation by *P. aeruginosa* revealed that sulfate reduction is a two step reaction catalyzed by two enzymes PAPS reductase and SIR of assimilatory sulfate reduction pathway. Two genes, *cysH* and *cysI* encoding PAPS reductase and SIR respectively have been identified, cloned and overexpressed in *E. coli* BL 21 AI™ cells.
Further recombinant *E. coli* BL 21 AI™ cells were used for the synthesis of cdS, ZnS and PbS. Wild type *E. coli* BL 21 AI™ cells without *cysH* and *cysI* gene inserts were unable to synthesize CdS, ZnS or PbS nanoparticles indicating the necessity of PAPS reductase and SIR during the biological synthesis of metal sulfide nanocrystallites.

### 4.3 Part II: Biosynthesis of iron sulfide and silver sulfide nanoparticles using *Actinobacter* spp.

This part of the chapter describes biosynthesis of magnetic iron sulfide e.g. greigite and silver sulfide nanoparticles using *Actinobacter* spp. Additionally preliminary biological mechanism for biosynthesis of iron sulfide and silver sulfide nanoparticles operational in *Actinobacter* spp. has been discussed.

#### 4.3.1 Experimental details

The Gram positive bacterium *Actinobacter* spp. was isolated and identified as described in chapter 3. For the cultivation of bacteria in 100 ml batch, bacterial seed culture was grown overnight in small volume (~2 ml) of Luria broth (LB) medium. This seed culture was then inoculated in 100 ml of LB medium without NaCl in 500 ml Erlenmeyer flask for bacterial propagation. For the biosynthesis of iron sulfide nanoparticles, the bacterial cells were freshly grown in 100 ml of LB medium (without NaCl) in 500 ml Erlenmeyer flask and incubated on the rotary shaker (200 rpm) at 37°C for ~ 48 h until the stationary phase is attained. Next, filter sterilized aqueous solution of ferric citrate and ferrous sulfate was added to the bacterial culture at the final concentration of 2:1 M ratio.

Silver sulfide nanoparticles were synthesized by adding the filter sterilized 10⁻³ M aqueous solution of Ag₂SO₄ to the bacterial culture in separate flasks. Both the flasks were incubated on the rotary shaker (200 rpm) at 37 °C for 48 to 72 h. The bacterial culture supernatants containing iron sulfide and silver sulfide nanoparticles were isolated by separating the bacterial biomass by centrifugation at 5000 rpm. The culture supernatant containing iron sulfide and silver sulfide nanoparticles were characterized by TEM, XRD, FTIR, UV-vis spectroscopy, TGA, Photoluminescence analysis and magnetic measurements etc. Magnetization as a function of the field at different temperatures was recorded by varying the applied field between -50 KOe to 50 KOe.
Temperature dependent magnetization of iron sulfide nanoparticles was studied in the ZFC (Zero-field-cooled) and FC (field cooled) modes for as-synthesized as well as calcined nanoparticles. Extracellular protein profile of the bacterial culture supernatant was checked for the induction of new protein/s upon metal salt addition. To check the induction of new protein/s, extracellular protein profile of *Actinobacter spp.* culture supernatant was analyzed in the presence and the absence of metal salts. Proteins were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) as well as native polyacrylamide gel electrophoresis on 12% gels (12% T and 2.7% C).

For analysis of the proteins bound to the surface of the nanoparticles, iron sulfide and silver sulfide nanoparticles were separated from the culture supernatant. First, bacterial biomass was removed by centrifugation at 5000 rpm (2560 × g) and the culture supernatant containing nanoparticles was collected. This culture supernatant was again subjected to centrifugation at 20000 rpm (20579 × g) for 30 minutes. After centrifugation the supernatant was discarded and the black pellet of iron sulfide and silver sulfide each were washed thrice with 50mM sodium phosphate buffer (pH 7.4) before dissolving in 100 μl of the same buffer. Proteins were separated from the nanoparticles by treatment with 8M urea and 1% SDS. The solutions were then heated at 60 °C for 10 minutes and then centrifuged at 20,000 rpm for 30 minutes. The supernatant was analyzed on SDS-PAGE for the presence of proteins as described above. To study the association of PAPS (phosphoadenosyl phosphosulfate) reductase in the reduction of sulfate to sulfide, biosynthesis of iron sulfide and silver sulfide by *Actinobacter spp.* was carried in the presence of 100 μM of chromium ions as chromium ions are known to inhibit PAPS reductase activity. The reaction was carried out for around four days. For the isolation and sequencing of the sulfate reductase genes from *Actinobacter spp.*, genomic DNA was isolated from *Actinobacter* spp. by standard phenol/chloroform extraction method as described previously. One of the genes (PAPS reductase) for sulfate reduction pathway was amplified with paps-F (5’ – GTT CTG CGT GAC CTC CTC CAT – 3’) and paps-R (5’–GTC CTC GCC CTC CAG CAC AC– 3’) for the amplification of sulphate reductase (PAPS reductase). The primers were designed from the available sequences of cysH (PAPS reductase gene) in *Sterpromyces avermitilis* MA-4680 genome (NC 003155). The PCR conditions used were an initial denaturation at 94 °C for two minutes, followed by
35 cycles of denaturation at 94 °C for one minute, annealing at 60 °C for one minute and extension at 72 °C for 30 seconds and final extension at 72 °C for 10 minutes. From the amplified PCR product bands with desirable sizes were excised from the gel by QIAquick gel extraction kit according to manufacturer’s instructions (Qiagen). The gel eluted PCR products were then sequenced from both the directions using same set of primers on automated DNA analyzer 3730 of Applied Biosystem BigDye terminator chemistry. The sequences so obtained were analyzed at the NCBI server using different BLAST programs.

4.3.2 Biosynthesis of magnetic iron sulfide nanoparticles

4.3.2.1 TEM analysis

Figure 4.17 shows the TEM analysis of iron sulfide nanoparticles synthesized by reaction between iron salts and *Actinobacter spp*. A number of quasi–spherical nanoparticles of iron sulfides are uniformly distributed on the grid area.

![Figure 4.17 TEM images of as-prepared iron sulfide nanoparticles synthesized by Actinobacter spp. iron salts reaction mixture after 48 h of reaction (A to E). The inset in A shows SAED pattern of as-synthesized iron sulfide nanoparticles. Letters P and G represent pyrite and greigite phase of iron sulfide. (D) Particles size distribution analysis of as-synthesized iron sulfide nanoparticles. (E – F) TEM images of as-synthesized iron sulfide nanoparticles after calcination at 300° C for 2 h. Inset in E shows SAED pattern of the calcined iron sulfide nanoparticles.](image-url)
Images A to E in Figure 4.17 corresponds to the as-synthesized iron sulfide nanoparticles after reaction of 48 h. Nearly monodisperse, well separated nanoparticles are clearly observed with the average diameter of 19 nm as indicated by the particle size distribution analysis (Figure 4.17D). Higher magnification TEM images of as-synthesized nanoparticles showed the presence of twin boundaries within nanoparticles (Figure 4.17C). SAED analysis performed on as-synthesized iron sulfide nanoparticles shows a well defined electron diffraction pattern (inset, Figure 4.17A). The diffraction pattern was indexed on the basis of mixed phases of iron sulfide, i.e. greigite \((\text{Fe}_3\text{S}_4)\) and pyrite \((\text{FeS}_2)\). Most of the diffraction rings (indicated by subscript g in diffraction pattern, inset in Figure 4.14A) were analyzed on the basis of greigite \((\text{Fe}_3\text{S}_4)\) phase of iron sulfide while two rings (indicated by subscript p) show excellent match with pyrite \((\text{FeS}_2)\) [41]. TEM Images E and F in Figure 4.14 correspond to iron sulfide nanoparticles after calcination at 300 °C for 2 h. The average size of iron sulfide nanoparticles remains unaltered after heat treatment, though some degree of aggregation is observed among the nanoparticles. This is probably due to the fact that some of the organic matter is retained even after the calcination at 300 °C (see TGA data for the detailed discussion) that prevents the nanoparticles from getting aggregated.

SAED pattern observed from calcined iron sulfide nanoparticles as well could be indexed on the basis of the mixed phases of iron sulfide namely greigite and pyrite (inset in Figure 4.14E greigite-g, pyrite p).

**4.3.2.2 FTIR and Thermogravimetric (TG) analysis**

Figure 4.18A illustrates the FTIR spectroscopic analysis performed on the as-synthesized, purified iron sulfide nanoparticles after 48 h of reaction between *Actinobacter spp.* and iron salts. FTIR analysis shows the presence of characteristic Fe-S bond vibrations [42]. The broad peak in the region between 620 cm\(^{-1}\) and 850 cm\(^{-1}\) is assigned to Fe-S bending vibrations while the small sharp band at 1160 cm\(^{-1}\) could be ascribed to the stretching of Fe-S bonds. These Fe-S bonds are accompanied with the presence of amide I and II vibration bands, which appear at 1660 and 1540 cm\(^{-1}\) respectively, indicating the presence of proteins along with the nanoparticles (Figure. 4.18A). The nanoparticles are probably stabilized by capping of the proteins secreted by *Actinobacter spp.*
TGA was performed on as-synthesized iron sulfide nanoparticles to calculate the amount of bio-organic molecules present with iron sulfide nanoparticles. TGA carried out on as-prepared sample of iron sulfide shows a weight loss in two major regions (Figure 4.18B). The first region is centered between 100°C to 500°C showing a gradual weight loss this can be assigned to the evaporation of the water molecules present in the sample and decomposition of protein molecules.

A sharp weight loss is observed after around 450°C, which is probably due to the rapid degradation of organic matter present in the sample. The gross weight loss observed by TGA is calculated to be about 64%.

4.3.2.3 X-ray diffraction analysis

Figure 4.19 represents the XRD pattern obtained from the solution cast film of as-synthesized and calcined (300°C, 2h) iron sulfide nanoparticles on the glass substrate. A number of strong Bragg reflections originating from the solution cast films are observed. The diffraction pattern obtained from as-synthesized and calcined iron sulfide nanoparticles was indexed on the basis of the mixed phase structure of iron sulfide. Most of the reflections were indexed on the basis of greigite (Fe₃S₄) phase of iron sulfide, which are indicated as “Δ” in the Figure 4.19. Rest of the reflections were assigned to
pyrite ($\text{FeS}_2$) phase of iron sulfide and indicated as “○” in Figure 4.19. The curve 1 (gray curve) in Figure 4.19 corresponds to the as – synthesized iron sulfide nanoparticles, while curve 2 (black curve) in Figure 4.19 represents the diffraction pattern obtained from iron sulfide nanoparticles after calcination at 300 °C for 2 h. Both curves are almost identical when superimposed with very little observable variation.

\[ \text{Figure 4.19 The XRD pattern obtained from iron sulfide nanoparticles synthesized by the reaction between iron salts and Actinobacter spp. after 48 h of reaction. Curve 1 (grey curve) represents XRD pattern of as – synthesized iron sulfide nanoparticles, while curve 2 (black curve) indicates the XRD pattern of iron sulfide nanoparticles after calcination at 300° C for 2 h.} \]

This observation indicates that calcination treatment does not alter the indigenous crystal structure of as – synthesized iron sulfide nanoparticles. However, enhancement in the crystallinity of individual domains of the nanoparticles can not be ruled out. On the basis of X-ray diffraction analysis it can be deduced that the iron sulfide synthesized by Actinobacter spp. gives $\text{Fe}_{x-1} \text{S}_x$ kind of crystal structure. The respective $d$ values for the corresponding $hkl$ planes are as follows: for greigite (marked as $\Delta$): 2.86 (222), 2.09 (422), 1.59 (620), 1.47 (444), 1.43 (731) and for pyrite (marked as ○): 3.12 (111), 2.21 (221), 1.75 (221), 1.60 (311) [41].
4.3.2.4 Magnetic measurements

Magnetic measurements were performed on dried iron sulfide nanoparticles before and after calcination. Magnetization as a function of the field at different temperatures was recorded by varying the applied magnetic field between -50 kOe to 50 kOe. Temperature dependent magnetization of magnetite nanoparticles is studied by ZFC (Zero-field-cooled) and FC (field cooled) modes for as prepared as well as calcined nanoparticles. Figure 4.20A shows the magnetization curves (M-H curves) obtained from as synthesized powdered iron sulfide nanoparticles at 5 K, 20 K, 150 K and 250 K, respectively.

![Figure 4.20](image)

**Figure 4.20** (A) M – H curve obtained from as – synthesized iron sulfide nanoparticles at various temperatures. Different experimental temperatures are displayed by different symbols. (□ - 5 K, ○- 20 K, △- 50 K, ▽- 150 K and ◊- 250 K). (B) FC and ZFC curves obtained from as – synthesized iron sulfide nanoparticles at the external applied magnetic field of 1000 Oe.

Magnetization studies carried out on as synthesized iron sulfide nanoparticles show weak magnetic response at higher temperature (above 50 K) and the induced magnetization in the nanoparticles is negligible indicating the paramagnetic behavior (Figure 4.20A, curves corresponding to 150 K and 250 K in). However, as the temperature is lowered, a gradual increase in the induced magnetization value was observed. Iron sulfide nanoparticles show superparamagnetism at lower temperatures (Figure 4.20A, curves corresponding to 20 K and 5 K) and the opening of a hysteresis loop was not observed even at the applied field of 50 kOe. Figure 4.20B shows the magnetization as the function of temperature studied by field cooled (FC) and Zero field cooled (ZFC) curves obtained from as synthesized iron sulfide nanoparticles. ZFC and
FC curves almost superimpose with each other indicating the absence of blocking phenomena [43].

Figure 4.21 shows the magnetization studies performed on iron sulfide nanoparticles after the calcination at 300 °C for 2 h. Magnetization curves obtained from calcined iron sulfide nanoparticles at various temperatures are shown in Figure 4.21A. As observed with as – synthesized iron sulfide nanoparticles, the calcined nanoparticles also exhibited weak magnetic response at higher temperatures and tend to be paramagnetic (at 150 K and 250 K). However at lower temperatures enhanced magnetization signal is observed in the nanoparticles.

![Figure 4.21](image)

*Figure 4.21* (A) M-H curves obtained from iron sulfide nanoparticles after calcination at 300 °C for 2 h at various temperatures. Different experimental temperatures are shown with various symbols as 5 K-□, 20 K-○, 50 K-Δ, 150 K-▲, 250 K-◊. (B) FC and ZFC curves obtained from calcined iron sulfide nanoparticles at the external applied magnetic field of 1000 Oe.

Iron sulfide nanoparticles after calcination shows superparamagnetic behavior as indicated by increasing induced magnetization at the applied magnetic filed as high as 60 KOe. The increase in the magnetization signal of the calcined sample is attributed to the removal of diamagnetic bio-organic molecules with the proteins from the surface of nanoparticles. This result is quite consistent with TGA studies, which indicated that bio-organic molecules are present in considerable amount along with maghaemite nanoparticles. The value for maximum magnetization per unit mass ($\sigma_s$) under given experimental conditions for iron sulfide nanoparticles synthesized by *Actinobacter* spp. is found to be around 3.8 emu/g while the coercivity was calculated to be ~ 120 Oe at 5 k.
Chapter IV

The low magnetization and Coercivity value obtained for iron sulfide nanoparticles are due to the small size of nanoparticles and presence of non-magnetic phases of iron sulfide like pyrite that reduces the overall magnetization value.

Figure 4.21B shows temperature dependence of magnetization of calcined iron sulfide nanoparticles studied by ZFC and FC curves. ZFC and FC curves show clear divergence at around 225 indicating that the blocking temperature of iron sulfide nanoparticles can be around 200 K. This result is consistent with the magnetization curves above 150 K where nanoparticles show almost paramagnetic behavior.

4.3.3 Biosynthesis of silver sulfide nanoparticles (Ag$_2$S)

4.3.3.1 TEM analysis

Figure 4.22 shows the TEM analysis carried out on the solution cast film of Ag$_2$S nanoparticles synthesized using *Actinobacter* spp. 24 h after reaction. Image A and B shows the low magnification TEM images of Ag$_2$S nanoparticles and clearly reveal the nanoparticles of apparently two different sizes. The bigger nanoparticles 10 to 20 nm in diameter are also observed in all the images and seem to be embedded in a kind of bio–organic matrix. The bigger nanoparticles are highly irregular in shape and are observed to be surrounded by a dense population of smaller sized nanoparticles. The smaller
nanoparticles are spherical in the shape, 3 to 5 nm in diameter and seem to be distributed in a regular order. However, it is very difficult to predict about the arrangement and the exact shape of the smaller nanoparticles with the existing TEM machine used for analysis. Ag$_2$S nanoparticles synthesized by *Actinobacter* spp. are highly crystalline as indicated by the well defined SAED pattern. Image F in Figure 4.22 shows SAED pattern obtained from as–synthesized Ag$_2$S nanoparticles and the diffraction spots were indexed on the basis of hexagonal structure of α-Ag$_2$S [44].

**4.3.3.2 UV-vis spectroscopic and PL analysis**

Ag$_2$S is a direct band gap semiconductor with a band gap energy value of ~1 eV and shows a featureless absorption in infrared region of the electromagnetic spectrum [45]. The optical properties of Ag$_2$S nanoparticles are highly size and shape dependent. Figure 4.23 depicts the optical properties of Ag$_2$S nanoparticles synthesized using *Actinobacter* spp. A time dependant UV-vis absorption spectrum of Ag$_2$S nanoparticles is shown in Figure 4.23A and indicates the progress of reaction. At the initial stage, after 6 h of reaction the UV-vis spectrum does not show any sharp feature, and rather shows a monotonous increase in the absorption at lower wavelength at around 390 nm (curve 1 in Figure 4.23A). After about 12 h of reaction a broad absorption onset is observed in the region of 380 nm to 440 nm with the absorption maxima centered at 400 nm (curve 2 in Figure 4.23A). The appearance of this weak shoulder peak in the UV-vis spectrum corresponds to the exitonic transitions in the Ag$_2$S nanoparticles [46]. As the progress of reaction continues the shoulder peak around 380 nm increases with intensity and becomes narrower (curve 3 in Figure 4.23A). Further evolution of the reaction brings the sharper features in the UV-vis spectrum and after 24 of reaction a well defined shoulder peak is observed with absorption maxima centered at ~ 390 nm (curve 4, Figure 4.23A). A large blue shift in the absorption spectra of Ag$_2$S nanoparticles synthesized using *Actinobacter* spp. is observed in comparison to the optical band edge of bulk Ag$_2$S. This large change in the absorption of Ag$_2$S is due to the change in the electronic properties that occurs when the diameter of the particles approaches the excitonic diameter of Ag$_2$S. As the size of Ag$_2$S particles decreases, a widening of the forbidden band occurs resulting in a blue shift in the absorption spectrum. The formation of Ag$_2$S nanoparticles is highly dependent on the presence of exogenous sulfate ions in the reaction medium. Sliver
sulfide nanoparticles are only synthesized when the bacterium was reacted with silver sulfate.

![Figure 4.23](image)

**Figure 4.23** (A) UV-vis spectra obtained from Ag$_2$S nanoparticles after different time intervals of reaction between Actinobacter spp. and silver sulfate. Curves 1 to 4 are obtained after 6, 12, 18 and 24 h of reaction. The inset in A shows that Ag$_2$S nanoparticles are synthesized only in the presence of exogenous sulfate ions. Curve 1 in black represents UV-vis spectrum obtained by reaction of the bacterium with 1mM Ag$_2$SO$_4$ while curve 2 in grey shows the formation of Ag nanoparticles when reacted with 1 mM AgNO$_3$. (B) PL spectra of Ag$_2$S nanoparticles obtained by exciting the aqueous solution of the nanoparticles at 400 nm (curve 1 in grey) and 350 nm (curve 2 in black).

When the same reaction was carried out in the presence of 1 mM AgNO$_3$ formation of silver nanoparticles are observed surprisingly instead of silver sulfide. Inset in Figure 4.20A shows the UV-vis spectra obtained from the reaction mixture between Actinobacter spp. and different silver salts. The curve 1 in black represents UV-vis spectrum obtained from the reaction between Actinobacter spp. and 1mM Ag$_2$SO$_4$, while curve 2 in grey shows the formation of Ag nanoparticles by the reaction between Actinobacter spp. and 1 mM AgNO$_3$. A sharp peak at 420 nm in the curve 2 is due to the surface plasmon resonance band, which arises due to the oscillation of the surface electrons, while the absorption onset at ~ 390 nm is due to the excitonic transitions in Ag$_2$S nanoparticles.

This result indicates that Ag$_2$S nanoparticles are synthesized only in the presence of exogenous sulfide ions, which are provided by silver sulfate. Figure 4.20B shows the room temperature PL spectra obtained from as–synthesized Ag$_2$S nanoparticles after 24 h of reaction exhibiting the luminescent nature of biogenic Ag$_2$S nanoparticles. PL
spectra were obtained by exciting the aqueous solution of the nanoparticles at 350 nm and 400 nm respectively. Ag\(_2\)S nanoparticles exhibit size dependent luminescent properties and PL spectrum shifts towards lower wavelengths as the size of nanoparticles is decreased. Curve 1 (grey curve) in Figure 4.23 B represents the PL spectrum obtained by exciting Ag\(_2\)S nanoparticles at 400 nm, which shows a sharp emission band with emission maxima centered at 440 nm. This emission of Ag\(_2\)S nanoparticles falls in the blue- green region of the visible wavelength of the electromagnetic spectrum. Curve 2 (black curve) in Figure 4.23 B shows the emission spectrum obtained by exciting Ag\(_2\)S nanoparticles at 350 nm. A sharp emission peak with the maximum intensity centered at 450 nm is observed. The peak position in both the PL spectra is almost independent of the excitation energy suggesting that Ag\(_2\)S are solely responsible for the observed luminescence. However, there is a large difference in the emission intensities, which could be assigned to the simultaneous excitation of all Ag\(_2\)S nanoparticles present in the solution.

4.3.3.3 X-ray diffraction (XRD) analysis

The crystalline nature of silver sulfide nanoparticles can be analyzed by the X-ray diffraction studies. Figure 4.24 illustrates the X-ray diffraction pattern (XRD) obtained by as–synthesized silver sulfide nanoparticles.

![XRD pattern](image)

**Figure 4.24** The XRD pattern of Ag\(_2\)S nanoparticles synthesized by reaction between silver sulfate and Actinobacter spp. The XRD pattern was indexed on the basis of \(\alpha\)-Ag\(_2\)S. The Bragg reflections arising from the nanoparticles film are indexed with the respective crystal planes.
A large number of intense Bragg reflections are observed originating from the solution cast film sample of Ag\textsubscript{2}S nanoparticles on the glass substrates. The XRD pattern obtained from Ag\textsubscript{2}S nanoparticles confirm their highly crystalline nature. All intense peaks originating from the nanoparticles film can be indexed with the monoclinic \(\alpha\) – Ag\textsubscript{2}S phase of silver sulfide \[43\]. The XRD pattern obtained from silver sulfide nanoparticles well corroborates with the SAED analysis performed on silver sulfide nanoparticles. The respective \(d\) values obtained for the corresponding \(hkl\) planes are as follows: 3.08 (111), 2.83 (-112), 2.21 (031), 2.04 (103), 1.96 (-123), 1.79 (-221), 1.69 (041), 1.61 (114), 1.40 (-144) \[44\].

4.3.4 Biochemical insight into metal sulfide formation by \textit{Actinobacter} spp.

\textit{Actinobacter} spp. can synthesize iron sulfide and silver sulfide nanoparticles when reacted with the respective iron and silver salts containing sulfate ions. However, the synthesis of iron sulfide and silver sulfide are highly specific to the exogenous sulfate ion source and in the absence of exogenous sulfate ions synthesis of respective metal sulfide nanoparticles does not occur. In the absence of sulfate ion source \textit{Actinobacter} spp. synthesizes iron oxide nanoparticles as described in the previous chapter, while the same bacterium capitate metallic silver nanoparticles in the absence of sulfate ions. This results indicate that a highly specific and sulfate dependent biological mechanism is responsible for the biosynthesis of iron sulfide and silver sulfide nanoparticles. We believe that the presence of exogenous sulfate ions trigger the formation of sulfate reductases in \textit{Actinobacter} spp. As indicated in the preceding section of this chapter, the sulfate reductases involved in the formation of iron sulfide and silver sulfide nanoparticles are most likely to be a component of assimilatory sulfate reduction pathway, since \textit{Actinobacter} spp. is a highly aerobic bacterium and also the formation of metal sulfate nanoparticles occurs in total aerobic conditions.

Figure 4.25 A shows the image of the agarose gel electrophoresis pattern of DNA fragment corresponding to the gene encoding PAPS (3’ phosphoadenosine 5’ phosphosulfate) reductase in \textit{Actinobacter} spp. after PCR amplification. A sharp and intense single band of the gene fragment at 0.9 kb is observed on 1% agarose gel after
PCR amplification with specific primers designed from *Sterpromyces avermitilis* MA-4680 genome (NC 003155).

Figure 4.25 Agarose gel electrophoresis pattern of putative PAPS reductase gene fragment amplified by PCR reaction. Lane 1 represents a single DNA band of 0.9 kb while lane M represents the DNA molecular weight marker with the respective molecular weight. Gene Sequence of the putative PAPS reductase gene fragment from Actinobacter spp.

The gene fragment with 0.9 kb size could be assigned to a gene encoding for the enzyme PAPS reductase based on molecular weight as studied before (NCBI GenBank accession no. NC 003155). PAPS reductase is a first enzyme in the sulfate reduction that converts sulfate into sulfite. When this 0.9 kb gene fragment was sequenced and analyzed on BLAST server no significant match in NCBI database was obtained. However, a domain of 102 bases showed the similarity with putative reductase gene from *S. avermitilis* in TBLASTX program at NCBI. Further, the role of PAPS reductase in the formation of iron and silver sulfide was analyzed by reacting the iron and silver salts in the presence of chromate ions, which are known to inhibit sulfate reduction pathway [47]. When sulfate salts of iron and silver were reacted with *Actinobacter* spp. in the presence of 100 μM chromate ions the formation of iron sulfide or silver sulfide nanoparticles was not observed even after eight days of reaction. Chromate ions were not found to inhibit
the bacterial growth, since *Actinobacter* spp. could grow normally with the chromate ion concentration used in this experiment (100 μM). This result together with the PCR amplification and sequencing of PAPS reductase gene fragment shows the presence and active role of PAPS reductase in sulfate reduction and the formation of iron sulfide and silver sulfide nanoparticles via sulfate reduction pathway. Presence of metal salts in excess amounts (usually more than μM concentration) generally induces the synthesis of new proteins in bacterial cells to nullify the toxic effect exerted by metal salts.

When the culture supernatant of *Actinobacter* spp. grown in the presence and absence of iron salts was assayed by SDS-PAGE, two proteins are found to be induced in the presence of iron salts. Figure 4.26A shows the comparative SDS-PAGE analysis of the extracellular protein profile of *Actinobacter* spp. grown in the presence and absence of iron salt.

![Figure 4.26](image)

**Figure 4.26** (A) SDS-PAGE analysis of the extracellular protein profile of *Actinobacter* spp. obtained by growing the bacterium in the presence (lane Ex) and the absence (lane C) of iron salts. Presence of the two extra bands in lane Ex corresponds to the induction of new proteins with the molecular weight of 55 KDa and 120 kDa respectively, which are absent in the lane C. Lane M corresponds to the standard protein molecular weight marker. (B) SDS-PAGE analysis of the proteins bound to the surface of iron sulfide nanoparticles (lane 1). Lane 2 corresponds to the standard protein molecular weight marker with the respective molecular weight in kDa. (C) SDS-PAGE analysis of the proteins bound to the surface of Ag$_2$S nanoparticles (lane 2). Lane 1 shows the standard protein molecular weight marker with the respective molecular weight in kDa.

The lane Ex in Figure 4.26A corresponds to the extracellular protein profile of *Actinobacter* spp. grown in the presence of iron salts while the lane C in Figure 4.26A corresponds to the extracellular protein profile of *Actinobacter* spp. grown in the absence
of iron salts. Induction of the two new proteins with molecular weights of 120 kDa and 55 kDa are observed after the addition of iron salt to Actinobacter spp (shown by arrows in lane Ex) culture medium. Lane M in Figure 4.26A corresponds to the molecular weight marker (the respective molecular weights in kDa from top to bottom are as follows: 97.4, 68, 43, 29 and 20). The proteins present on the surface of iron sulfide nanoparticles were also analyzed by SDS-PAGE. Figure 4.26B shows SDS-PAGE analysis of the proteins present on the surface of iron sulfide nanoparticles. SDS-PAGE analysis of the surface bound proteins reveal the presence of five different surface bound proteins with the respective molecular weights of 15, 16, 20, 23 and 27 kDa (lane 1 in Figure 4.26B). Lane 2 in Figure 4.26B indicates the standard molecular weight marker with the respective molecular weights. The association of these proteins with the nanoparticles surface indicates that they can be responsible for the stabilization of maghaemite nanoparticles. However, the exact function of these proteins is yet to be understood completely at this stage of time.

Figure 4.26C shows SDS-PAGE analysis of the proteins isolated from the surface of Ag$_2$S nanoparticles. The presence of five different proteins was observed on 12% resolving gel with the respective molecular weights of 97, 32, 30, 16 and 12 kDa. Lane 1 in Figure 4.26C represents the standard molecular weight marker, while lane 2 in Figure 4.26 shows the well separated capping proteins isolated from the surface of Ag$_2$S nanoparticles. These proteins could be silver binding proteins which bind to silver ions to nullify the toxic effects exerted by silver ions. However, some of these proteins can act as capping agents, which binds and stabilize Ag$_2$S nanoparticles. One of these proteins with a molecular weight of 16 kDa is also found to be associated with the surface of iron sulfide nanoparticles. Presence of this protein on the surface of iron sulfide as well as silver sulfide nanoparticles indicate that it, could have an affinity for sulfide moiety present in both, iron sulfide and silver sulfide nanoparticles.

4.3.5 Discussion

This section of the chapter explains the biological synthesis of iron sulfide has silver sulfide nanoparticles using Actinobacter spp. Iron sulfide nanoparticles are composed of the mixture of greigite (Fe$_3$S$_4$) and pyrite (FeS$_2$) as indicated by XRD analysis. Magnetic measurements revealed the superparamagnetic nature of iron sulfide
nanoparticles at low temperature. Biogenic silver sulfide nanoparticles exhibited quantum confinement within the nanoparticles. *Actinobacter* spp. exhibited high specificity towards iron and silver salts, since sulfide nanoparticles are synthesized only in the presence of respective sulfate salts of iron and silver i.e. FeSO$_4$ and Ag$_2$SO$_4$. In the absence of exogenous sulfate salts synthesis of iron sulfide and silver sulfide was not observed. Biochemical insight into metal sulfide formation by *Actinobacter* spp. indicated that sulfide formation proceeds via the sulfate reduction pathway. Presence of the gene for PAPS reductase has been conclusively shown to be present in *Actinobacter* spp. by PCR amplification and DNA sequencing of the gene. Presence of chromate ions inhibited the formation of iron sulfide and silver sulfide nanoparticles further indicating that the synthesis of iron sulfide or silver sulfide occurs via sulfate reduction pathway. Extracellular protein profile of *Actinobacter* spp. grown in the presence and absence of iron salts indicated the formation of two new proteins with the molecular weights of 120 kDa and 55 kDa, which are probably synthesized in response to the iron salt stress. Five different proteins are found to be associated with the surface of iron sulfide nanoparticles with the respective molecular weights of 15, 16, 20, 23 and 27 kDa. Analysis of proteins associated with the surface of silver sulfide nanoparticles also showed the presence of five different proteins with the respective molecular weights of 97, 32, 30, 16 and 12 kDa.

### 4.4 Conclusions

This chapter describes the biological synthesis of metal sulfide nanoparticles using bacteria. Nanocrystalline semiconductor sulfides such as CdS, ZnS and PbS were synthesized using a Gram negative aerobic bacterium *Pseudomonas aeruginosa*. All the semiconductor sulfide nanocrystallites were in quantum size regime and exhibited the phenomenon of quantum confinement. All the biogenic sulfide nanocrystallites showed excellent optical properties that are comparable to the semiconductor sulfide nanocrystallites synthesized by chemical means. In comparison with the earlier reports on the biosynthesis of CdS, ZnS and PbS, the formation of semiconductor sulfide nanoparticles was rapid using *P.aeruginosa*. The biosynthesis of semiconductor metal sulfide occurs by the assimilatory sulfate reduction pathway operating in *P.aeruginosa*.
cells. Two genes encoding the two enzymes namely PAPS reductase and SIR reductase responsible for the reduction of sulfate have been identified, cloned and overexpressed in *E. coli* cells. All the semiconductor sulfide nanocrystallites synthesized by overexpressed sulfate reductase genes show excellent optical properties with the size quantization effect. Another part of this chapter describes the biological synthesis of iron sulfide and silver sulfide nanoparticles using a Gram positive aerobic bacterium *Actinobacter* spp. Biogenic iron sulfide nanoparticle was found to consist of mixed phases, greigite (Fe$_3$S$_4$) and pyrite (FeS$_2$). Iron sulfide nanoparticles exhibited superparamagnetic behavior at low temperature with overall weak magnetic response. Silver sulfide nanoparticles synthesized by *Actinobacter* spp. show of monoclinic $\alpha$-Ag$_2$S crystal structure. The large blue shift in the absorption and emission spectra obtained from silver sulfide nanoparticles were observed indicating the phenomenon of quantum confinement. Biochemical insight of metal sulfide nanoparticles by *Actinobacter* spp. indicated that sulfate reductases are responsible for the formation of respective sulfide nanoparticles. These metal sulfide nanoparticles were found to be stabilized by different capping proteins, which are present on the surface of sulfide nanoparticles.
4.5 References

(b) Alivisatos, P. A. Science 1996, 271, 933.


[34] The XRD pattern was indexed with reference to wurtzite and cubic phase of CdS


[38] The XRD pattern was indexed with reference to wurtzite and sphalerite phases of ZnS from JCPDS – International Center for Diffraction Data PCPDFWIN version 1.30, 10-0434 and 05-0566 respectively.

[39] The XRD pattern was indexed with reference to the cubic and galena phase of PbS from JCPDS – International Center for Diffraction Data PCPDFWIN version 1.30, 05-0592 and 03-0614 respectively.


[41] The XRD pattern was indexed with reference to the crystal structure of iron sulfide from JCPDS – International Center for Diffraction Data PCPDFWIN version 1.30, 16-0713 for greigite (Fe3S4) and 42-1340 for pyrite (FeS2).


[44] The XRD pattern was indexed with reference to α- Ag2S phase of silver sulfide from JCPDS – International Center for Diffraction Data PCPDFWIN version 1.30, 14-0072.

