Chapter III

Bacteria and fungi mediated biosynthesis of magnetic iron oxide nanoparticles

This chapter describes a biological route for the synthesis of magnetic iron oxide nanoparticles mediated by bacteria and fungi. A bacterium, serendipitously isolated from our laboratory, was explored for the synthesis of magnetite and maghaemite nanoparticles. Two fungi, namely, *Fusarium oxysporum* and *Verticillium* sp. were assessed for the biosynthesis of magnetite. In all processes, synthesis of magnetic iron oxide nanoparticles is shown to occur under aerobic conditions, which was not demonstrated in microorganisms until now. Magnetic measurements performed on the iron oxide nanoparticles synthesized by the methods described here show superparamagnetic behavior. Preliminary biochemical analysis of all the reactions showed the induction of new proteins in bacterial cells as well as in fungi. Also, the time required for the synthesis of iron oxide nanoparticles by the methods described here is considerably smaller than existing biosynthetic methods involving magnetotactic bacteria and iron reducing bacteria.

3.1 Introduction

Oxide nanoparticles occupy an important place among nanomaterials due to their enormous applications ranging from catalysis to electronics to biomedics [1]. Among the vast range of oxide nanoparticles, iron oxide nanoparticles like magnetite and maghaemite are unique due to their technological interest. Magnetic iron oxides are a versatile class of material that enables a wide range of technologies, many of which are contingent with their distinct magnetic properties. Hence, there is a great interest in fabricating iron oxide based magnetic materials. Iron based magnetic oxides have been exploited for magnetic recording and multi-terabit magnetic storage devices [2], ferrofluids [3], as contrast enhancers in magnetic resonance imaging [4] and in other biomedical applications like separation [5], diagnostics [6] and drug delivery [7].

Current chemical protocols in vogue for the synthesis of magnetic iron oxides include sol-gel, forced hydrolysis, sonochemical and electrochemical methods [1]. Some of these methods require harsh experimental conditions of high temperature and pressure and therefore are considered to be energy intensive. Most of chemical synthesis procedures employ specialty chemicals and often yield particles in non-polar organic solutions [8] thereby precluding biomedical applications. Toxic and environmentally harmful chemicals like surfactants are uniformly utilized for size and shape controlled synthesis of iron oxide nanoparticles. There are few reports which describe formulation of magnetic iron oxides in aqueous medium [9]. However, these methods require stringent control over reaction conditions and employ strong alkalis like sodium hydroxide.

In contrast to chemical synthesis methods, biological synthesis of iron based inorganic material is characterized by ambient experimental conditions of temperature, pH and pressure. Iron is the most essential trace element for all the forms of life since it is at the center of most of the biochemical processes that occur inside the living cell [10]. Many unicellular as well as multicellular living forms synthesize iron oxide nanoparticles [11]. Biomineralization of magnetite has been shown to occur in diverse range of organisms like algae, insects, mollusks, fish, birds and even humans [12]. However, biological synthesis of iron oxide by microorganisms has been extensively studied. Considerable amount of magnetite is deposited by magnetotactic and iron reducing
bacteria in nature. Magnetotactic bacterium was first discovered by R. P. Blackmore in 1975 as a motile, aquatic life form that swims along geomagnetic field lines of the earth [13]. Magnetite synthesis in magnetotactic bacteria occurs in a unique intracellular structure called magnetosomes. Magnetosome is a lipid bilayer structure, which houses highly ordered magnetite crystals aligned parallel to the cellular axis [14]. The magnetite synthesized by magnetotactic bacteria shows species specific morphology and size variation [14].

Magnetotactic bacteria are a heterogeneous group of fastidious prokaryotes that display a myriad of cellular morphologies including coccoid, rod shaped, vibrioid, helical and even multicellular [15]. Magnetite crystals are typically 35-120 nm long, which is a permanent single domain size range for magnetite. In most of the magnetotactic bacteria, the magnetosomes are arranged in single or multiple chains. Magnetic interactions between the magnetosomes in chain cause their magnetic dipole moments to orient parallel to each other along the length of the chain. Thus, the overall magnetic moment is maximized by linear arrangement of magnetosomes enabling cells as tiny, self-propelled magnetic compass needles [16]. Magnetite crystals synthesized inside the magnetosomes shows unusual crystal morphologies [14].

Magnetite formation in magnetotactic bacteria is a complex process which involves numerous discrete steps. The first step in magnetite synthesis appears to be synthesis of magnetosome vesicles. However, it has recently been shown that magnetosome vesicles can exist before magnetite biomineralization [17]. The formation of magnetosome vesicles is followed by iron reduction from Fe (III) to Fe (II) with its subsequent uptake and transport inside the vesicle. Iron uptake in magnetotactic bacteria is believed to occur either by siderophores or by iron reduction [18]. The last step in magnetite formation seems to be the controlled biomineralization of magnetite.

Siderophores are low molecular weight (0.5-1.5 kDa) ferric iron chelating molecules synthesized by most bacteria under iron limiting conditions [19]. Most magnetotactic bacteria are known to synthesize one or more types of siderophore [18, 20]. Iron is likely to be taken by a cation efflux protein, Mag A in *Magnetospirillum magneticum* AMB-1 in an energy dependent process. Mag A protein was found to be present on cellular as well as magnetosome membrane and function as a H⁺/Fe(II)
antiporter [16]. Once iron is taken inside the cell, it is thought to be reoxidized into hydrous Fe (III) oxide, which is similar to the mineral ferrihydrite by the action of membrane bound iron oxidase. In the final step of magnetite formation, one third of the Fe (III) ions in the hydrous oxide are reduced and finally dehydrated to magnetite [21]. Thus, magnetite formation in magnetotactic bacteria is studied in great depth to understand the biomolecular mechanism underlying the process. Matsunaga et al have given a detailed account of genetic and biochemical basis of magnetite formation in a magnetotactic bacterium Magnetospirillum magneticum AMB-1[22].

Another group of bacteria, generally referred to as iron reducing bacteria, is also known to synthesize magnetite nanoparticles. Unlike magnetotactic bacteria, iron reducing bacteria synthesize magnetite extracellularly in the surrounding environment [23]. Iron reducing bacteria generally precipitate ultra-fine magnetite granules under strictly anaerobic conditions. However, unlike magnetotactic bacteria, iron reducing bacteria synthesize magnetite for energy generation. Magnetite is synthesized by coupling the oxidation of organic matter to the reduction of ferric iron during the metabolism of the bacteria [24]. Generally, most of the iron reducing bacteria utilizes poorly crystalline ferrihydrite as an electron acceptor which in turn is reduced to magnetite.

The morphology and size of magnetite synthesized by iron reducing bacteria is quite distinct from that synthesized by magnetotactic bacteria. There is no evidence of association of any cellular material (e.g. lipid bilayer in case of magnetotactic bacteria) with the magnetite that is extracellularly precipitated. However, the association of exopolysaccharides with magnetite has been observed in S. putrefaciens [25]. The magnetite particles are usually round and oval in shape and range in size from 10-50 nm [24]. The mechanism of magnetite formation by iron reducing bacteria is much simpler than that of magnetotactic bacteria. Geobacter metallireducens GS-15 utilizes ferric iron as a terminal electron acceptor and reduces it to tabular single domain magnetite [26]. Most of the iron reducing bacteria like Shewanella, Geobacter, Desulfovibrio and Thiobacillus contain a membrane bound Fe (III) reductase that reduces ferric iron. Also, these bacteria are shown to contain several membrane bound c-type cytochromes which are involved in the electron transport [27]. Since magnetite formation is an end-product
of an energy generating metabolism, on per cell basis, iron reducing bacteria like *Geobacter* generates 5000 times more magnetite than a magnetotactic bacterium [28].

**Outline of the present work:**

The current chapter describes the biological synthesis of magnetic iron oxide nanoparticles using bacteria and fungi. Until now, biosynthesis of magnetite has been considered the domain of magnetotactic and iron reducing bacteria. In this work, it is shown that extracellular biosynthesis of magnetite at room temperature can occur *under totally aerobic conditions*. The first part of the chapter describes biosynthesis of iron oxides like magnetite (Fe$_3$O$_4$) and maghaemite (γ-Fe$_2$O$_3$) by using a bacterium *Actinobacter* spp. The magnetic iron oxides so formed show superparamagnetic behavior at room temperature. The second part of the chapter describes biosynthesis of magnetite (Fe$_3$O$_4$) nanoparticles using fungi. Magnetite nanocrystals thus formed show ferromagnetic transition at low temperatures. The most unusual and remarkable feature of the biosynthetic protocols that are described here is the use of non-conventional microbes for the synthesis of nanomaterials.

**Part I. Biosynthesis of magnetite and maghaemite nanocrystals using *Actinobacter* spp.**

**3.2 Isolation and identification of *Actinobacter* spp.**

A bacterium *Actinobacter* spp. was discovered in a serendipitous manner which was growing in aqueous mixture of potassium ferri/ferrocyanide salts. Typically, *Actinobacter* spp. was isolated from 2:1 molar aqueous solution of K$_3$Fe(CN)$_6$ and K$_4$Fe(CN)$_6$ respectively, which was stored over a period of week. The bacterial growth was observed as turbidity showing the presence of some suspended matter in the mixture. That this indeed is a microbial growth could be conclusively shown by streaking a 50μl of ferri/ferrocyanide mixture on Luria agar plate. The bacterium was preserved in solution of 30% (v/v) glycerol and Luria broth (1:1 ratio). The culture was further maintained on Luria agar slant at 4 °C.

For identification, the bacterium was freshly cultured on Luria agar, incubated at room temperature and analyzed with molecular taxonomy method of 16S rRNA sequencing using bioinformatics tools. DNA extraction for 16S rRNA amplification and
sequencing was carried out using standard phenol-chloroform extraction procedure [29]. The gene for 16S rRNA was amplified by Polymerase Chain Reaction (PCR) by a set of eubacterial universal bacterial primers [30] and then sequenced from 3’ end. The sequence was initially analyzed at NCBI server using BLAST program. Similarity matrix of BLAST resulted sequence was prepared using Dnadist program in PHYLIP analysis package using Jukes Cantor corrections. The region of 16S rRNA gene used for analysis gives reliable information about phylogenetic affiliation [31]. Around 789 bases from 3’ end of PCR amplified gene was used for phylogenic analysis. The sequence showed maximum similarity (98.2%) with *Actinobacter* strain EC5 (Genbank accession no. AY 337600). The isolate thus could be assigned to the genus *Actinobacter* (GenBank accession no. AY 864333).

Figure 3.1 Phylogenetic affiliation based on 16S rRNA gene sequence comparisons over 900 bases showing the relationship between closest cultivable relatives and isolate MB1 NCL. The bar represents distance values calculated in MEGA 3.1 and values at node represent percentage of 1000 bootstrap replicates. Number in parenthesis represents GenBank accession numbers.

Figure 3.1 represents phylogenetic affiliation of *Actinobacter* spp. with the closest cultivable strains. The analysis was performed by sequencing the first 943 bases of 16 S r- RNA gene. Based on this analysis it is clear that our isolate shows maximum similarity with the group *Actinobacterium* and therefore could be considered as a species of the genus *Actinobacter*. 

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3.3 Biosynthesis of nanocrystalline magnetite (Fe₃O₄)

This part of the chapter describes the extracellular, aerobic biosynthesis of magnetite nanoparticles using Actinobacter spp.

3.3.1 Experimental details

In a typical experiment, seed culture of Gram-positive bacterium Actinobacter spp. was prepared in Luria broth (LB) for 24 h as an inoculum. The inoculum was then transferred to 100 ml of LB and incubated at 37 °C for 24 h at 150 rpm till the bacterium entered into the late log phase of the growth cycle. Filter sterilized aqueous mixture of K₃Fe(CN)₆ and K₄Fe(CN)₆ was then added to the bacterial culture and the flask was incubated for another 72 h on a shaker (150 rpm) at 37 °C. Magnetite synthesized in culture medium was harvested from bacterial biomass by centrifugation at 5000 rpm (2560 X g). The supernatant containing magnetite was then lyophilized and used for further analysis by Transmission electron microscopy (TEM), X-ray diffraction analysis (XRD), Fourier transformed infrared spectroscopy (FTIR), Magnetic force microscopy (MFM), Mossbauer spectroscopy, magnetic measurements etc. The biochemical investigation of magnetite formation was carried out by analysis of protein profile and enzymatic assays.

3.3.2 Transmission electron microscopic analysis

Figure 3.2 shows TEM images of magnetite nanoparticles synthesized after 48 h and 72 h of reaction between ferri/ferrocyanide salts and Actinobacter spp., respectively. After 24 h of reaction, a number of quasi-spherical particles were observed with 10-20 nm size range (Figure 3.2A and B). These nanoparticles appear to be well separated from each other since they are stabilized by the proteins present on the surface. In most of the magnetite nanocrystals, twinning was observed indicating the presence of defects in the crystal structure (Figure 3.2B). When the bacterial culture is incubated further along with the ferri/ferrocyanide mixture, after about 72 h of the reaction the quasi-spherical structure of magnetite nanoparticle is completely lost. Prolonged incubation yielded uniform cubical nanoparticles of edge length of 50-150 nm (Figure 3.2C) suggesting the assembly of spherical nanoparticles into cubic superstructures. High magnification image of the cubes reveal voids that could arise from aggregation of smaller spherical nanoparticles (inset, Figure 3.2C). Even under these conditions of aggregation, the
magnetite nanoparticles were extremely stable in solution for weeks suggesting stabilization of the particle surface with bio-organic molecules secreted by *Actinobacter* spp.

![Figure 3.2](image)

**Figure 3.2** TEM images of as synthesized magnetite nanoparticles synthesized after 24 h (A & B) and 72 h (C) of reaction between Actinobacter spp. and ferri/ferrocyanide mixture. SAED pattern (D) shows that the particles are single crystals and the diffraction spots could be indexed on the basis of the magnetite structure. Calcination of the as-synthesized nanoparticles leads to aggregation with drastic change in overall morphology (E and F). Inset in (E) shows SAED pattern obtained from calcined nanoparticles.

Selected area diffraction analysis showed the presence of well defined diffraction spots indicating that nanoparticles are crystalline. The diffraction spots could be indexed on the basis of magnetite crystal structure [32].

While the as-synthesized powdered magnetite nanoparticles were calcined at 350 °C for 3 h, the morphology of nanoparticles was drastically altered. The cubic assembly of the magnetite nanoparticles is completely destroyed and the particles are uniformly dispersed over the grid surface. Some amount of sintering of the particles also appears to have occurred with the growth of individual nanocrystallites indicating the formation of aggregates with dimensions around 20 nm (Figure 3.2E and G). This change in the morphology of as-synthesized nanoparticles could be attributed to partial loss of surface capped proteins. Also, in this case the SAED pattern showed the presence of diffraction
rings indicating the polycrystalline nature of the particles that show excellent match with magnetite crystal structure [32].

### 3.3.3 X-ray diffraction analysis

The X-ray diffraction pattern recorded from the solution cast magnetite film on glass surface is shown in Figure 3.3. A number of strong Bragg reflections are observed originating from the sample which could be indexed on the basis of magnetite crystal structure. The high intensity of (220) Bragg reflection indicates preferential orientation of these crystals. Along with magnetite, reflections from the crystal planes marked as “•” were found to show a match with the maghaemite (γ-Fe₂O₃) phase of iron oxide.

![Figure 3.3 XRD pattern of magnetite nanoparticles synthesized after 72h of reaction between Actinobacter spp. and ferri/ferrocyanide salts. XRD pattern could be indexed on the basis of mixed phases of iron oxide, magnetite being the major phase. Curve 1 in black shows XRD pattern of as synthesized sample, while curve 2 in grey shows XRD pattern obtained from the sample that was calcined at 350 °C for 3h.](image)

All the peaks shown with the symbol “*” denote reflections originating from magnetite nanoparticles while one peak marked as “○” can be assigned to FeO [32]. Curve 1 in Figure 3.3 (black curve) shows the XRD pattern recorded from as-synthesized magnetite nanoparticles. XRD pattern recorded for as-synthesized nanoparticles which were calcined at 350 °C for 3 h also shows excellent match with
magnetite crystal structure (curve 2 in grey, Figure 3.3). XRD pattern recorded from as synthesized and calcined nanoparticles showed a little variation and could be superimposed. This indicates that the heat treatment does not cause any crystallite growth and there is no change in the overall composition of the maghaemite particles either by way of sintering of the particles or by oxidation. However, a small improvement in the crystallinity of each individual domain cannot be ruled out. This observation is well supported by TEM analysis wherein a partial aggregation of nanoparticle is seen due to calcination which caused an individual crystallite growth. The $d$ values and the respective $hkl$ planes are as follows: For magnetite marked as “*” 2.86 (220), 2.44 (311), 2.02 (400), 1.64 (422) 1.26 (622), 0.96 (642). For maghaemite ($\gamma$-Fe$_2$O$_3$) marked as “•”: 3.23 (205), 2.20 (113), 1.63 (511) and for FeO marked as “○”: 1.51 (104).

3.3.4 FTIR spectral characterization and TGA analysis

Figure 3.4A shows the FTIR spectrum of culture supernatant containing magnetite nanoparticles before (curve 1 Figure 3.4A) and after (curve 2 Figure 3.4A) synthesis. Curve 1 in Figure 3.4A shows the FTIR spectrum of Actinobacter spp. ferri/ferrocyanide reaction mixture, recorded immediately after the addition of ferri/ferrocyanide salts to the bacterial suspension. This spectrum does not show any Fe–O vibration bands specific to iron oxide.

Curve 2 in Figure 3.4A depicts the FTIR spectrum of powdered magnetite nanoparticles synthesized after 72 h of reaction between Actinobacter spp. and ferri/ferrocyanide salts. Spectrum 2 (black curve) shows a number of vibration bands that arise due to the stretching and bending mode of Fe-O bonds in magnetite. A strong vibration band at around 610 cm$^{-1}$ along with a small band at 547 cm$^{-1}$ is due to the Fe-O bending mode while, a broad peak at around 840 cm$^{-1}$ is due to Fe-O stretching vibrations (solid lines in the spectrum) [33]. Along with these vibration bands the spectrum shows the presence of two peaks centered around 1650 and 1560 cm$^{-1}$. These bands could be assigned to the vibrations due to amide I and II bands that arise most probably due to the presence of proteins (dotted lines spectrum 2, Figure 3.4A). This analysis suggests that there is association of proteins with the surface of magnetite nanoparticles.
Thermogravimetric analysis (TGA) of as-synthesized magnetite nanoparticles was carried out to quantify the bio-organic loading of magnetite nanoparticles. Figure 3.4B shows the TGA profile recorded from carefully weighed powdered magnetite sample. As-synthesized magnetite nanoparticles display a gradual weight loss of c.a. 65% around 500 °C.

A steady weight loss around 200 °C can be attributed to removal of water that is associated with magnetite nanoparticles. A sharp and steady weight loss in the temperature range of 250 °C to 500 °C can be attributed to desorption and decomposition of the surface bound bio-organic molecules present along with the magnetite nanoparticles. After 500 °C the curve shows no further weight loss, indicating that around 35% of inorganic matter, most of which could be magnetite is left by 600 °C.

**3.3.5 Magnetic measurements**

Magnetic measurements were performed on dried magnetite nanoparticles before and after calcination. 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 magnetite nanoparticles is studied by ZFC (Zero-field-cooled) and FC (field cooled) modes for as prepared as well as calcined nanoparticles. Figure 3.5A shows the magnetization curves (M-H curves) obtained from as synthesized powdered magnetite nanoparticles at 5 K, 20 K, 150 K and 250 K, respectively. Magnetic particles below a certain size regime exist as single domain particles displaying properties characteristic of superparamagnetism [34]. In these size regimes the thermal energy, above a temperature termed as blocking temperature ($T_B$), is sufficient enough to make the remanent magnetization overcome the barriers dictated by any of the different anisotropies, making the moments jump between two stable orientations of magnetization. Consequently, above $T_B$, the M-H curves of the superparamagnets show no hysterisis, while below this temperature the curves open up displaying curves very similar to those observed for ferromagnets.

![M-H curve of as prepared magnetite samples measured at various temperatures (5K-black, 20K-red, 50K-green, 150K-blue and 250K-orange respectively) indicate the superparamagnetic nature of the particles (Figure 3.5A). ZFC (curve 1) and FC (curve 2) measurements (Figure 3.5B) show that both curves almost superimpose above 60 K.](image)

Magnetite has a complex spinel structure with one Fe$^{2+}$ ion and two Fe$^{3+}$ ions per formula unit. In each unit cell there are eight molecules of Fe$_3$O$_4$ and two distinct types of sites; 64 tetrahedral sites and 32 octahedral sites, which the iron ions can occupy. In these, only 16 of the octahedral sites are occupied and are shared by Fe$^{3+}$ and Fe$^{2+}$ ions and only 8 of the 64 possible tetrahedral sites are occupied by the other trivalent ion. In a very simple way it can be explained that the two trivalent Fe$^{3+}$ ions interact antiferromagnetically in Fe$_3$O$_4$ and hence their magnetic moments cancel each other out.
and the net moment observed, comes from the remaining divalent iron [35]. Magnetite nanoparticles prepared using *Actinobacter* spp. clearly reveal the superparamagnetic nature. Here, as-synthesized nanoparticles showed a weak magnetic signal at room temperature (orange colored curve in Figure 3.5A), which is attributed to the presence of high amount of proteins along with the nanoparticles. However, at low temperature magnetization is greatly enhanced and showed a characteristic superparamagnetic behavior (black and red curve for 5 and 20 K respectively in Figure 3.5A). Figure 3.5B shows the zero field cooled and field cooled (ZFC-FC) measurements performed at an applied external field of 1000 Oe. FC and ZFC curves almost superimpose on each other suggesting the absence of blocking phenomenon in the nanoparticles. This could most probably be due to the considerable size variation in the as synthesized magnetite nanoparticles by *Actinobacter* spp.

Figure 3.6A shows the magnetization curves recorded from magnetite nanoparticles, which were calcined at 350 °C for 3 h. These calcined nanoparticles showed enhanced magnetic response under magnetic field with the signature of superparamagnetic behavior at room temperature (Figure 3.6A brown curve). At low temperature (5 K), a characteristic hysteresis loop was observed with opening of the loop indicating ferrimagnetic behavior of the nanoparticles. The increase in the magnetization signal in calcined sample is attributed to the removal of diamagnetic proteins from the surface of nanoparticles, a result consistent with TGA studies, which indicated that there is heavy loading of bio-organic molecules on magnetite nanoparticles. For magnetic nanoparticles, as the capping molecules become more and more bulky the overall magnetization of the nanoparticles is reduced [36].

The maximum magnetization and the coercivity values calculated for magnetite synthesized by *Actinobacter* spp. shows considerable variation from the reported literature. Maximum magnetization value ($\sigma_s$) per unit mass under given experimental conditions for magnetite is found to be ~ 9 emu / g while the coercivity was calculated to be ~ 600 Oe at 5 k. The decrease in the saturation magnetization could be due to either the presence of magnetically dead layer of iron oxide on the nanoparticle surface or due to the very small size of the particles [37]. A small hysteresis loop is observed in the M-H curve that measured at 275 K. However, at lower temperature (below 50 K) the hysteresis
loop is sizable with clear opening indicating ferromagnetic transition in the nanoparticles. Also the hysteresis loop is symmetric about the centre, which is a characteristic of superparamagnetic behavior [38].

![Figure 3.6 (A) M-H curve of calcined magnetite sample measured at various temperatures (5 K-black, 20 K-green, 50 K-red, 150 K-blue and 250 K-brown respectively). The inset in Figure 3.6A shows clear opening of hysteresis loop at 5 K (black curve) while at room temperature superparamagnetism is restored (red curve). ZFC (red curve) and FC (blue curve) measurement (Figure 3.6B) shows that both curves almost superimpose at 275 K indicating that blocking temperature could be around 275 K. Inset in Figure 3.6B is a Mossbauer spectrum obtained from the calcined magnetite sample.](image)

In contrast to ZFC-FC behavior of as prepared magnetite nanoparticles, calcined sample exhibited a sharp divergence in ZFC-FC curves (Figure 3.6B). After calcination, the sample showed clear separation in ZFC (red curve, Figure 3.6B) and FC (blue curve, Figure 3.6B) curves with a blocking temperature of around 275 K. This is probably due to the enhanced interaction between the small nanoparticles after the removal of surface capped protein or could be due to the fact that the defects in the nanocrystals are annealed after calcination. To ascertain the crystalline phase of iron oxide as magnetite, Mossbauer spectroscopic analysis was performed on the calcined powder. Inset in Figure 3.6B shows the Mossbauer spectroscopic analysis performed on room temperature. Mossbauer spectrum shows the presence of several sextets resulting from the longer relaxation time or due to the poor statistical fitting of the spectral data points. Mossbauer spectrum clearly shows the superparamagnetic nature of magnetite nanoparticles and the six lined pattern with peak broadening clearly indicates the wide size distribution of magnetite nanoparticles. The corresponding Mossbauer resonance parameters such as isomer shift
are (δ) 0.28 mm/s for 64 % of magnetized iron and 0.21 mm/s for 36 % of relaxed iron component.

Figure 3.7 shows the magnetic force microscopy (MFM) image of the magnetite nanoparticles calcined at 350 °C for 3 h. The surface topographical (height) and magnetic force images (frequency) were recorded on a mica surface, which indicates a detectable contrast in both the images. Figure 3.7A corresponds to the topography of the aggregated nanoparticles while Figure 3.7B shows the magnetic force image. In frequency mode (MFM) the black contrast corresponds to the magnetic domains associated with the aggregated magnetite nanoparticles due to the attractive force between tip and the sample surface. MFM images with a fair contrast can be influenced by the sample topography. However, the shape and size of magnetite nanoparticles is a little different in topographical image and magnetic force image.

![Figure 3.7](image)

**Figure 3.7** AFM analyses of magnetite nanoparticles indicating the particle topography and magnetic contrast. There is a considerable contrast variation between topographical (height) image (Figure 3.6A) and magnetic (frequency) image (Figure 3.6B) due to the attractive force between tip surface and magnetic domains in the sample. Scale bar in both the images is 5μM and frequency range for magnetic image is 380 Hz.

MFM images with fair contrast can be influenced by the sample topography. However, the shape and size of magnetite nanoparticles is little different in topographical image and magnetic force image. This indicated that the observed contrast is due to the domain structures present in the sample. In the frequency image discontinuous changes in
contrast are observed, which can be possibly due to the distortion of domain structure under the influence of the tip since the stray field of the tip is larger than the coercive field [39].

3.3.6 Biochemical insight into magnetite formation by *Actinobacter* spp.

Magnetite formation in magnetotactic bacteria is extensively studied. There is a fair understanding of the process of magnetite formation at biomolecular level. Iron reducing bacteria like *Geobacter metallireducens* are also investigated for the process of magnetite formation at biomolecular level. The biological basis of magnetite synthesis in iron reducing bacteria lies in the energy production for the bacterial metabolism and growth [27], while magnetotactic bacteria synthesize magnetite for magnetotaxis to sense oxic-anoxic zones [16]. Biomolecular foundation of magnetite synthesis by *Actinobacter* spp. seems to be totally different from magnetotactic bacteria and iron reducing bacteria. *Actinobacter* spp. is a gram positive and aerobic bacterium, which synthesizes magnetite under fully aerobic conditions, contrary to magnetotactic and iron reducing bacteria, which requires strict anaerobic conditions for magnetite biosynthesis.

Figure 3.8 UV-vis spectrum showing the hydrolysis of ferricyanide/ferrocyanide complex by *Actinobacter* spp. Curve 1 shows the absorption due to ferricyanide/ferrocyanide complex, while curve two shows that absorption at 440nm completely disappears while absorption hump at 325nm is evolved, which is due to iron oxide.
Magnetite biosynthesis by *Actinobacter* spp. depends on its capacity to grow in the presence of ferricyanide/ferrocyanide reaction mixture. It should be noted that *Actinobacter* spp. was isolated from potassium ferricyanide/ferrocyanide reaction mixture and was able to grow on a Luria agar (LA) plate supplied with potassium ferricyanide/ferrocyanide salts in 2:1 molar ratio respectively. *Actinobacter* spp. could synthesize magnetite when reacted with cyanide complexes of iron and indicates the fact that the bacterium has the ability to hydrolyze iron-cyanide complexes, which is followed by magnetite formation.

**Figure 3.9** (A) SDS-PAGE analysis of extracellular proteins secreted by *Actinobacter* spp. in the presence (lane2) and absence (lane3) of ferri/ferrocyanide reaction mixture. Lane 1 shows standard molecular weight marker with respective molecular weights. (B) UV-vis spectroscopic assay for ferric/ferrocyanide hydrolysis by inducible proteins with molecular weight of 70 and 100 kDa after elution from gel. A prominent absorbance peak at 440 nm (curve 1) is due to ferri/ferrocyanide salt, which is considerably dampened in the presence of inducible proteins with subsequent evolution of a shoulder peak around 325 nm, due to formation of iron oxide (curve 2). (C) TEM image of magnetite nanoparticles synthesized by reaction between inducible proteins eluted from native polyacrylamide gel and $K_3$Fe(CN)$_6$ - $K_4$Fe(CN)$_6$ mixture. The inset shows a SAED pattern corresponding to magnetite structure [$\Delta$-(220), □-(311), ○-(400) lattice planes].
Microorganisms such as bacteria and fungi are known to synthesize various cyanide hydrolyzing enzymes that hydrolyze metal cyanide complexes or free cyanide [40]. Figure 3.8 shows the UV-vis spectroscopic analysis of hydrolytic features of iron cyanide complex before and after reaction with *Actinobacter* spp. In most bacteria, an enzyme called cyanidase or cyanide dihydratase is synthesized in response to metallocyanide complexes, which hydrolyze metal cyanide complexes [41]. For microorganisms and most of the plant, cyanide can serve as a carbon and nitrogen source. We believe that *Actinobacter* spp. can also use cyanide as a source of carbon and nitrogen, since in stationary growth phase bacterium has to rely on alternative energy source to support its metabolic activities. Therefore it extracellularly synthesizes cyanidase, which can hydrolyze iron cyanide complexes.

The process of magnetite formation is extracellular and it is quite possible that iron-cyanide hydrolyzing protein is secreted in the surrounding medium. Once the iron is removed from cyanide complex, it is most probably converted into magnetite by the action of some other protein. Figure 3.9A shows the extracellular protein profile of culture supernatant of bacterium grown in the presence and absence of K$_3$Fe(CN)$_6$ - K$_4$Fe(CN)$_6$ mixture. Induction of two new proteins with molecular weight of around 100 kDa and 70 kDa is observed when the bacterium was grown in the presence of ferri/ferrocyanide salt mixture (lane 2, Figure 3.9A). Figure 3.9B shows the UV-vis spectroscopic assay for ferri/ferrocyanide hydrolysis by inducible proteins with molecular weight of 70 and 100 kDa after elution from native polyacrylamide gel. Also, these proteins are not observed in the culture supernatant, when the bacterium was grown in the absence of ferri/ferrocyanide salt. This indicated that the new proteins are synthesized in response to the iron salts. We believe that one of inducible protein is actually cyanidase, an enzyme that catalyzes the hydrolysis of ferri/ferrocyanide salts. Indeed the inducible protein fractions when eluted from native polyacrylamide gel and reacted with iron cyanide complexes 10 mM sodium phosphate buffer (pH 6.4), could hydrolyze and subsequently synthesize magnetite. Curve 1 in Figure 3.9B shows a prominent absorbance at 440 nm, which is due to K$_3$Fe(CN)$_6$ - K$_4$Fe(CN)$_6$. After reaction with gel eluted purified proteins, considerable dampening in the intensity of ferry/ferrocyanide absorbance is observed (curve 2). This, along with the concomitant evolution of a
shoulder peak around 325 nm (due to formation of iron oxide) suggest that hydrolysis of $K_3Fe(CN)_6 - K_4Fe(CN)_6$ salts lead to formation of iron oxide nanoparticles in the presence of two inducible proteins.

Figure 3.9C shows the TEM image of magnetite nanoparticles synthesized by reaction between inducible proteins, eluted from native polyacrylamide gel and $K_3Fe(CN)_6 - K_4Fe(CN)_6$ mixture in 10 mM sodium phosphate buffer (pH 6.4) after 12 h incubation. TEM analysis indicates the presence of aggregates of around 50 nm, which are composed of smaller magnetite nanoparticles. That these nanoparticles are indeed magnetite is confirmed by the selected area electron diffraction pattern, which could be indexed on the basis of magnetite crystal structure. These results indicate that magnetite formation by Actinobacter spp. is mediated by at least two proteins, one of which is responsible for the hydrolysis of ferri/ferrocyanide salt releasing ionic Fe. Subsequently other protein transforms Fe ions into magnetite. However, other proteins secreted by the bacterium in tandem can also be involved in the synthesis and stabilization of magnetite.

3.3.7 Discussion

This part of the chapter describes the biosynthesis of magnetite by Actinobacter spp., which is a gram positive, aerobic bacterium. Actinobacter spp. was isolated from $K_3Fe(CN)_6 - K_4Fe(CN)_6$ reaction mixture. The bacterium was able to grow in the presence of these iron salts and could even hydrolyze them. It was identified as a species of genus Actinobacter by molecular taxonomic method of 16S rRNA sequencing using bioinformatics tools. The most remarkable feature of this extracellular, room temperature biosynthesis method is that magnetite nanocrystals are formed under complete aerobic conditions. The magnetite so formed is characterized by TEM, XRD, FTIR, TGA and magnetic measurements. Biological mechanism of magnetite formation in Actinobacter spp. is substantially different from earlier reports of magnetotactic bacteria and iron reducing bacteria, which requires anaerobic conditions for magnetite biosynthesis. Magnetic measurements carried out on as synthesized and calcined magnetite nanoparticles indicated that they are superparamagnetic in nature. Biochemical analysis of the process indicated that there are at least two proteins, which can play an important role in the magnetite biosynthesis. These two proteins with molecular weight of around 70 kDa and 100 kDa are induced in the presence of ferri/ferrocyanide salts. One of the
proteins is with the hydrolase activity that hydrolyzes ferri/ferrocyanide complex. We suspect that this enzyme could be a cyanidase, which hydrolyze the iron cyanide complex. We believe that the other protein with a molecular weight c.a. 100 kDa is responsible for the conversion of Fe ions into magnetite. However, these results are preliminary and require further insight.

3.4 Biosynthesis of nanocrystalline maghaemite ($\gamma$-Fe$_2$O$_3$)

This part of the chapter describes Actinobacter spp. mediated biological synthesis of maghaemite nanoparticles. Like magnetite, biosynthesis of maghaemite nanoparticles also occurs at room temperature and under fully aerobic conditions.

3.4.1 Experimental details

In a typical experiment seed culture of Actinobacter spp. was grown in the 2ml of LB medium, which was subsequently propagated into 100 ml of the same medium. The flask was then incubated at room temperature (~ 35 °C) on a rotary shaker (150 rpm). After 48 h of the bacterial growth, filter sterilized solution of ferric citrate or ferric chloride was added to the pre-grown Actinobacter spp. to the final concentration of 1mM. The flask was then further incubated for another 48-72 h on a rotary shaker (150 rpm) at 37 °C. Maghaemite synthesized in culture medium was harvested from bacterial biomass by centrifugation at 5,000 rpm (2560 X g). Supernatant containing maghaemite was then lyophilized and further analyzed using TEM, MFM, XRD, FTIR, Mossbauer spectroscopy and magnetic measurements etc. as discussed in section 3.3.1.

The biochemical investigation of maghaemite formation was carried out by genetic and protein analysis and by enzyme assays for ferric iron reductase. The extracellular protein profile of the bacterial culture supernatant was checked for the induction of the new protein/s upon iron salt addition. 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 the analysis of the proteins bound to the surface of the nanoparticles, Maghaemite and iron sulfide nanoparticles were separated from the culture supernatant. First, the bacterial biomass was removed by centrifugation at 5,000 rpm (2560 X g) and the culture supernatant containing nanoparticles was collected. This culture supernatant was again subjected to centrifugation at 15,000 rpm (20579 X g) for 30 minutes. After centrifugation, the
supernatant was discarded and the black pellet of $\gamma$-Fe$_2$O$_3$ was washed thrice with 50 mM sodium phosphate buffer (pH 7.4) before dissolving in 100 $\mu$l 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 $^\circ$C for 10 minutes and then centrifuged at 15,000 rpm for 30 minutes. The supernatant was then extensively dialyzed against 50 mM sodium phosphate buffer (pH 7.4), and then analyzed on SDS-PAGE for the presence of proteins as described above.

Iron reductase activity was measured by the standard method of Dailey [42] with slight modification. The method involves use of ferrous iron chelator, ferrozine [3-(2-pyridyl)-5, 6- bis (4-phenylsulphonic acid)-1,2,4-triazine]. Ferrozine reacts with ferrous iron to form a purple colored chelator with a molar extinction coefficient of 28,000 at 562 nm. The assay mixture in the final volume of 2 ml contained 50 $\mu$l of NADH, 100 $\mu$l of ferrozine, 50 $\mu$l of 5 mM ferric citrate, 200 $\mu$l of bacterial culture supernatant in 20mM phosphate buffer (pH-7.2).The reaction was initiated by addition of the culture supernatant containing enzyme and incubated at room temperature for five hours. The spectral measurement was carried out on a JASCO dual beam spectrophotometer (model V-570) operated at a resolution of 1nm. In a control experiment for assay, all the ingredients were kept unaltered, while the bacterial culture supernatant obtained from bacterial growth in absence of iron precursor was added with rest of the ingredients. To elucidate the role of iron reductase in the formation of maghaemite, Actinobacter spp. was reacted with ferric ions in the presence of 100 $\mu$M of Zn$^{2+}$ ions, which inhibit iron reductase activity [43]. Iron reductase activity in presence of Zn$^{2+}$ ions was assayed as discussed above.

Isolation and sequencing of iron reductase gene from Actinobacter spp. was performed by isolating genomic DNA was isolated from Actinobacter spp. by standard phenol/chloroform extraction method as described previously [29]. The genes encoding iron reductase was amplified using a polymerase chain reaction with specific primers as fhuf-F ($5'$ – GAC CCC GAT CTC ACC GCA CT -3') and fhuf-R ($5'$ – GAA ACA GCA GTC GCC GCA -3') designed from S. Sterpromyces avermitilis MA-4680 genome (NCBI Genbank no. NC 003155). The PCR conditions used were an initial denaturation at 94 $^\circ$C for two minutes, followed by 35 cycles of denaturation at 94 $^\circ$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 products, 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 NCBI server using different BLAST programs.

A number of control experiments were carried out to assess the authenticity of iron mineral biosyntheses by Actinobacter spp. In the first control experiment, iron precursors were mixed with the culture medium in the absence of Actinobacter spp. and the experiment was carried out as described above. In yet another control experiment, a number of other bacteria like Bacillus subtilis, Aeromonas spp., Micrococcus spp. and E.coli were used to check the synthesis of nanoparticles.

3.4.2 Transmission electron microscopic analysis

Figure 3.10 shows the representative TEM images of as synthesized (A-C) and calcined (350 °C, 2 h) maghaemite nanoparticles (D-F). It is clearly seen from low magnification images that particles tend to organize into clusters of roughly 100 nm size (Figure 3.10 A). High magnification image of these clusters show smaller particles with the dimension of around 5-10 nm (Figure 3.10C) that are capped possibly by the proteins secreted by Actinobacter spp. Selected area diffraction pattern obtained from one of the images (Figure 3.10A, inset) show well defined diffraction spots indicating the crystalline nature of the nanoparticles and the lattice parameter obtained were consistent with maghaemite crystal structure [44].

After the calcination of as-synthesized sample at 350 °C for 2 h, little change in morphology of nanoparticles was observed. Maghaemite nanoparticles tend to sinter together because of the partial removal of the surface bound proteins resulting in the formation of aggregates with 20-30nm diameter. The aggregation within nanoparticles is also due to the magnetic exchange interaction now much prominent due to the removal of proteins present on the surface of maghaemite nanoparticles. Moreover, the calcination treatment further broadens the size distribution in maghaemite nanoparticles leading to size irregularity
Figure 3.10 TEM images of as-synthesized maghaemite nanoparticles after 72h of reaction between Actinobacter spp. and ferric citrate (A-C). The inset in image A shows SAED pattern obtained from maghaemite nanoparticles. Calcination of as-synthesized nanoparticles leads to aggregation with little change in overall morphology (D - F). The inset in image D shows SAED pattern obtained from calcined nanoparticles.

Nevertheless some well separated particles were also observed with overall dimension between 10-20 nm along with the aggregates of individual nanoparticles (Figure 3.10D-F) The SAED pattern is much more discernible now which was clearly indexed on the basis of $\gamma$-$\text{Fe}_2\text{O}_3$ crystal structure (inset in Figure 3.10E).

3.4.3 X-ray diffraction and Mossbauer spectroscopic analysis

Figure 3.11A shows the X-ray diffraction pattern of as synthesized and 350 °C calcined maghaemite ($\gamma$-$\text{Fe}_2\text{O}_3$) nanoparticles, which were recorded from a solution cast maghaemite film on glass surface. The XRD pattern of as prepared maghaemite nanoparticles shows a number of Bragg reflections originating from the sample (curve 1 in Figure 3.11). Most of the peaks showed excellent match with the reported values of maghaemite crystal structure. Apart from the peaks marked as “○”, which correspond to maghaemite, a small number of peaks corresponding to other crystal phases of iron oxide, namely $\gamma$-$\text{FeOOH}$ (goethite) and FeO are also observed. The peak marked as “●” is due to
γ-FeOOH, while symbol “*” indicates Bragg reflections due to FeO. Curve 2 in Figure 3.11 shows the XRD pattern recorded from maghaemite nanoparticles, which were calcined at 350°C for 2 h.

![Figure 3.11](image-url) (A) XRD patterns of maghaemite nanoparticles synthesized after 72h of reaction between Actinobacter spp. and ferric citrate. Grey curve represents the XRD pattern of as-synthesized sample while black curve indicates the XRD pattern of maghaemite sample that was calcined at 350°C for 2h. (B) Mossbauer spectroscopic analysis of calcined maghaemite sample.

There is little variation in the XRD pattern of calcined nanoparticles in comparison with the XRD pattern of as synthesized nanoparticles indicating the fact that heat treatment does not alter the crystallinity of as synthesized nanoparticles. However, small improvement in individual crystal domains due to the partial removal of capped proteins can be expected. The d values and the respective hkl planes for γ-Fe₂O₃, γ-FeOOH and FeO are as follows: For maghaemite (γ-Fe₂O₃) peaks marked as “○”: 3.42 Å (211), 2.79 Å (221), 2.52 Å (311), 2.24 Å (321), 1.83 Å (421), 1.61 Å (511), 1.45 Å (530), 1.28 Å (533). For γ-FeOOH peaks marked as “•”: 2.95 Å (210) and for FeO peak marked as “*” 1.44 Å (110) [44].

Figure 3.11B shows Mossbauer spectroscopic analysis of calcined maghaemite nanoparticles performed at room temperature. Mossbauer spectroscopic analysis showed a weak sextet peak different that of magnetite, which shows the presence of two sextet peaks. Presence of quadrupole doublet corresponding to the relaxation time arises due to the superparamagnetic nature of maghaemite nanoparticles. The broad lines of the magnetic sextets and the smooth inner slopes suggest the existence of a wide size...
distribution of the obtained maghaemite nanoparticles [45]. The corresponding hyperfine parameters obtained are as follows- isomer shift (δ) = 0.35 mm/s, quadrupole shift (Δ) = 0.722. Values obtained for isomer shift and quadrupole shift are near to typical values of iron (III) [45].

3.4.4 FTIR spectroscopic analysis

Figure 3.12A shows FTIR spectrum of powdered maghaemite nanoparticles synthesized after 72 h of the reaction between Actinobacter spp. ferric citrate. The spectrum shows a number of vibration bands that arise due to the stretching and bending mode of Fe-O bonds present in maghaemite.

Figure 3.12A FTIR spectrum of as synthesized maghaemite nanoparticles after 72 h of the reaction. A number of Fe-O bond vibrations are observed which correspond to maghaemite. The presence of amide I and amide II bonds in the spectrum suggests the association of proteins along with nanoparticles. (B) FTIR kinetics indicating the progress of reaction at various stages. Curve 1-6 corresponds to the spectral measurements after 12, 24, 36, 48, 60 and 72 h of the reaction showing that the conversion of ferric citrate into maghaemite occurs via iron oxyhydroxide as an intermediate.

A strong and broad vibration band at around 620 cm$^{-1}$ along with a small band at 916 cm$^{-1}$ is due to the Fe-O bending vibrations while, a peak at around 840 cm$^{-1}$ is due to Fe-O stretching vibrations (solid lines in the spectrum) [33]. A strong peak around 1030 cm$^{-1}$ is due to the presence of iron oxyhydroxide which can be assigned to γ-FeOOH. Along with these vibration bands, two peaks centered around 1650 and 1560 cm$^{-1}$ could
be assigned to the vibrations due to amide I and II bands those arise most probably because of the presence of proteins (dotted lines in the spectrum). This analysis suggests that there is association of proteins on the surface of maghaemite nanoparticles.

Conversion of ferric citrate into maghaemite by *Actinobacter* spp. was studied by FTIR spectroscopy as a function of time. Figure 3.12B shows FTIR analysis of the reaction mixture at various stages of the reaction. It is clearly revealed that the synthesis of $\gamma$-Fe$_2$O$_3$ occurs through the formation of iron oxyhydroxide as an intermediate stage. After first 12 h of the reaction, a prominent peak at 1030 cm$^{-1}$ is observed indicating the presence of iron oxyhydroxide which can be assigned to $\gamma$-FeOOH [44] (curve 1, Figure 3.12B). Along with this peak presence of two small peaks centered around 770 cm$^{-1}$ and 910 cm$^{-1}$ could be ascribed to $\beta$-FeOOH [46]. In between 60 to 72 h of the reaction iron oxyhydroxide is almost converted into iron oxide which is indicated by the presence of Fe-O band vibrations at 620 cm$^{-1}$ (curve 6 Figure 3.12B). The small peak at 840 cm$^{-1}$ is due to Fe-O vibrational band, which show shifts towards 810 cm$^{-1}$ after 48 hr (curve 4, 5, 6 Figure 3.12B) of the reaction.

### 3.4.5 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) of as synthesized maghaemite nanoparticles was carried out to calculate the gross amount of maghaemite in the reaction mixture. Figure 3.13 shows the TGA curve recorded from powdered maghaemite nanoparticles. The curve shows many regions at which, weight loss seems to occur, however only regions corresponding to major weight loss are discussed here. The curve shows overall gradual weight loss in the temperature region between 100 °C to 550 °C, which is ca. 67%. The initial, slow loss of weight at 100 °C can be due to the removal of water molecules associated with maghaemite powder.

There is a broad region of a gradual weight loss in between 250 °C to 550 °C which can be assigned to the decomposition and desorption of bioorganic molecules associated with maghaemite nanoparticles. After 550 °C no further decrease in weight loss is observed indicating that rest of the material is inorganic maghaemite powder, which is found to be approximately 33% by weight. Association of bio-organic proteinaceous materials with maghaemite nanoparticles is evident by their retention at 500 °C.
Figure 3.13 Thermogravimetric analysis of as synthesized, powdered maghaemite nanoparticles. The curve shows a gradual weight loss of ~ 67% which is due to decomposition of biomolecules present along with magnetite nanoparticles by 550 °C. After 550 °C the curve does not show further weight loss.

Thus, TGA analysis indicates that there is a very high amount of organic material of biological origin associated with maghaemite nanoparticles.

3.4.6 Magnetic measurements

As reported earlier (section 3.3.5), in this case also magnetic measurements were performed on dried maghaemite nanoparticles before and after the calcination. Magnetization as a function of external magnetic field was recorded by varying the applied field between -50 KOe to 50 KOe at different temperatures. Temperature dependence of magnetization in maghaemite nanoparticles is studied by ZFC (Zero-field-cooled) and FC (Field cooled) modes for as-prepared and the calcined nanoparticles at constant applied external field (1000 Oe). Figure 3.14A shows the magnetization curves (M-H curves) obtained from as synthesized powdered maghaemite nanoparticles at range of temperatures like 5 k, 20 k, 150 k and 250 k respectively.

As expected, as-synthesized maghaemite nanoparticles show very weak magnetization at higher temperatures indicating the paramagnetic response (brown curve, corresponding to 275 k, Figure 3.14A) owing to the fact that there is a considerable diamagnetic contribution due to the presence of proteins and other bioorganic
components with maghaemite nanoparticles. This observation is quite consistent with the TGA results, which indicated a very high amount of bioorganic molecules associated with maghaemite nanoparticles. However, like magnetite nanoparticles (section 3.3.5) maghaemite nanoparticles shows enhanced magnetization signal at lower temperatures and at 5 k it shows clear superparamagnetic behavior. The enhanced magnetization signal at lower temperature could be due to the fact that magnetic moments in maghaemite overcomes the thermal energy barrier and align themselves in the direction of magnetic field.

Figure 3.14(A) M-H curve obtained from as-prepared maghaemite samples at various temperatures (5k-black, 20k-red, 50k-green, 150k-blue and 250k-brown respectively) indicate the superparamagnetic nature of the nanoparticles. (B) The ZFC (red curve) and FC (blue curve) measurements show that both curves almost superimpose indicating the absence of blocking phenomenon.

Figure 3.14B shows the ZFC-FC measurements performed on as-synthesized maghaemite nanoparticles at an external applied magnetic field of 1000 Oe. ZFC and FC curves almost superimpose each other suggesting that the absence of blocking phenomenon. This could be most probably due to the even lower blocking temperature, which was not achieved by SQUID magnetometer that was used in this study. Absence of blocking phenomenon could arise due to a very small size of the nanoparticles with the considerable size variation [37]. Figure 3.15A shows M-H curves obtained from 350 °C calcined powdered maghaemite nanoparticles at various temperatures like 5k, 20k, 150k and 250 k respectively (various magnetization curves are indicated by different color coded symbols corresponding to specific temperature as discussed in figure).
Figure 3.15 (A) M-H curve obtained from the calcined maghaemite nanoparticles measured at various temperatures (5 k-black, 20 k-red, 50 k-green, 150 k-blue and 250 k-cyan respectively) indicate the superparamagnetic nature of the particles at room temperature. The inset in (A) shows the clear opening of hysteresis loop at 5 k. (B) ZFC (curve 1) and FC (curve 2) measurements showing that both curves superimpose at 275 k indicating that the blocking temperature is around 275 k.

A sharp rise in the induced magnetization is observed at all temperatures in comparison with as-synthesized maghaemite nanoparticles. At room temperature nanoparticles exhibit superparamagnetic behavior (Figure 3.14A, cyan curve). However, at low temperature (5 k) clear opening of hysteresis loop was observed indicating the ferromagnetic behavior of the nanoparticles (inset in Figure 3.15A).

The increase in the magnetization signal of the calcined sample is attributed to the removal of diamagnetic bio-organic molecules with the proteins present on 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 maghaemite nanoparticles synthesized by Actinobacter spp. is found to be around 8.5 emu/g while the coercivity was calculated to be ~ 540 Oe at 5 k. The lower saturation magnetization value reported here for maghaemite nanoparticles is due to the superparamagnetic nature of nanoparticles [37]. M-H curve shows clear opening of hysteresis loop at lower temperature (5 k) indicating the ferromagnetic transition of the nanoparticles. Figure 3.16 shows the magnetic force microscopic image of the maghaemite nanoparticles after calcined at 350 °C for 2 h.
Figure 3.16 Atomic force microscopy analysis of maghaemite nanoparticles showing the particle topography and magnetic contrast in MFM mode. A significant contrast variation between topographical (height) image (figure 3.16 A) and magnetic (frequency) image (figure 3.16 B) due to the attractive force between tip surface and magnetic domains in the sample can be seen. Scale bar in both the images is 6.3 μM and frequency range for magnetic image is 380 Hz.

The surface topographical (height) and magnetic force images (frequency) were recorded on a mica surface as indicated above in section 3.3.5. In this case also, topography and height images show a detectable contrast in both images. Figure 3.16 A corresponds to the topography of the aggregated nanoparticles while Figure 3.16B shows the magnetic force image. In frequency image (MFM), black contrast corresponds to the magnetic domains associated with the aggregated maghaemite nanoparticles due to the attractive force between the tip and sample surface. Though the presence of magnetic domain is observed in the frequency image, due to the presence of proteinaceous organic matter with nanoparticles, clarity of the image is considerably affected.

3.4.7 Biochemical analysis of maghaemite formation by *Actinobacter* spp.

Biological synthesis and stabilization of maghaemite nanoparticles by *Actinobacter* spp. seems to be a complex, multistep reaction. Like magnetite, maghaemite biosynthesis occurs under aerobic conditions suggesting that conversion of ferric salts into iron oxides does not result in energy generation as observed in iron reducing bacteria. The very fact that *Actinobacter* spp. was isolated from aqueous iron cyanide
complex suggests that the organism could withstand high iron concentration than that is normally required for cellular metabolism (usually ~ 6 μg).

We believe that the bacterium overcomes the toxic effect of excess iron by converting it into iron oxides, in this case maghaemite. The first and key step of the process is the reduction of Fe$^{3+}$ to bio-active Fe$^{2+}$, followed by the formation of iron oxyhydroxide which then is converted into maghaemite by dehydration. In the first step ferric iron (Fe$^{3+}$) is reduced extracellularly into ferrous (Fe$^{2+}$) form by the action of the enzyme ferrisiderophore reductase. This step is very crucial, since inhibition of ferrisiderophore reductase activity stops the synthesis of maghaemite nanoparticles. Once reduced, Fe$^{2+}$ is then converted into iron oxyhydroxide either via chemical way or by the action of iron oxidase. In the last step iron oxyhydroxide seems to be transformed to maghaemite by the action of dehydratase enzyme. Extracellular protein profile of *Actinobacter* spp. indicated the induction of a new protein with the molecular weight of around 55 kDa. Four different proteins with the molecular weights of 15, 16, 20 and 23 kDa respectively are found to be associated with the surface of maghaemite nanoparticles.

### 3.4.7.1 Iron reductase assay and identification of iron reductase gene

Most of the bacteria are known to synthesize iron reductase enzyme for the assimilatory metabolism of iron. *Actinobacter* spp. synthesizes iron reductase extracellularly. We believe that iron reductase of *Actinobacter* spp. is a ferrisiderophore reductase as it scavenges the Fe$^{3+}$ extracellularly. Figure 3.17A shows the assay of iron reductase enzyme that is induced by *Actinobacter* spp. in the presence of excess ferric iron. Ferrisiderophore reductase assay showed the iron reductase activity as indicated by the strong purple colour of assay mixture. A strong absorbance at 560 nm was observed, which indicates the formation of ferrozine-ferrous iron complex (Figure 3.17A). Ferrisiderophore reductase concentration increases in the presence of excess iron salt. However, no ferric reductase activity was observed in the supernatant obtained from bacterial culture grown in the absence of iron salts (curve1, Figure 3.17A). To elucidate the role of iron reductase in the formation of maghaemite, the reaction was carried out in the presence of zinc ions. Synthesis of iron oxide was not observed at all in the presence
of zinc ions indicating that iron reductase is required for the synthesis of iron oxide and at the initial stage.

Figure 3.17B shows the PCR analysis of iron reductase gene on 1% agarose gel. When *Actinobacter* spp. genome was analyzed for the presence of iron reductase gene using a set of specifically designed gene primers (see section 3.4.1), a single band of 675 base pairs was obtained corresponding to iron reductase (*fhu F*) gene. The size of *fhu F* gene in the bacterium from which primers for PCR amplification were designed (*Streptomyces avermitilis* MA-4680 genome, NCBI Genbank no. NC 003155) is around 700 base pairs. The size of PCR amplified fragment is near to the reported value of *fhu F* gene in *Streptomyces avermitilis* MA-4680 and therefore the amplified gene fragment may be considered as *fhu F* homologue in *Actinobacter* spp.

This result along with iron reductase assay indicates the presence of iron reductase in *Actinobacter* spp. Inability for maghaemite synthesis by the bacterium in the presence of zinc ions, which inhibits iron reductase activity, shows that iron reductase is required for the biosynthesis of maghaemite nanoparticles. However, the exact function of iron reductase during maghaemite nanoparticles is not identified yet, as it not known.
whether this enzyme specifically synthesized in the presence of excess ferric iron or is a part of regular bacterial metabolism.

### 3.4.7.2 Analysis of extracellular and surface bound proteins

To study the induction of new protein/s due to iron salt stress, extracellular protein profiles of culture supernatant from the bacterium grown in the presence and absence of ferric citrate were analyzed on polyacrylamide gels in native and denaturing conditions (SDS-PAGE). The denaturing (SDS-PAGE) gel electrophoresis profile (Figure 3.18A) shows the induction of a new protein with molecular weight c.a. 55 kDa in the presence of ferric citrate.

![Figure 3.18(A) SDS-PAGE analysis of extracellular proteins secreted by Actinobacter spp. in the presence (lane Ex) and absence (lane c) of ferric citrate. Induction of a protein with molecular weight of 55 kDa is observed in the presence of iron salt (lane Ex, marked by arrow), which are absent in the culture supernatant of bacterium grown in the absence of iron salt (lane C). Lane M is standard molecular weight marker with respective molecular weights. (B) SDS-PAGE analysis of the proteins bound to the surface of maghaemite nanoparticles. Lane Ex shows the surface bound protein of maghaemite nanoparticles. Lane M is standard molecular weight marker (three bold bands from bottom to top in lane M are of 14 kDa, 20 kDa, and 29 kDa).](image)

This protein might be playing an important role in the formation of maghaemite nanoparticles. The proteins present on the surface of maghaemite nanoparticles were also analyzed by SDS-PAGE. This analysis revealed the presence of four different surface bound proteins with the respective molecular weights of 15, 16, 20 and 23 kDa (Figure 3.18B). The association of these proteins with the nanoparticles surface indicates that
they may be responsible for the stabilization of maghaemite nanoparticles. However, exact function of these proteins is yet to be understood completely. The work described here shows the preliminary results for the biochemical analysis of maghaemite formation by *Actinobacter* spp. Based on these results it can be hypothesized that initially Fe$^{3+}$ ions are reduced extracellularly to Fe$^{2+}$ ions by the action of ferrisiderophore reductase. Immediately after, Fe$^{2+}$ ions are reoxydized to iron oxyhydroxide (ferrihydrite). FTIR kinetics of the reaction progress (Figure 3.12B) revealed the presence of iron oxyhydroxide as an intermediate in the synthesis of maghaemite. This step can be biotic or abiotic. It is possible that ferrous iron can bind to bacterial cell wall, which has anionic organic molecules exposed on its surface. It has been shown that iron show extremely high affinity for polymeric materials present on the bacterial cell wall due to its valence, hydrated radius, hydration energy and electronegativities [47]. Once bound to cell surface, Fe$^{2+}$ can be oxidized to iron oxyhydroxide by the action of membrane bound iron oxidase enzyme. However, it is also possible that Fe$^{2+}$ ions can spontaneously get oxidized into iron oxyhydroxide (ferryhydrite) under aerobic conditions at circumneutral pH. Lastly, the iron oxyhydroxide thus synthesized is dehydrated to maghaemite by the action of bacterial dehydratase enzyme.

3.4.8 Discussion

This part of the chapter describes biosynthesis of nanocrystalline maghaemite (γ-Fe$_2$O$_3$) by *Actinobacter* spp. from ferric citrate. Like magnetite, maghaemite formation by *Actinobacter* spp. occurs under aerobic conditions. The biogenic maghaemite nanoparticles are found to be of 5-7 nm in diameter. Magnetic measurements performed on as-synthesized and calcined maghaemite nanoparticles indicated the superparamagnetic behavior with coercivity value 540 Oe at 5 K. MFM measurements performed on maghaemite nanoparticles after calcination clearly showed contrast variation in topographical and magnetic image due to the presence of magnetic domains. Mossbauer analysis was in line with the SQUID measurement indicating that iron oxide nanoparticles are indeed composed of maghaemite phase. Biochemical analysis of maghaemite formation in *Actinobacter* spp. indicated that the maghaemite synthesis is a complex multistep process, which begins with the reduction of ferric iron into ferrous iron by the action of ferrisiderophore reductase. Inhibition of ferrisiderophore reductase
activity by the addition of zinc ions completely stops the biosynthesis of maghaemite. Fe$^{2+}$ reduction is followed by the conversion of ferrous ions into iron oxyhydroxide. Conversion of ferric citrate into maghaemite by Actinobacter spp. was studied by FTIR spectroscopy as a function of time, which clearly indicated the presence of iron oxyhydroxide. In the last step of the reaction iron oxyhydroxide is dehydrated into maghaemite by the action of bacterial dehydratase.

**Part II. Synthesis of nanocrystalline magnetite by eukaryotic microorganism**

### 3.5 Fungi mediated biological synthesis of nanocrystalline magnetite (Fe$_3$O$_4$)

This part of the chapter describes biological synthesis of magnetite nanoparticles using eukaryotic microorganisms like fungi. Previous studies from this laboratory illustrated beautifully that, the interaction of metal ions with fungi like *Fusarium oxysporum* and *Verticillium* sp. can lead to the synthesis of metal nanoparticles by enzymatic pathway [48]. We have recognized an important advance in developing this strategy to encompass synthesis of oxide nanoparticles using fungi.

#### 3.5.1 Experimental details

Fungi such as *Fusarium oxysporum* and *Verticillium* sp. were maintained on potato-dextrose-agar (PDA) slants. Stock cultures were maintained by subculturing at monthly intervals. After growing at pH 7 and 27 °C for four days the slants were preserved at 15 °C. From an actively growing stock culture, subcultures of both the fungi were made on fresh slants and after four days of incubation at pH 7 and 27 °C were used as the starting materials for the experiments. Seed inoculums were made from the slants which further are inoculated in 500 ml Erlenmeyer flasks containing 100 ml MGYP medium. After adjusting the pH of the culture medium to 7, the cultures were grown under continuous shaking on a rotary shaker (200 rpm) at 27 °C for 96 hours. After 96 hours of growth, mycelia of the respective fungi were separated from the culture broth by centrifugation (5000 rpm) for 20 minutes at 20 °C and then the mycelia were washed thrice with distilled water under sterile conditions. 20 g (wet weight) of *F. oxysporum* and *Verticillium* sp. each was then resuspended in 100 ml aqueous solutions of K$_3$Fe(CN)$_6$ and K$_4$Fe(CN)$_6$ (pH 3.1) in a 2 : 1 molar ratio in 500 ml Erlenmeyer flasks and kept on a shaker (200 rpm) at 27 °C. The reaction was carried out for a period of 120 h. In a control
experiment, all the experimental conditions were kept similar, except that 20 g of each fungal biomass was separately exposed to sterilized deionised water instead of iron complex. The bio-transformed products were collected by separating the fungal mycelia from the aqueous extract by filtration under sterile conditions. Filtrate was characterized for the presence of magnetite nanoparticles by TEM, FTIR, XRD and magnetic measurements etc. M vs H curves and $\chi^{-1}$ vs temperature plots were obtained for Fe$_3$O$_4$ nanoparticles synthesized using *Fusarium oxysporum* and *Verticillium* sp. Magnetization data were collected on frozen colloidal solutions containing magnetite nanoparticles using a vibrating Sample Magnetometer (Oxford Instruments) with superconducting iron magnet generating an external magnetic field of 12 T. FC-ZFC measurements were performed by cooling the samples from 150 K to 2 K in the absence and presence of external magnetic field of 50 Oe respectively.

Biochemical analysis of magnetite formation by *Verticillium* sp. was carried out by analyzing extracellular proteins from *Verticillium* sp. with and without its exposure to the iron precursors. The extracellular extracts thus obtained from *Verticillium* sp. were lyophilized, dialyzed (12 kDa cut off) and their protein profiles were compared by 10% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) carried out at pH 8.2. In a separate experiment, the above two protein profiles were compared by native PAGE and the extra protein bands obtained in the presence of iron precursor ions were eluted from native gel. The hydrolytic activity of these eluted proteins was checked by TEM and SAED analysis. Hydrolysis of ferri/ferrocyanide complex by *Verticillium* sp. was analyzed by UV-vis spectroscopic analysis for the activity of cyanide hydratase.

### 3.5.2 Transmission electron microscopic analysis

Figure 3.19 shows the TEM images of magnetite nanoparticles synthesized using *F. oxysporum* after the reaction between fungal biomass and aqueous solution of ferri/ferrocyanide salts for 120 h. Representative TEM images recorded at different magnifications of magnetite nanoparticles from the *F. oxysporum* - iron precursor reaction medium after 120 h of reaction are shown in Figure 3.19A and B. The particles formed are irregular in shape presenting an overall quasi-spherical morphology. The particles range in size from 20–50 nm. SAED analysis of the individual particles...
indicated that they are crystalline; the diffraction pattern could be indexed on the basis of the magnetite ($\text{Fe}_3\text{O}_4$) structure (inset in Figure 3.19B).

High magnification of these images indicated that nanoparticles are imbedded in a matrix like structure, which could be a proteinacious material secreted by the fungus. Nanoparticles appear to be well separated from each other, since they are capped by the proteins present on the surface. Figure 3.19C and D shows TEM images of magnetite nanoparticles after calcined at 400 °C for 2 h indicating a little variation in morphology. Nanoparticles appeared to be well separated with little sintering and the overall dimension was found to be around 20-50 nm. However, the crystallinity of nanoparticles dramatically improved due to the heat treatment. This is reflected in the SAED pattern of the calcined nanoparticles, which can be indexed on the basis of magnetite structure quite radially [32].

![TEM micrographs of iron oxide nanoparticles synthesized by reaction between Fusarium oxysporum and iron cyanide precursors before (A & B) and after calcination at 400 °C for 3 h (C & D) The insets in B and C are SAED patterns recorded from the particles shown in the main images respectively.](image)

Figure 3.19 TEM micrographs of iron oxide nanoparticles synthesized by reaction between Fusarium oxysporum and iron cyanide precursors before (A & B) and after calcination at 400 °C for 3 h (C & D) The insets in B and C are SAED patterns recorded from the particles shown in the main images respectively.

Figure 3.20 shows TEM micrograph of magnetite nanoparticles obtained by the reaction of Verticillium sp. and iron cyanide precursors after 120 h. A number of cubo-octahedrally shaped particles ranging in size from 100–400 nm are observed (Figure 3.20)
A). Careful analysis of these images indicated that the cubic shape of the nanoparticles is actually an assembly of individual nanoparticles and proteins secreted by *Verticillium* sp. SAED analysis of the particles indicated the spot diffraction pattern that could be indexed on the basis of magnetite crystal structure of iron oxide (inset in Figure 3.20A) [32]. Figure 3.20C and D show the TEM images of magnetite nanoparticles after the calcination at 400 °C for 2 h. Unlike the magnetite nanoparticles synthesized by *F. oxysporum*, the iron oxide nanoparticles synthesized using *Verticillium* sp. undergo a detectable change in overall particle morphology and form large irregularly shaped aggregates (Figure 3.20C & D).

*Figure 3.20* TEM micrographs of iron oxide nanoparticles synthesized by reaction between *Verticillium* sp. and iron cyanide precursors before (A & B) and after calcination at 400 °C for 3 h (C & D) The insets in A and D are SAED patterns recorded from the particles shown in the main images respectively.

This supports our inference that the cubic structures seen in the as-prepared particles are indeed due to a loosely bound assembly of smaller magnetite particles held together due to the proteins secreted by the fungus. The SAED analysis of iron oxide nanoparticles synthesized using *Verticillium* sp. after the calcination shows that the particles still possess magnetite structure (inset in Figure 3.20D).
3.5.3 X-ray diffraction analysis

Figure 3.21 shows X-ray diffraction pattern of iron oxide nanoparticles synthesized by *F. oxysporum* and *Verticillium* sp. respectively. XRD pattern shows a number of Braggs reflections originated from solution cast film of reaction filtrate. These XRD patterns show excellent match with magnetite structure [32]. All the peaks marked as “o” corresponds to magnetite while peaks marked as “•” could be indexed on the basis of \( \gamma \)-Fe\(_2\)O\(_3\) structure of the iron oxide.

![XRD pattern](image)

**Figure 3.21** XRD patterns recorded from iron oxide nanoparticles synthesized by the reaction of iron cyanide precursors with *F. oxysporum* (curve 2 in grey) and *Verticillium* sp. (curve 1 in black) respectively. The peaks marked with “o” correspond to the Fe\(_3\)O\(_4\) and “•” to \( \gamma \)-Fe\(_2\)O\(_3\). The \( d \) values and the respective \( hkl \) planes are as follows: For magnetite marked as “o” 2.86 (220), 2.44 (311), 2.02 (400), 1.64 (422) 1.26 (622), 0.96 (642). For maghaemite (\( \gamma \)-Fe\(_2\)O\(_3\)) marked as “•” 3.23 (205), 2.20 (113) respectively [32].

3.5.4 FTIR spectral characterization

Figure 3.22 illustrates the FTIR spectra of iron oxide nanoparticles synthesized after 120 h of reaction between *F. oxysporum* and *Verticillium* sp. FTIR analysis of the nanoparticles from *Fusarium oxysporum* - iron precursor reaction medium showed the presence of prominent resonances at ca. 522, 568 and 627 cm\(^{-1}\) (curve 1, Figure 3.22) and are attributed to excitation of Fe-O stretching vibration [33]. Two absorption bands
centered at 1638 and 1540 cm\(^{-1}\) (amide I and II bands respectively; curve 1, Figure 3.22B) attest to the presence of proteins in the quasi-spherical magnetite particles. The FTIR spectrum recorded from iron oxide nanoparticles synthesized using \textit{Verticillium} sp. showed the presence of absorption bands centered around 522 cm\(^{-1}\) and 627 cm\(^{-1}\) (Figure 3. 22A, curve 2), which are characteristic of Fe-O-Fe vibrational modes. As observed earlier in case of biogenic iron oxide synthesized using \textit{Fusarium oxysporum}, the presence of proteins in the iron oxide nanoparticles synthesized using \textit{Verticillium} sp. is indicated by the amide I and II bands in the FTIR spectrum (curve 3, Figure 3. 22B).

\textbf{Figure 3.22}(A) FTIR spectra recorded from the powdered iron oxide synthesized by the reaction between iron cyanide complex and fungi \textit{F. oxysporum} (curve 1) and \textit{Verticillium} sp. (curve 2) respectively. A number of Fe-O absorption bands are observed which are due to the bending and stretching mode of Fe-O bonds. (B) Presence of amide bands suggests that proteins are associated with the nanoparticles. (curve 1-as synthesized magnetite by \textit{F. oxysporum}, curve 2-calcined magnetite synthesized by \textit{F. oxysporum}. curve 3- As synthesized magnetite \textit{Verticillium} sp, curve 4- calcined magnetite synthesized by \textit{Verticillium} sp.).

Calcination treatment of as synthesized iron oxide nanoparticles does not show the presence of amide bands (curve 2 and 4 in Figure 3.22B) indicating the association of proteins along with nanoparticles.

\textbf{3.5.5 Magnetic measurements}

Magnetic measurements were performed on the frozen colloidal solutions containing iron oxide nanoparticles synthesized by both fungi namely, \textit{F. oxysporum} and
Verticillium sp. respectively. Magnetization data from the particles synthesized using the two fungi are shown in Figure 3.23. The magnetization isotherm measurements (M vs H at fixed temperature) were carried out up to ±5 T at 2 k. There was no observation for the spontaneous magnetization in iron oxide nanoparticles synthesized by both the fungi. However, at low magnetic field M vs H data (Figure 3.23 A) shows sharp rise in the magnetization (M), particularly for magnetite particles synthesized using Verticillium sp. This possibly signifies the presence of interparticle dipolar interaction in the ensemble of nanoparticles [49].

Figure 3.23 Magnetization data collected on frozen colloidal solution containing magnetite nanoparticles synthesized by F. oxysporum and Verticillium sp. respectively. (A) M-H curve of magnetite nanoparticles showing initial magnetization as a function of applied external field at 2.1 k. Induced magnetization is higher in case of magnetite synthesized by Verticillium sp. (black curve) in comparison with magnetite synthesized by F. oxysporum (grey curve). (B) ZFC-FC plot for magnetite particles synthesized using Verticillium sp. at an external applied magnetic field of 50 Oe. (Black curve – FC plot, red curve-ZFC plot).

Figure 3.23B shows ZFC-and FC plots for the magnetite sample synthesized by Verticillium sp. ZFC-FC plots do not superimpose and show clear branching at a temperature of around 4k. Such behavior has been earlier observed in many ferrofluid systems [50]. On the other hand, the value of branching temperature obtained in our study is quite low compared to the earlier observation for 10 nm magnetite nanoparticles [48]. Thus, from the magnetization data it may be inferred that the correlated magnetic domains are much smaller than the 10 nm magnetite nanoparticles that form larger particles (~ 400 nm) as observed in TEM pictures (Figure 3.20B). For such a small magnetic domains, one expects very low moments for the system.
ZFC-FC plot for iron oxide nanoparticles synthesized using *Fusarium oxysporum* did not show any measurable branching. This may be due to the fact that the measurements were carried out at 0.05 T field, since the reliable data could not be collected with a low field of 0.005 T as used for particles synthesized using *Verticillium* sp. The application of higher fields can cause suppression of blocking phenomenon. The $\chi^{-1}$ vs T data for magnetite nanoparticles, synthesized by *F. oxysporum* clearly exhibit a sharp fall below 5 K, however for high temperature region the plot follows the Curie-Weiss law $\chi = C/(T-\theta)$ (dotted line, Figure 3.24 A) yielding a value of $\theta = -32.9$K. Curie-Weiss fit ($\chi^{-1} = (T-\theta)/C-b/(T-\theta)$) above the transition temperature $\theta_P$ gives the value of 1.0 K (solid line, Figure 3.24A). The corresponding values for particles obtained from *Verticillium* sp. are found to be -68.5 K and 3.0 K respectively (dotted and solid lines, Figure 3.24B respectively). The higher values of $\theta$ and $\theta_P$ obtained for *Verticillium* sp. implies stronger antiferromagnetic exchange interaction [51].

![Figure 3.24](image.png)

*Figure 3.24* $\chi^1$ vs T plots for Fe$_3$O$_4$ particles synthesized using fungi *Fusarium oxysporum* (A) and *Verticillium* sp. (B) respectively. Doted lines in figure indicate Currie–Weiss law, while solid lines are the fitted data. Note that the plot for the magnetite nanoparticles synthesized by *Verticillium* sp. shows ferromagnetic behavior.

This result is consistent with the fact that nanoparticles synthesized by this fungus are composed of much smaller particles. Thus, the magnetic measurements indicate the presence of interparticle interaction. M-H curves along with FC-ZFC measurements indicate that iron oxide nanoparticles synthesized by *Verticillium* sp. are superparamagnetic at low temperature with a signature of ferromagnetic transition.
3.5.6 Biochemical analysis

The fact that iron cyanide complexes are not toxic to the fungi indicates that fungi are capable of hydrolyzing these iron complexes subsequently converting them into iron oxides. Many microorganisms including fungi are known to degrade cyanide and metal cyanide complexes [52]. Fungi hydrolyze metal cyanide complex by the action of enzymes like cyanide hydratase or nitrilase, which hydrolyses metal cyanide bond, releasing the free metal moiety. It is also well known that fungi like F. oxysporum synthesize low molecular weight ferric iron chelating compound called as siderophores [19]. We believe that the biochemical mechanism of iron oxide formation in both the fungi under consideration is similar. However, there could be structurally different capping proteins, since we observed substantial difference in the morphology of iron oxide nanoparticle synthesized by F. oxysporum and Verticillium sp. respectively. We have considered magnetite formation by Verticillium sp. for biochemical characterization owing to the superior results that we obtained as comparison with F. oxysporum.

One of the easier ways to analyze the biochemical mechanism for the extracellular biosynthesis of nanoparticles is to study the protein profile of culture filtrate/supernatant of the fungal biomass incubated with and without precursor salts. To identify the fungal protein(s) responsible for hydrolysis of aqueous precursor complexes into magnetite particles, the extracellular extracts obtained from Verticillium sp. with and without its exposure to the iron precursors after 120 h of incubation were analyzed by 12% denaturing polyacrylamide gel electrophoresis (SDS-PAGE).

Exposure of Verticillium sp. to the anionic iron complexes resulted in the induction of the proteins with the molecular weights of ca. 13 kDa and 55 kDa (lane Ex, Figure 3.25A), which are absent in the fungal extract profile obtained in the absence of anionic iron complexes (lane C, Figure 3.25A). These two protein profiles, when compared on native PAGE, shows the similar results as observed in SDS-PAGE. When two induced proteins were eluted from the gel and checked for their activity, the formation of iron oxide from its precursors was observed (Figure 3.25B). TEM analysis and SAED pattern of these nanoparticles confirmed that they are magnetite (Figure 3.25B along with inset). We believe that the hydrolysis of iron cyanide complex is mediated by the action of an enzyme cyanide hydratase, which is synthesized by Verticillium sp.
extracellularly. The fact that exposure of iron cyanide complex to *Verticillium* sp. results in the induction of two proteins, which have the ability to synthesize iron oxide nanoparticles *in vitro* suggests that one of the inducible protein has cyanide hydratase activity.

![Figure 3.25](image)

**Figure 3.25** (A) Silver stained SDS-PAGE (Sodium dodecyl sulphate- polyacrylamide gel electrophoresis) data showing the extracellular protein profile of *Verticillium* sp. on 12% resolving gel. Lanes C and Ex correspond to the extracellular proteins from *Verticillium* sp. obtained by lyophilization of the fungal filtrate in the absence and presence of iron oxide precursors, respectively. Lane M corresponds to standard protein molecular weight markers with respective molecular weight. (B) TEM micrographs of iron oxide nanoparticles synthesized using two induced proteins fraction obtained from *Verticillium* sp. and eluted from native PAGE. Inset in image shows the SAED pattern. (C) UV-vis spectroscopic analysis of the hydrolysis of iron cyanide complex by *Verticillium* sp. Grey curve shows the absorption at 420 nm due to ferricyanide/ferrocyanide complex, while black curve shows the absorption hump at 330 nm, due to the presence of iron oxide.

It is quite possible that fungus can utilize cyanide as a source of carbon and nitrogen for its own metabolism under the starving conditions as discussed in section 3.3.6. Therefore based on the preliminary results it can be concluded that the exposure of iron cyanide to *Verticillium* sp. results in the induction of two new proteins.
Figure 3.25C shows the UV-vis spectral analysis of degradation of ferri/ferrocyanide reaction mixture and the formation of iron oxide nanoparticles by *Verticillium* sp. It is clearly observed that iron cyanide complex, which shows absorption at 420 nm (Grey curve, Figure 3.25C) is hydrolyzed due to the metal cyanide hydrolyzing enzyme secreted by *Verticillium* sp. Instead, an absorption hump at 330 nm is developed indicating the presence of iron oxide nanoparticles (black curve, Figure 3.25C). One of the proteins has cyanide hydratase activity while other may be involved in the conversion of released Fe ions into magnetite. It should be noted here also that after the incubation of the fungal biomass with iron salts pH of the reaction medium becomes slightly alkaline, which may help in the formation of magnetite. Though preliminary analysis of magnetite biosynthesis suggests that cyanide hydrolyzing enzyme cyanidate hydratase is involved in the biosynthesis of magnetite, it further entails detailed investigation of the process.

### 3.5.7 Discussion

This part of the chapter illustrates the use of eukaryotic microorganisms such as fungi for the synthesis of magnetite nanoparticles from anionic iron cyanide complexes. TEM analysis indicated that magnetite nanoparticles synthesized by using *F. oxysporum* were 20-50 nm in diameter. On the other hand TEM analysis of magnetite synthesized by *Verticillium* sp. indicated cubic superassembly with edge lengths between 100-400 nm, which is destroyed after calcination treatment indicating that is composed of smaller nanoparticles. X-ray analysis of magnetite nanoparticles indicated the presence of mixed phases of iron oxides, magnetite being the dominant one. Magnetic measurements suggest that magnetite nanoparticles synthesized by *Verticillium* sp. and *F. oxysporum* showed superparamagnetic and antiferromagnetic exchange interactions at low temperature. Biochemical analysis of magnetite formation using *Verticillium* sp. indicates that exposure of iron cyanide complex to *Verticillium* sp. induces the synthesis of two new proteins with molecular weights of 55 kDa and 13 kDa. One of these proteins harbored cyanide hydratase activity, which degrades metal cyanide complex as indicated by UV-vis spectroscopic analysis of the reaction mixture. Further in-vitro reaction of inducible proteins and iron precursors resulted in the magnetite formation.
3.6 Conclusions

This chapter describes the biological synthesis of iron oxide nanoparticles by using different microorganisms. A bacterium isolated from our lab, which was latter identified as Actinobacter spp. was explored for the biological synthesis of iron oxides like magnetite (Fe$_3$O$_4$) and maghaemite ($\gamma$- Fe$_2$O$_3$). It has been conclusively shown that Actinobacter spp. synthesizes different oxides of iron when reacted with the different iron precursors. The reaction with aqueous K$_3$Fe(CN)$_6$ and K$_4$Fe(CN)$_6$ mixture in 2:1 M ratio resulted in the synthesis of nanocrystalline magnetite, while exposure to 1mM ferric citrate could yield maghaemite nanoparticles. The TEM analyses of magnetite nanoparticles indicated the overall dimensions of 10-20 nm, whereas maghaemite nanoparticles were found to be 5-7 nm in dimensions. Magnetic measurements performed on both the samples showed superparamagnetic nature of nanoparticles. Biochemical analysis of magnetite nanoparticle synthesis indicated the implication of the enzyme cyanidase, a metal cyanide hydrolyzing enzyme. Extracellular protein analysis showed the presence of two new proteins with molecular weight of 100 kDa and 70 kDa in the culture supernatant of the bacteria exposed to iron cyanide complexes. Maghaemite formation involved iron reduction, followed by hydroxylation and dehydration. Ferrisiderophore reductase synthesized by Actinobacter spp. is found to be responsible for Fe$^{3+}$ reduction. Inhibition of its activity blocked the synthesis of maghaemite nanoparticles. Induction of a 55 kDa protein is found to occur extracellularly after the exposure of Actinobacter spp. to iron salt. Four different low molecular weight proteins were isolated from the surface of maghaemite nanoparticle with the molecular weights of 15, 16, 20, 23 kDa respectively. Fungi such as F. oxysporum and Verticillium sp. are also found to yield magnetite nanoparticles when challenged with aqueous solution of K$_3$Fe(CN)$_6$ and K$_4$Fe(CN)$_6$. Magnetite nanoparticles 20-40 nm in diameter were synthesized by F. oxysporum. On the other hand cubical super assembly of smaller magnetite nanoparticles with edge length of 100-400 nm is resulted using Verticillium sp. with superparamagnetic and antiferromagnetic exchange interqctions. Biochemical analysis of magnetite formation in Verticillium sp. shows the involvement of the enzyme cyanide hydratase. However involvement of other proteins can not be ruled out at this point of research.
3.7 References


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