CHAPTER 6

General Discussion and Conclusions.
Summary

This chapter presents the major outcome of the thesis and gives the general conclusions of the extracted results from the work done in this particular thesis. It also emphasizes on the potential scope for future work required to be done in this field.
6.1 Summary of the work

The work done in this thesis mainly focuses on the biological synthesis of oxide nanoparticles using microorganisms. The details of the work have been described in different chapters and the summary for each chapter with their conclusions has been described here. This thesis mainly revolves around three microorganisms: i) thermophilic fungus *Humicola* sp. ii) mesophilic fungus *Fusarium oxysporum* and iii) alkalothermophilic actinomycete *Thermomonospora* sp. The reasons why each of the above mentioned microorganisms are used for the synthesis of specific inorganic nanomaterials are as follows:

i) We have screened a number of mesophilic, thermophilic, alkalophilic and alkalothermophilic microorganisms in order to synthesize oxide nanoparticles of medical importance such as gadolinium oxide and cerium oxide nanoparticles at different pH and temperatures. Out of the several microorganisms screened, only thermophilic fungus *Humicola* sp. produces protein capped water dispersible extracellular gadolinium and cerium oxide nanoparticles at just 50°C.

ii) Our group has already shown *Fusarium oxysporum*-based bioleaching approach towards the room temperature synthesis of oxide nanoparticles using cheap naturally available raw materials (white sand and zircon sand) as well as agro-industrial by-products (rice husk). This led us into thinking towards the possibility of extraction of protein capped nanoparticles such as SiO$_2$ from fly-ash.

iii) We had already reported the extracellular biosynthesis of monodispersed gold nanoparticles from the whole cells of a novel extremophilic actinomycete, *Thermomonospora* sp. In order to know the exact mechanism of synthesis of monodispersed gold nanoparticles, we decided on further investigating this extremophilic actinomycete.

In this thesis, we have shown that the fungus *Humicola* sp. can be used for the extracellular biosynthesis of lanthanide nanoparticles such as gadolinium and cerium. The choice for these metals may be justified by the fact that these metals started gaining their importance in biomedical applications but their biosynthetic routes have not been explored yet. We have shown that when the aqueous solutions of oxide precursors such as Gadolinium chloride (GdCl$_3$) and Cerium (III) nitrate hexahydrate (CeN$_3$O$_9$.6H$_2$O) were exposed to fungus *Humicola* sp., the fungus was able to synthesize oxide nanoparticles of gadolinium and cerium respectively. Transmission
electron microscopic analysis confirmed that Gd$_2$O$_3$ nanoparticles are in the range of 3-8 nm with an average size of 6 nm, whereas CeO$_2$ nanoparticles are in the range of 12-20 nm with 16 nm as average diameter. Surface characterization techniques such as X-ray diffraction and X-ray photoemission spectroscopy also confirm the synthesis of Gd$_2$O$_3$ and CeO$_2$ nanoparticles with the aforementioned sizes. The highly fluorescent protein capped Gd$_2$O$_3$ nanoparticles were radiolabelled with Technicium-99m and injected into rats in order to see biodistribution. These nanoparticles reach out to the liver and kidneys and pass through urine very fast. Since these nanoparticles reach out to the liver, they can be used for targeted drug delivery for liver cancer. In order to avoid any side effects of taxol, we conjugated taxol with protein capped Gd$_2$O$_3$ nanoparticles with the help of EDC coupling protocol. To achieve proper conjugation, taxol was first derivatized and modified to 1, 6 hexane diamine taxol and electrostatically conjugated to free carboxyl groups of capping peptide involved in the capping of the Gd$_2$O$_3$ nanoparticles using EDAC coupling reaction. The conjugation was then confirmed with the help of UV-vis spectroscopy and flourimetric analyses. Unbound drug molecules were removed by HPLC which finally gave the purified Gd$_2$O$_3$-taxol conjugate. Cytotoxicity of Gd-taxol conjugate was checked on THP-1 cell lines (cancer cell lines) to access its potential in the treatment of cancer. IC$_{50}$ values of taxol and Gd-taxol conjugate showed that Gd-taxol conjugate is more effective in killing cancer cells than taxol alone. It can be concluded that the conjugation of taxol to Gd$_2$O$_3$ nanoparticles enhances its hydrophilicity so that the drug can easily penetrate deep inside the cells with the help of endocytosis, phagocytosis or other receptor mediated internalization processes. We believe this work could open new doors for nanosized drug delivery applications in cheap treatment of cancers.

In an attempt to obtain oxide nanoparticles purely through eco-friendly routes and negate the requirement of chemical precursors, we have extended the concept of fungal bioleaching from agro-based by-products to waste material such as fly-ash. We have shown that the fungus *Fusarium oxysporum* when exposed to fly-ash was able to leach crystalline silica nanoparticles extracellularly in solution. Hence, a cheap and environment friendly approach has been derived to obtain commercially important oxide (silica) nanoparticles out of waste materials using the fungus.
In a very novel discovery of its kind, we have shown that the fungus *Humicola* sp. can be used for the biotransformation of shape, size and phase of bulk anatase type TiO$_2$ particles. When we treated the fungus *Humicola* sp. with disc shaped micron size anatase type TiO$_2$ particles, to our pleasant surprise we found that the fungus could successfully transform the disc shaped micron size anatase type TiO$_2$ particles into circular brookite type TiO$_2$ nanoparticles. It is to be noted here that the phase transformation of TiO$_2$ is an extremely difficult process and requires very high temperature to occur successfully. However, we were able to achieve this task at just 50°C.

Mechanistic aspect of formation of nanomaterials through biological route which is still seldom encountered in literature has been discussed in details in this thesis. We have purified an NADPH dependent sulphite reductase enzyme and capping protein from *Thermomonospora* sp. to synthesize gold nanoparticles. We chose gold because one can easily track the formation of gold nanoparticles synthesis through visual inspection and could further confirm it by UV-vis spectroscopy which is rather difficult in case of oxide nanoparticles. When we incubated the purified sulphite reductase enzyme along with NADPH, capping protein, HAuCl$_4$ and sodium sulphite, the enzyme could synthesize gold nanoparticles within just 4h of reaction. The electrons required for the reaction were provided by NADPH. Transmission electron microscopic (TEM) analysis showed that the particles were almost monodispersed and were in the range of 2-4nm. Capping protein which was also added in the reaction mixture provided the required stability and prevented aggregation of nanoparticles in solution. The role of capping protein in providing stability was further confirmed when the same reaction mixture was incubated without capping protein and when observed under TEM, the gold nanoparticles were found to be agglomerated and tend to cluster. Here, we have shown that by purifying the biomolecules and capping entities associated with the synthesis and capping of nanomaterials, one can synthesize nanoparticles *in vitro* with desired size.

### 6.2 Scope for future work

Biological synthesis of oxide nanomaterials using microorganisms is still a new field and the oxide nanoparticles synthesized in this thesis were chosen according to the growing applications of these materials especially in biology. For example,
gadolinium finds its application in magnetic resonance imaging technique and cerium oxide nanoparticles are being used in various biomedical applications such as wound healing, inhibition of cellular ageing, treatment of neurodegenerative diseases, etc. However, there is still a long way to go when these biosynthesized oxide materials start replacing conventional chemically synthesized materials. The foremost question which needs to be answered is the scale-up method of biosynthesized products. The underlying mechanism involving the formation of oxide nanoparticles needs to be completely worked out in order to synthesize tailor made nanomaterials and gain access to control over shape and size. Complete biosynthetic pathways needs to be elucidated and a series of enzymes and proteins responsible for the synthesis need to be purified and can be genetically engineered in some other sources to maximize the production of desired valuable products. Time dependent kinetics need to be carried out for maximum production of nanomaterials. Selection of the type of microorganisms for obtaining desired nanomaterials needs to be done. One can broaden the vision on fungal bioleaching and look for waste materials to obtain valuable products by employing microorganisms. However, certain protocols need to be standardized in order to get maximum output in lesser time. Pharmacokinetic and pharmacodynamic studies in case of conjugation of nanoparticles to drug need to be performed. Quantitative studies of drug release kinetics inside the cancer cells will help us in designing better strategies for conjugation of nanomaterials to different anticancer drugs.

We have already made significant progress towards biological synthesis of inorganic nanoparticles of different sizes, shapes and chemical compositions along with complete characterization of nanomaterials and synthesis of nanoparticle-drug conjugates. Since nanomaterials which we are making from microbial routes are capped with natural proteins and are water dispersible, they may bind to cell adhesion molecules (CAMs) like integrins or VEGFs (vascular endothelial growth factors). Therefore, targeting CAMs such as integrins and VEGFs is a novel anti–angiogenesis strategy for targeting of solid tumor. The nanomaterials which we are synthesizing using fungal routes may also bind to various receptors such as LHRH (Leutinizing hormone releasing hormone), EGFR (Epidermal growth factor receptor) and EpCAM (Epithelial cell adhesion molecule) without a targeting agent. Hence, these nanoparticles which are until now considered only as delivery agents in targeted drug
delivery treatment, can also be taken into consideration to be used directly as a drug in future; with no side-effects and much cheaper than the ones currently being used.