Bioleaching—an approach for synthesizing functionalized nanoformulations for agricultural use

by

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M.Sc.

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

Deakin University

April, 2016
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Dedicated to the Almighty, my family and loving husband........
Acknowledgement

“I'm a success today because I had a friend who believed in me and I didn't have the heart to let him down.”

— Abraham Lincoln

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viii
# Table of Contents

List of conferences attended ................................................................. viii  
List of publications in preparation ........................................................ viii  
List of Tables ...................................................................................... xii  
List of Figures .................................................................................... xiii  
List of abbreviations .......................................................................... xviii  
Abstract ......................................................................................... xxiii  

## Chapter 1: Introduction and Literature review  
1.1 Introduction ................................................................................... 2  
1.2 Essential nutrients and natural toxicity in Agriculture and Food safety .......... 3  
  1.2.1 Nutrient Deficiencies ................................................................. 4  
    1.2.1.1 Zinc Deficiency .................................................................. 4  
    1.2.1.2 Iron Deficiency ................................................................. 6  
  1.2.2 Understanding waste as a resource ........................................... 8  
    1.2.2.1 Jarosite .............................................................................. 12  
    1.2.2.2 Iron Ore Tailings ............................................................... 14  
1.3 Treatment of Waste ........................................................................ 15  
  1.3.1 Bioleaching of Zinc ................................................................. 19  
  1.3.2 Bioleaching of Iron ................................................................. 23  
1.4 Biosynthesis of Nanoparticles ......................................................... 25  
  1.4.1 Zinc Nanofactories ................................................................. 26  
  1.4.2 Iron Nanofactories ................................................................. 28  
1.5 Application of Zn and Fe nanoparticles as nanonutrients ....................... 31  
1.6 Conclusion .................................................................................... 35  
1.7 Aims of the study .......................................................................... 36

## Chapter 2: Isolation, screening, selection and characterization of fungal isolates for the biosynthesis of nanoparticles from jarosite and iron ore tailings  
2.1 Introduction ................................................................................... 39  
2.2 Materials and Methods ................................................................. 42  
  2.2.1 Chemicals used ....................................................................... 42  
  2.2.2 Sampling site .......................................................................... 42  
  2.2.3 Elemental analysis and particle size imaging of Jarosite and Iron Ore Tailings ......................................................................................... 43  
  2.2.4 Isolation and purification of fungal strains .................................. 45  
  2.2.5 Bioleaching screening of fungi ................................................ 45
Chapter 3: Bioleaching and biosynthesis of nanoparticles from Jarosite and Iron ore tailings

3.1 Introduction ........................................................................................................ 69

3.2 Materials and Methods ....................................................................................... 71
  3.2.1 Chemicals used ............................................................................................ 71
  3.2.2 Fungal growth kinetics study through Ergosterol estimation ...................... 71
  3.2.3 Microorganism and growth ......................................................................... 72
  3.2.4 Optimization of bioleaching and biosynthesis of nanoparticles using fungal cell-free extract ......................................................................................... 72
    3.2.4.1 Effect of reaction time on the bioleaching and subsequent biosynthesis of nanoparticles .......................................................... 73
    3.2.4.2 Effect of different concentrations of cell-free extract and substrate i.e. jarosite/iron ore tailings along with change in shaker speed......................... 73
  3.2.5 Bioleaching and biosynthesis of nanoparticles using fungal cell-free extract from jarosite and iron ore tailings ........................................... 74
    3.2.5.1 UV-Vis Spectroscopy ........................................................................... 74
    3.2.5.2 Fourier transform infrared spectroscopy (FTIR).................................... 75
    3.2.5.3 Determination of zeta-potential ............................................................ 75
    3.2.5.4 TEM, HRTEM, EDX and XRD analysis ............................................. 75
  3.3 Results and Discussion ....................................................................................... 76
    3.3.1 Fungal growth kinetics study ...................................................................... 76

Chapter 2: Bioleaching and biosynthesis of nanoparticles using fungal cell-free extract

2.2.5.1 Microorganism and growth .................................................................. 46
  2.2.5.2 Bioleaching and biosynthesis of nanoparticles ........................................ 46
  2.2.5.3 Characterization technique using TEM and EDX ..................................... 46
  2.2.6 Characterization of fungi ............................................................................. 47
    2.2.6.1 Scanning electron microscopy (SEM) .................................................. 47
    2.2.6.2 Molecular characterization of fungi ..................................................... 48
  2.3 Results and Discussion ....................................................................................... 52
    2.3.1 Elemental analysis and particle size imaging of jarosite and iron ore tailings .......................................................... 52
    2.3.2 Isolation of fungi ......................................................................................... 54
    2.3.3 Bioleaching and biosynthesis of nanoparticles ........................................... 56
      2.3.3.1 Jarosite .................................................................................................. 56
      2.3.3.2 Iron ore tailings .................................................................................... 59
    2.3.4 Characterization of fungi ............................................................................. 62
      2.3.4.1 Morphological characterization through SEM ..................................... 62
      2.3.4.2 Molecular characterization of fungal isolates ...................................... 64
  2.4 Conclusion .......................................................................................................... 67

Chapter 3: Bioleaching and biosynthesis of nanoparticles from Jarosite and Iron ore tailings
3.3.2 Optimization of bioleaching and biosynthesis of nanoparticles using fungal cell-free extracts ................................................................. 79

3.3.3 Bioleaching and biosynthesis of nanoparticles from jarosite and iron ore tailings .................................................................................................. 81

3.3.3.1 Visual Observations ............................................................................. 81

3.3.3.2 UV-Vis Spectroscopy ........................................................................... 82

3.3.3.3 FTIR ..................................................................................................... 83

3.3.3.4 Zeta potential ........................................................................................ 84

3.3.3.5 TEM, HRTEM, EDX and XRD analysis ............................................. 86

3.4 Conclusion .......................................................................................................... 89

Chapter 4: Application of biosynthesized nanoparticles as plant nanonutrients ................................................................. 91

4.1 Introduction ........................................................................................................ 92

4.2 Materials and method ........................................................................................ 94

4.2.1 Chemicals used ............................................................................................ 94

4.2.2 Surface modification of biosynthesized nanoparticles (synthesized by A. terreus strain J4 cell-free extract) using polymers ........................................ 94

4.2.2.1 Determination of Zeta potential ........................................................... 96

4.2.2.2 TEM and EDX analysis ........................................................................ 96

4.2.3 Application of nanoparticles as nanonutrients ............................................ 97

4.2.3.1 Surface sterilization of seeds ................................................................ 97

4.2.3.2 Preparation of seeds with treatments .................................................... 97

4.2.3.3 Seed germination study of biosynthesized nanoparticles ..................... 98

4.2.3.4 In vitro nutrient assimilation studies .................................................... 98

4.3 Results and Discussion ..................................................................................... 101

4.3.1 Surface modification of biosynthesized nanoparticles ................................ 101

4.3.2 Evaluation of seed germination using varying treatments ....................... 103

4.3.3 In vitro nutrient use efficiency of nanostructured jarosite ........................ 105

4.3.3.1 Growth parameters ............................................................................. 105

4.3.3.2 Confocal Microscopy ......................................................................... 109

4.4 Conclusion ........................................................................................................ 113

Chapter 5: Summary and Future Directions .................................................... 114

References ............................................................................................................ 119
List of Tables

Chapter 1:

Table 1.1 Crops susceptible to Zinc deficiency.................................6
Table 1.2 Iron deficient crops..........................................................7
Table 1.3 World mine production of Zinc and Iron............................12
Table 1.4 Nanoparticles and their Microbial Nanofactories...............30

Chapter 2:

Table 2.1 Chemical analysis of Jarosite for metal content..................53
Table 2.2 Chemical analysis of Tailings for metal content....................54

Chapter 3:

Table 3.1 Effect of different parameters on the bioreaching and biosynthesis of nanoparticles from jarosite and iron ore tailings using fungal cell-free extract.........................................................80

Chapter 4:

Table 4.1 Preparation of working solutions of surface modified biosynthesized nanoparticles for seed treatments..........................97
Table 4.2 Effect of surface modified biosynthesized nanoparticles on wheat seed germination.........................................................104
List of Figures

Chapter 1:

Figure 1.1 Global scenario of Zinc deficiency………………………………………5
Figure 1.2 Process of waste generation according to European Union……………..8
Figure 1.3 Types of solid waste described by Environmental Protection Department and World Bank………………………………………………………………………………9
Figure 1.4 Global quantum of waste generation per annum……………………………10

Chapter 2:

Figure 2.1 Working of Zinc extraction plant……………………………………………40
Figure 2.2 Mining process and generation of waste……………………………………40
Figure 2.3 Jarosite sample collection site (24°35'58"N 73°49'8"E)………………………43
Figure 2.4 Iron ore tailings collection site (15°20'28"N 74°8'8"E)……………………..43
Figure 2.5 Transmission electron micrograph and EDX spectrum of controls (A&B) Jarosite particles with an average size of 400nm & (C & D) Iron ore tailings with an average size of around 500nm and more………54
Figure 2.6 Colony morphology of 5-days old pure fungal isolates from Jarosite on PDA plates at 28±20C…………………………………………………………………………………55
Figure 2.7 Colony morphology of 5-days old pure fungal isolates from Iron ore tailings on PDA plates at 28±200C…………………………………………………………………………………56
Figure 2.8 Graph indicating the bioleaching efficiency of the fungal isolates from jarosite…………………………………………………………………………………………………………………………..57
Figure 2.9 TEM micrographs and their respective EDX spectra of bioleachate indicating the presence or absence of zinc bioleaching and biosynthesis of nanoparticles from Jarosite by J1-J3 fungal isolates……………….58
Figure 2.10 TEM micrographs and their respective EDX spectra of bioleachate indicating the presence or absence of zinc bioleaching and biosynthesis of nanoparticles from Jarosite by J4-J5 fungal isolates……………59
Figure 2.11 Graph indicating the bioleaching efficiency of the fungal isolates from tailings………………………………………………………………………………………………………………………………………………..60
Figure 2.12 TEM micrographs and their respective EDX spectra of bioleachate (T1-T3 fungal isolates) indicating the presence or absence of iron bioleaching and biosynthesis of nanoparticles from tailings. 61

Figure 2.13 TEM micrographs and their respective EDX spectra of bioleachate indicating the presence or absence of iron bioleaching and biosynthesis of nanoparticles from tailings by T4-T6 fungal isolates. 62

Figure 2.14 (A) Colony morphology on PDA plate; SEM images showing structural morphology of fungal mycelia (B) and spores (C) of \textit{A. flavus} strain J2. 63

Figure 2.15 (A) Colony morphology on PDA plate; SEM images showing structural morphology of fungal mycelia (B) and spores (C) of \textit{A. terreus} strain J4. 64

Figure 2.16 (A) Colony morphology on PDA plate; SEM images showing structural morphology of fungal mycelia (B) and spores (C) of \textit{A. nomius} strain T4. 64

Figure 2.17 (A) Colony morphology on PDA plate; SEM images showing structural morphology of fungal mycelia (B) and spores (C) of \textit{A. aculeatus} strain T6. 64

Figure 2.18 Agarose gel electrophoresis of the plasmid digested samples using EcoR1. Lane 1 denotes ladder (100 bp to 1000 bp); Lane 2 to 5 – J2; Lane 12 to 16 - T4; Lane 19 to 22 – J4 and Lane 24 to 27 – T6. 65

Figure 2.19 Phylogenetic relationship of the 18s rRNA sequences of fungal isolates from Jarosite based on their similarity to closely related sequences. 66

Figure 2.20 Phylogenetic relationship of the 18s rRNA sequences of fungal isolates from tailings based on their similarity to closely related sequences. 66

\textbf{Chapter 3:}

Figure 3.1 (A) Standard ergosterol graph; (B & C) Growth kinetics study of \textit{A. flavus} strain J2 and \textit{A. terreus} strain J4, respectively, by Ergosterol assay. 77

Figure 3.2 (A) Standard ergosterol graph; (B & C) Growth kinetics study of \textit{A. nomius} strain T4 and \textit{A. aculeatus} strain T6, respectively, by Ergosterol assay. 78
Figure 3.3 pH of MilliQ with washed biomass recorded at various time intervals to confirm the release of organic acids.................................80

Figure 3.4 Protocol for bioleaching and biosynthesis of nanoparticles from Jarosite................................................................................81

Figure 3.5 Biosynthesized nanoparticles from (A) Jarosite-colour changed from white yellow to brick red, and (B) Iron ore tailings- colour shifted to a darker tone.................................................................82

Figure 3.6 UV-spectra of biosynthesized nanoparticles using cell-free extract of (A, B) *A. flavus* strain J2 and *A. terreus* strain J4; (C, D) *A. nomius* strain T4 and *A. aculateus* strain T6 from jarosite and iron ore tailings, respectively.................................................................83

Figure 3.7 FTIR spectrum of bio-synthesized nanoparticles using fungal cell-free extract (A) *A. flavus* strain J2; (B) *A. terreus* strain J4; (C) *A. nomius* strain T4 and (D) *A. aculateus* strain T6, showing different peaks representing a range of functional groups......................................................84

Figure 3.8 Zeta potential of nanoparticles biosynthesized from jarosite using cell-free extract of (A) *A. flavus* strain J2; (B) *A. terreus* strain J4.............85

Figure 3.9 Zeta potential of nanoparticles biosynthesized from iron ore tailings using cell-free extract of (A) *A. nomius* strain T4 ; (B) *A. aculateus* strain T6........................................................................................................86

Figure 3.10 (A, D) TEM images; (B, E) EDAX based elemental composition; and HRTEM images (C, F) at scale of 5nm and 10nm showing lattice fringes indicating crystalline nature of biosynthesized nanoparticles from jarosite using cell-free extract of *A. flavus* strain J2 and *A. terreus* strain J4, respectively.................................................................88

Figure 3.11 (A, D) TEM images; (B, E) EDAX based elemental composition; and HRTEM images (C, F) at scale of 5nm and 10nm showing lattice fringes indicating crystalline nature of biosynthesized nanoparticles from iron ore tailings using cell-free extract of *A. nomius* strain T4 and *A. aculateus* strain T6, respectively......................................................89
Chapter 4:

Figure 4.1 Zeta potential graph showing the surface modification of negatively charged biosynthesized nanoparticles using Atlox Semkote E-135……96

Figure 4.2 Treated seeds kept on 0.8 % water agar supplemented with Hoagland solution……………………………………………………………………………………………………..99

Figure 4.3 Zeta potential of (A) nanoparticles biosynthesized from jarosite using cell-free extract of A. terreus strain J4, (B) PLL as control; (C) PLL-capped nanoparticles showing surface modification………………102

Figure 4.4 (A) TEM image; (B) EDAX based elemental composition of PLL-capped biosynthesized nanoparticles from jarosite using cell-free extract of A. terreus strain J4, respectively…………………………………………………103

Figure 4.5 (A) Different treatments of seeds; (B) effect as observed on seed germination…………………………………………………………………………………………………104

Figure 4.6 Effect of respective treatments on wheat plant growth (A) Control; (B) Raw jarosite as control; (C) PLL as control; (D) Bulk Zn-Fe as control; (E) 10 ppm nanoparticles; (F) 20 ppm nanoparticles; (G) 30 ppm nanoparticles; (H) 40 ppm nanoparticles and (I) 50 ppm nanoparticles………………………………………………………………………………………………………………………..106

Figure 4.7 Effect of surface modified biosynthesized nanoparticles on root and shoot length of wheat………………………………………………………………………………107

Figure 4.8 Effect of surface modified biosynthesized nanoparticles on fresh and dry weight of roots…………………………………………………………………………108

Figure 4.9 Effect of surface modified biosynthesized nanoparticles on fresh and dry weight of shoots…………………………………………………………………….109

Figure 4.10 Control stained roots (A) and leaves (B) respectively…………………110

Figure 4.11 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 10ppm nanoparticles…………………………………………………………………110

Figure 4.12 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 20ppm nanoparticles………………………………………………………………110

Figure 4.13 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 30ppm nanoparticles………………………………………………………………110

Figure 4.14 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 40ppm nanoparticles………………………………………………………………111
Figure 4.15 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 50ppm nanoparticles ................................................................. 111
Figure 4.16 Control stained roots (A) and leaves (B) respectively .................. 111
Figure 4.17 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 10ppm nanoparticles ................................................................. 111
Figure 4.18 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 20ppm nanoparticles ................................................................. 112
Figure 4.19 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 30ppm nanoparticles ................................................................. 112
Figure 4.20 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 40ppm nanoparticles ................................................................. 112
Figure 4.21 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 50ppm nanoparticles ................................................................. 112
<table>
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Cu  - Copper
CuFeS₂ - Chalcopyrite
DMSO - Dimethyl sulfoxide
DNA - deoxyribonucleic acid
dNTP - deoxyribonucleoside triphosphate
DTPA - diethylenetriamine penta-acetic acid
EDTA - Ethylenediaminetetraacetic acid
EDX - Energy dispersive X-ray spectrum
ENP - Engineered nanoparticles
EPA - Environmental Protection Agency
epd - Environmental Protection Department
FAAS - Flame Atomic Absorption Spectroscopy
Fe  - Iron
Fe₂O₃ - Haematite
Fe₂O₄ - Magnetite
Fe₃S₄ - Greigite
FeCO₃ - Siderite
FeS₂ - Pyrite
FeSO₄ - Iron sulphate
FeSO₄.7H₂O - Iron sulphate heptahydrate
FP - Forward primer
FTIR - Fourier Transform Infrared Spectroscopy
g L⁻¹ - Grams per litre
g - Grams
H₂SeO₃ - Selenious acid
H₂SO₄ - Sulphuric acid
HCl - Hydrochloric acid
HgCl₂ - Mercuric chloride
HNO₃ - Nitric acid
HPLC - High Pressure Liquid Chromatography
hrs - Hours
HRTEM - High Resolution TEM
HZL - Hindustan Zinc Limited
i.e. - that is
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<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>Mn</td>
<td>Manganese</td>
</tr>
<tr>
<td>Mn₂O₃</td>
<td>Manganese (III) oxide</td>
</tr>
<tr>
<td>Mn₃O₄</td>
<td>Manganese (II, III) oxide</td>
</tr>
<tr>
<td>MQ</td>
<td>Milli Q</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MSW</td>
<td>Municipal Solid Waste</td>
</tr>
<tr>
<td>MT</td>
<td>Million tonnes</td>
</tr>
<tr>
<td>mV</td>
<td>Milli Volt</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotubes</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NERC</td>
<td>National Environment Research Council</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>Ni</td>
<td>Nickel</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>Pb</td>
<td>Lead</td>
</tr>
<tr>
<td>PbSO₄</td>
<td>Lead sulphate</td>
</tr>
<tr>
<td>PCBs</td>
<td>Printed Circuit Boards</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PCs</td>
<td>Personal Computers</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato dextrose agar</td>
</tr>
<tr>
<td>PDB</td>
<td>Potato dextrose broth</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly L-Lysine</td>
</tr>
<tr>
<td>pM</td>
<td>Picomolar</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>R²</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>RLE</td>
<td>Roast Leach Electrolysis</td>
</tr>
<tr>
<td>RLs</td>
<td>Rhamnolipids</td>
</tr>
<tr>
<td>RNase A</td>
<td>Ribonuclease A</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Se</td>
<td>Selenium</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimal Broth with glucose for Catabolite repression</td>
</tr>
<tr>
<td>sp.</td>
<td>Species</td>
</tr>
<tr>
<td>StEP</td>
<td>Solving the E-waste Problem</td>
</tr>
<tr>
<td>TEA</td>
<td>triethanolamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>U</td>
<td>Enzyme Unit</td>
</tr>
<tr>
<td>U.K.</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>U.P.</td>
<td>Uttar Pradesh</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>UV-Visible</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume by volume</td>
</tr>
<tr>
<td>w.r.t.</td>
<td>with respect to</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight by volume</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight by weight</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>XRD</td>
<td>X-Ray Diffraction</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
<tr>
<td>ZnO</td>
<td>Zinc oxide</td>
</tr>
<tr>
<td>ZnS</td>
<td>Zinc sulphide</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>Zinc sulphate</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>Zinc sulphate heptahydrate</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>γ-Fe₂O₃</td>
<td>Maghemite</td>
</tr>
</tbody>
</table>
Abstract

Solid waste such as jarosite from hydrometallurgical metallic zinc extraction processes of the zinc industry and iron ore tailings from iron mines are known to be potentially hazardous due to the presence and mobility of ecologically toxic heavy metals into the environment. In order to avoid the environmental problems caused by leaching of heavy metals from these wastes, researchers are developing bioremediation methods and technologies. In the last two decades, various waste management strategies have been developed for the disposal of this waste, such as the development of landfill and utilization of this waste for construction and ceramic materials. However, these management strategies are non-renewable and non-sustainable besides being economically non-feasible. Such deficiencies motivated us to develop an alternative that is an eco-friendly and may be economically viable waste management approach. These two wastes contain plant nutritive metal elements including Fe, Zn, Cu, Al and Si in sufficient quantities to make their extraction attractive. These nutrients are in demand as plant nutrients, have and are being used in a variety of agricultural fertilizer applications. We developed a novel biological approach called ‘myco-nano-mining’ which involved fungal secretome (cell-free extract) mediated bioleaching and conversion of bulk metallic elements into the nanostructured materials.

Jarosite was collected from Debari Zinc Smelter plant, Hindustan Zinc Limited in Udaipur, Rajasthan and iron ore tailings from Codli mines, Goa, India. The first objective of the study was to develop complete elemental and structural profile of the two waste materials using AAS, TEM and EDX analysis. It was concluded that jarosite contained zinc (~34,000 ppm), iron (~38,000 ppm), sulphur (~11,000 ppm)
and lead (~14,000 ppm) along with trace elements like copper and aluminium. The most abundant metals present in iron ore tailings were iron (~42,000 ppm) and aluminium (~34,000 ppm). TEM analysis showed that the particles were big agglomerations having an average size of ~400 nm for jarosite and ~500 nm for iron ore tailings. Among the five fungal isolates obtained based on the philosophy “solution lies where the problem is” from jarosite (J1-J5) and six from tailings (T1-T6) using culture enrichment technique, J2 (A. flavus strain), J4 (A. terreus strain) and T4 (A. nomius strain) and T6 (A. aculateus strain) showed maximum bioleaching and subsequent biosynthesis of zinc or iron nanoparticles. Morphological characterization of these four promising fungal isolates using SEM and molecular characterization using 18S rRNA gene sequence analysis was carried out.

The second study aimed at optimizing bioleaching and the biosynthesis of nanoparticles using the cell-free extracts from these isolates (A. flavus strain J2, A. terreus strain J4, A. nomius strain T4 and A. aculateus strain T6). Optimizing the concentration of substrate and cell-free extract, and the pH and time of reactions, it was concluded that 10 g of substrate with 100 mL of cell-free extract showed the greatest level of bioleaching and subsequent biosynthesis of nanoparticles after 96 hrs of reaction. The biosynthesized nanoparticles were characterized using UV-Vis spectroscopy, FTIR, TEM, HRTEM, EDX and Zeta analyser. The nanoparticles were stabilized by protein capping, as confirmed by UV-Vis spectroscopy (absorption around 270-280 nm indicating the presence of aromatic amino acids), FTIR (~1640 cm⁻¹, corresponding to the amide I functional group from the carbonyl stretch of proteins and ~3270-3310 cm⁻¹, the –NH group of amines) and zeta potential (-5.99 mV of J2, -10.2 mV of J4, -22.7 mV of T4 and -15.2 mV of T6).
TEM analysis indicated that the nanoparticles synthesized were in the size range of 10-50 nm, with a varying average size range of 45 ± 5 nm for J2, 15±5 nm for J4 and 15±5 nm for T4 and T6. Bioleaching of Zn and Fe was confirmed using EDX spectroscopy. HRTEM further confirmed the crystallinity of the biosynthesized nanoparticles.

The final aim of this research was to test these biologically synthesized nanoparticles as potential nanonutrients for the plant. This was a preliminary study which investigated nutrient adsorption, uptake and assimilation by the plant. Jarosite nanoparticles synthesized using the cell-free extract of *A. terreus* strain J4 were tested. Firstly, these negatively charged particles were subjected to surface modification using Poly L-Lysine (PLL) resulting in positively charged nanoparticles (+ 24.9 mV). Surface modified nanoparticles were tested for their efficacy in terms of seed germination and plant uptake in wheat (*Triticum aestivum*). Results showed 100 % seed germination and enhanced plant growth at 10 and 20 ppm, which decreased thereafter at higher concentrations. Confocal microscopy confirmed the uptake of Zn and Fe by the plant using the fluorescence indicators Zinpyr-1 and Perls stain, for Zn and Fe respectively. Further optimization of concentrations, nutrient uptake and plant imaging is required to confirm the utility of these nanoparticles for plant nutrient use.

Through this work, we have shown that myco-nano-mining can not only be used for the reduction of waste but also for overcoming plant nutrient deficiencies. Mining waste can therefore be a potential source of nanonutrients for agriculture.
Chapter 1

*Introduction and Literature review*
1.1 Introduction

In today’s world, rapid population growth and increased demand for resources leads to increase in waste generation. Unwarranted dumping and inappropriate waste handling results in many problems like contamination of water, spread of diseases through breeding of insects and rodents and increased chances of flooding due to blockage of drainage, canals or gullies. The contamination of groundwater and soil with the toxic/hazardous compounds released as waste by various industries is an environmental problem. For example, mining waste affects crop productivity due to undesirable changes in physico-chemical properties of soils. Thus, the foremost objectives of waste management should be safe disposal, and preferably safe utilization, for example the recovery of valuable metals from the wastes containing them (Mishra & Rhee, 2010). As described by the International Solid Waste Association (ISWA 2009): “The waste hierarchy is a valuable conceptual and political prioritization tool which can assist in developing waste management strategies aimed at limiting resource consumption and protecting the environment.”

Various methods have been reported for the treatment of wastes like Pyrometallurgical (Lee et al., 2007) and Hydrometallurgical methods (acid or caustic leaching of solid metals) (Park & Fray, 2009). These methods can incur environmental risks due to the usage of toxic reagents and the generation of large amount of by-products. To overcome these problems, bioleaching has emerged as a novel promising technology for the treatment of waste using microorganisms. Many of these organisms showing high metal tolerance have been reported to synthesize metal nanoparticles from the available metals (Bajaj et al., 2012; Dhanjal & Cameotra, 2010).
Over the last decade, the biosynthesis of nanoparticles has been widely investigated, due to their unique magnetic, electronic (Peto et al., 2002), optical (Krolikowska et al., 2003), chemical (Kumar et al., 2003) and catalytic properties. Nanoparticles possess greater surface area by weight compared to larger particles, which results in higher reactivity when compared to larger molecules (Prathna et al., 2010). The application of biologically synthesized nanoparticles is attracting research attention in areas such as targeted drug delivery, cancer treatment, magnetic resonance imaging (MRI) (Fan et al., 2009), antibacterial agents (Fayaz et al., 2010; Xie et al., 2011) and biosensors (Wang et al., 2010). Furthermore, the biosynthesis of metal nanoparticles obtained from waste, particularly those under the category of trace elements, may have future applications for the enhancement of agricultural productivity.

1.2 Essential nutrients and natural toxicity in Agriculture and Food safety

Mineral nutrients found in soil, water, air and plants have been classified as Macro and Micronutrients based on their utility and requirement (Marshner, 2012; Sperotto et al., 2014). In the present exploitive agricultural scenario, major constraints in the path towards sustainable food and crop production are being faced due to continued soil degradation and macro and micronutrient deficiencies. Soil degradation occurs due to various activities like excessive overburden created by industries, where release of pollutants into the water bodies lead to contamination of groundwater. The utilization of this groundwater for irrigation can deteriorate the fertility of soil. These altered soil conditions, in turn, affect the solubility of nutrients as well as their uptake by plants, leading to nutrient deficiencies.
1.2.1 Nutrient Deficiencies

Adequate levels of the seven trace elements- Zinc, Boron, Iron, Chlorine, Copper, Manganese, and Molybdenum, help in regulating activities like plant metabolism, reproductive growth, carbohydrates production, nutrient regulation, and are thus essential for the optimum growth and yield of plants as well as animals and humans (Tripathi et al., 2015). Thus, the inadequacy of these nutrients in soil and diet leads to developmental defects, poor growth and premature death. Zinc deficiency is the major deficiency globally targeting crops like maize, rice and wheat, followed by iron and boron deficiencies (Alloway, 2008). According to the 2002 report by World Health Organization (WHO), dietary zinc and iron deficiencies are serious global health risks (Alloway, 2008; Bell & Dell, 2008). It has also been reported that approximately two thirds of the world’s population is at the risk of nutrient deficiency (Stein, 2010; White & Broadley, 2009).

1.2.1.1 Zinc Deficiency

Zinc (Zn) is an essential micronutrient for plants. It is required in small quantities, but is essential for normal plant growth and development, and plays a major role in auxin activity, photosynthesis, enzyme activities and other processes. Zn deficiency in plants is widespread. Among all the micronutrients, Zn deficiency in plants is most directly related to deficiency in human (Alloway, 2004; Cakmak et al., 1999; Welch & Graham, 2004; White & Zasoski, 1999). There are many factors responsible for Zn deficiency in plants (Alloway, 2008):

- High soil pH (like calcareous, heavily limed soils)
- High phosphate applications
- Water-logging and flooding of soil (rice, paddy)
- High salt concentrations
- High soil organic matter content
- Low total zinc content in soil (sandy soils)
- Low manure applications

Some estimates report that almost 50 % of the agricultural soils in India, 30 % in China, 45 % in Turkey and over 8 million of hectares in Western Australia are zinc-deficient (Alloway, 2008; Frossard et al., 2000; Gupta, 2005) (Figure 1.1). Among the various plant crops prone to Zn deficiency (Table 1.1), maize and wheat are the most susceptible.

![Zinc Deficiency Affected Areas](image)

Figure 1.1 Global scenario of Zinc deficiency.
Table 1.1 Crops susceptible to Zinc deficiency (Alloway, 2008; Martens & Westermann, 1991).

<table>
<thead>
<tr>
<th>High</th>
<th>Medium</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bean</td>
<td>Barley</td>
<td>Alfalfa</td>
</tr>
<tr>
<td>Citrus</td>
<td>Cotton</td>
<td>Asparagus</td>
</tr>
<tr>
<td>Flax</td>
<td>Lettuce</td>
<td>Carrot</td>
</tr>
<tr>
<td>Fruit trees (deciduous)</td>
<td>Potato</td>
<td>Clover</td>
</tr>
<tr>
<td>Grapes</td>
<td>Soybean</td>
<td>Grass</td>
</tr>
<tr>
<td>Hops</td>
<td>Sudan grass</td>
<td>Oat</td>
</tr>
<tr>
<td>Maize (corn)</td>
<td>Sugar beet</td>
<td>Pea</td>
</tr>
<tr>
<td>Onions</td>
<td>Table beet</td>
<td>Rye</td>
</tr>
<tr>
<td>Pecan nuts</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sorghum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweetcorn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.2.1.2 Iron Deficiency

Iron (Fe) being an essential micronutrient for crops, is required in the chlorophyll manufacturing process and for some enzyme activities. About 30 % of cultivated area globally, with India (12 %), China (40 %) and large portions of Sub-Saharan Africa, are found to be deficient in Fe, predominantly in regions with a mediterranean climate and calcareous soils (Alloway, 2008). In India, deficiency is highest in the soils of Karnataka, followed by Himachal Pradesh (Gupta, 2005). Iron is present in most of these soils (3-5 % of soils), but it is mostly not bioavailable for plant absorption due to following factors (Meng et al., 2005):
• High pH soils

• Weather condition (cool, wet weather)

• Phosphorus, manganese and zinc as antagonists

• Poorly aerated and compacted soils

• Loamy soils low in organic matter

Fe-deficiency is particularly problematic for fruit trees (citrus), soyabean and peanut, but also negatively impacts many other groups (Table 1.2).

Table 1.2 Iron deficient crops (Alloway, 2008; Martens & Westermann, 1991).

<table>
<thead>
<tr>
<th>High</th>
<th>Medium</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>Barley</td>
<td>Rice</td>
</tr>
<tr>
<td>Bean</td>
<td>Corn</td>
<td>Grass</td>
</tr>
<tr>
<td>Citrus</td>
<td>Cotton</td>
<td></td>
</tr>
<tr>
<td>Linseed/Flax</td>
<td>Oat</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>Pea</td>
<td></td>
</tr>
<tr>
<td>Sorghum</td>
<td>Rice</td>
<td></td>
</tr>
<tr>
<td>Soyabean</td>
<td>Wheat</td>
<td></td>
</tr>
<tr>
<td>Sugar beet</td>
<td>Alfalfa</td>
<td></td>
</tr>
<tr>
<td>Spinach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grapes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tomato</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2.2 Understanding waste as a resource

With the dawn of industrialization, the generation of waste has increased dramatically. This tremendous rise in industry has led to the complex problem of waste removal. Under the Waste Framework Directive, the European Union defines waste as "an object the holder discards, intends to discard or is required to discard." (Figure 1.2) (European Directive 75/442/EC codified to Directive 2008/98/EC on waste).

![Diagram of waste generation process]

Figure 1.2 Process of waste generation according to European Union (Source: European Directive 75/442/EC codified to Directive 2008/98/EC on waste).

In pre-industrial history, low population density and minimal exploitation of natural resources resulted in low levels of waste generation by humans, although large amounts of plant burn-off for farming did lead to significant CO₂ release. Due to the development of varying industries and even health-care facilities, a huge amount of hazardous waste, including biomedical wastes, are being released, posing threats to the environment and human health. Types of solid waste as described by the Environmental Protection Department as well as the World Bank are illustrated below (Figure 1.3)
On the basis of their effect on human health and the environment, waste can be classified as hazardous and non-hazardous. The hazardous waste can cause threat to individuals or the environment if exposed, due to certain factors like toxicity, ignitability, corrosivity and reactivity. On the other hand, non-hazardous waste is generally refuse or municipal solid waste like paper, food scraps, plastics, metals, rubber, leather, textiles, wood, glass and other related material. As per the report by the World Bank’s Urban Development department, Municipal Solid Waste (MSW) is expected to increase from 1.3 billion tonnes per year in 2012 to 2.2 billion tonnes per year by 2025 (Hoornweg & Bhada-Tata, 2012), being an increase of about 900 million tons (MT) in about a decade. During the year 2010, the projected quantity of waste being generated globally was around 20 billion tonnes per annum [Figure 1.4, Source: (Pappu et al., 2011; Yoshizawa et al., 2004)].
Vital waste graphics (Baker, 2004) have reported that the extraction of metal through mining, irrespective of raw material is always a setback for the environment. Waste from extraction and processing of minerals include materials like topsoil, waste rock and tailings. The generation of these wastes is linked to the amount of ore production. There is a considerable usage of many toxic chemicals such as mercury, sulphuric acid etc. for separation of metal from ore. These chemicals are generally recycled, but their residues and the presence of heavy metals in the tailings create a major threat to the environment.

Various industries like electroplating, electronics, petrochemical, pharmaceutical, ferrous and non-ferrous, release toxic waste containing heavy metals into their surrounding environment. Metals like Zn, Cr, Cu, Ni, Pb and Co can be toxic for human health if they are discharged beyond permissible limits (Mishra & Rhee, 2010). According to V.K. Garlapati (Garlapati, 2016), an initiative known as “Solving the E-waste Problem (StEP)” projects that by 2017 the world will produce 33 % more e-waste, or 72 MT. As reported by Heeks and co-workers, China generates about 12.2 MT of e-waste, followed by the U.S. with about 11 MT (Heeks et al., 2014). Globally, 20-50 MT/year of e-waste is estimated (UNEP 2006), which
is equivalent to 1-3 % of predicted global civil waste production (Gaidajis et al., 2010).

As per the report by U.S. Environmental Protection Agency (EPA), approximately 500 million computers were discarded between the years 2000 and 2007, 2 MT of technology trash was dumped in landfills, and only 400 thousand tonnes were recycled. Ilyas et al. and Cui and Zhang have reported that this electronic waste is recycled, incinerated or disposed off in landfills (Cui & Zhang, 2008; Ilyas et al., 2007).

Since Zn and Fe are the most essential micronutrients, their widespread deficiency is of prime concern (Alloway, 2008; Das & Green, 2013). Thus, focusing on the waste containing these metals, could be a dual approach for management of these wastes and their subsequent utilization to combat their deficiency in soil. Here, Iron ore tailings and Jarosite are being examined for this purpose for the following reasons:

- High concentrations of these metals i.e. Fe and Zn.
- Large amount of dumping of these wastes in the ponds surrounding the mining/ extraction plant. Table 1.3 shows the global status of zinc and iron ore mining
- Not explored on commercialized basis to a great extent
Table 1.3 World mine production of Zinc and Iron

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>United states</td>
<td>832</td>
<td>58</td>
</tr>
<tr>
<td>Australia</td>
<td>1560</td>
<td>660</td>
</tr>
<tr>
<td>Bolivia</td>
<td>449</td>
<td>-</td>
</tr>
<tr>
<td>Brazil</td>
<td>-</td>
<td>320</td>
</tr>
<tr>
<td>Canada</td>
<td>353</td>
<td>41</td>
</tr>
<tr>
<td>China</td>
<td>4930</td>
<td>1500</td>
</tr>
<tr>
<td>India</td>
<td>706</td>
<td>150</td>
</tr>
<tr>
<td>Iran</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>Ireland</td>
<td>283</td>
<td>-</td>
</tr>
<tr>
<td>Kazakhstan</td>
<td>345</td>
<td>26</td>
</tr>
<tr>
<td>Mexico</td>
<td>660</td>
<td>-</td>
</tr>
<tr>
<td>Peru</td>
<td>1320</td>
<td>-</td>
</tr>
<tr>
<td>Russia</td>
<td>-</td>
<td>105</td>
</tr>
<tr>
<td>South Africa</td>
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<td>78</td>
</tr>
<tr>
<td>Sweden</td>
<td>-</td>
<td>26</td>
</tr>
<tr>
<td>Ukraine</td>
<td>-</td>
<td>82</td>
</tr>
<tr>
<td>Other countries</td>
<td>1860</td>
<td>131</td>
</tr>
</tbody>
</table>

1.2.2.1 Jarosite

Jarosite is released as a by-product in large quantity from zinc extraction plants. It is being disposed off in the form of solid residues. The main constituents are iron, sulphur, zinc, calcium, lead, cadmium and aluminium. The annual production of Zn globally was about 13 MT in 2011 (Fugleberg, 2014). Approximately 0.25 MT of such Zn residues are being disposed off per annum in India. The major constituents are FeSO₄, ZnSO₄ and PbSO₄. Jarosite, on an average, contains around 20,000 ppm of iron; 12,000 ppm of sulphur; 8,000 ppm of zinc and other elements like calcium, magnesium, sodium, potassium etc. are present in trace amounts (Pappu et al., 2011). The major producers of jarosite are Spain, Holland, Canada, France, Australia, Yugoslavia, Korea, Mexico, Norway, Finland, Germany, Argentina, Belgium and Japan (Arslan & Arslan, 2003). Hindustan Zinc Limited (HZL) (2003)
in India produces 3.49 MT of Zn (in turn 2, 34,000 tonnes of jarosite) per annum. The smelter plant at Debari Zinc, Rajasthan is one of the largest units. It produces around 49,000 tonnes of Zn metal per year, thus as an outcome a huge amount of jarosite (29,400 tonnes) is being released (Acharya et al., 1992; Pappu et al., 2011). These solid residues obtained, are dumped off and stored on the grounds in close proximity to the smelter plant itself. This accumulation and increase in annual production is of major concern with respect to the environmental pollution of soil, vegetation as well as aquatic life (Kerolli-Mustafa et al., 2015; Pappu et al., 2006). Thus, research has been going on for jarosite waste management using various processes, with the most widely used being the Jarofix process. Here hydrated lime is used, followed by the stabilization of jarosite with ordinary Portland cement before being disposed off in a pond (Raghavan et al., 2011). Hage and Schuiling have reported the use of sodium sulphide for stabilization of jarosite, which helped in the inhibition of leaching of toxic metals like Cu, Cd, Pb and also enhanced the physical properties of the waste. Mymrin and Vaamonde have shown that jarosite has potential for use as a construction material since the strength can be increased with 1-4 % CaO through compression moulding (Mymrin & Vaamonde, 1999). A report by Katsioti and group highlights the drawback of jarosite i.e. reduced settling time and compressive strength because of decreased concentration of water soluble SO₃ and increase in Cr³⁺ (Katsioti et al., 2006). As per the report by Central Pollution Control Board (CPCB 2010-11), utilization of jarosite by cement industries is not being carried out successfully due to the presence of high concentration of Si; as well as Zn and Cd (preventing construction purpose) (Hage & Schuiling, 2000). Therefore, the commercialization potential of jarosite is still to be explored.
Among the various issues related to the mining industries (Kerollí-Mustafa et al., 2015; Lottermoser, 2010), excessive dumping, degradation of land (i.e. removal of top soil), and deforestation have an indirect effect on the soil and groundwater quality, which in turn decreases the soil fertility in the long term. Thus, the regulated disposal of waste being generated and subsequent treatment is of major concern in today’s world.

1.2.2.2 Iron Ore Tailings

It has been reported in World Mineral Production by the National Environment Research Council (NERC) that world iron ore production reached 2611 MT during 2010 (Brown et al., 2014). Among 50 countries, Brazil is the largest producer of iron ore followed by China, Australia, India and Russia. Various types of waste released from iron ore mining have an effect on the environment. These are as follows:

- Top soil: Top soil being of superior quality containing plant nutrients, microbes and humus, can be of use in remediation of waste dumps and mined out areas
- Tailings from Ore Processing plant: Tailings are the residues of iron ore beneficiation plant which are fine particles. These are dumped in the tailing ponds/dams that are built for containment. The disposal of these tailings is a major threat to the environment
- Mining wastes/ Rejects: These include iron ores which have iron below the cut off point set up for approved quality to the ore processing
- Wastes from Service facilities: The metallic and non-metallic wastes like tubes etc. are saleable. The oil contaminated wastes like oil muck, oil filters
etc. are hazardous and are mostly to be burned or dumped in specially designed areas

According to the Central Pollution Control Board (CPCB) report 2007, there is a generation of approximately 14 MT of tailings every year by the iron ore beneficiation plant in India. Approximately 1800 MT of iron ore is being consumed globally which thus indicates generation of huge amount of mine waste (waste rocks, tailings) (Lottermoser, 2010). Thus, managing this enormous quantity of tailings stored in massive impoundments known as tailing dams is of primary concern in controlling pollution and for conservation of resources. Disposal of these materials in a regulatory manner or their utilization is an exigent task for the industry. One of the likely approaches could be to use these tailings as a value added product in the building industry. Reports are available that companies like Kundremukh Iron Ore Company (KIOCL) Ltd. have submitted a proposal to the Karnataka ministry for using tailings in products like tiles and bricks (Kulkarni 2012), however commercial utilization has not yet been reported.

1.3 Treatment of Waste

High concentration of heavy metals are present in wastes from mining and metal industries, residues from power stations and waste incineration plants. The extraction of such metals, through easy and economical process remains a challenge. Some of the physical and chemical methods for the remediation of these materials include:

- Extraction with mineral acids like nitric, sulphuric and hydrochloric acids (Andreottola et al., 2010; Arevalo et al., 2002; Naoum et al., 2001)
- Extraction with organic acids like oxalic, citric and acetic acids (Aung & Ting, 2005)
- Extraction with chelating agent like EDTA (Tandy et al., 2004; Tejowulan & Hendershot, 1998)
- Ultrasonic extraction (Narayana et al., 1997; Swamy & Narayana, 2001) and Electro dialysis (Hanay et al., 2009; Ottosen et al., 2003)
- Various coupled processes including the above methods and bioleaching i.e. using organisms like Acidithiobacillus ferrooxidans have also been carried out (Cheikh et al., 2010)

Researchers have been investigating recycling of electronic waste through mechanical and pyrometallurgical methods. Pyrometallurgical treatment raises concern regarding possible formation of brominated and chlorinated di-benzo furans and dioxins in burning processes due to the presence of halogens in the plastic parts of electronic waste (Tsydenova & Bengtsson, 2011). Hydrometallurgical processes can incur environmental risks due to the usage of toxic reagents and generation of large amount of by-products (Mecucci & Scott, 2002). These are expensive processes due to the requirement of more investment involved in efficient set up, and a high consumption of energy (Bosecker, 1997).

Coal fly ash is being considered as a challenging industrial waste released from coal-fired electric power stations. The problem is attributed to the presence of boron, arsenic and selenium present as trace elements. Kashiwakura et al. have developed an acid-wash process for the removal of selenium from coal fly ash. This chemical based process involves the treatment of fly ash with H₂SO₄ leading to the adsorption of selenious acid H₂SeO₃ on the surface of coal fly ash (Kashiwakura et al., 2011).
Thus, bioleaching, the extraction of metals from their ores and mineral concentrates with the help of living organisms (bacteria, fungi, yeasts), has been explored in the recent years as an efficient treatment of increasing industrial and municipal wastes, as well as recovery of metals from their low-grade ores and concentrates, because of the advantages listed below (Mishra & Rhee, 2010):

- **Economical**: It is a simpler and cheaper method to operate and maintain than the traditional processes as it has the potential to partially replace the extensive crushing and grinding which adds to the cost and energy consumption in a conventional process.

- **Environmental friendly**: The process is more environmental friendly than traditional extraction methods as it circumvents the usage of hazardous acids as well as reduces the release of by-products which may be toxic in nature.

- **Flexible process**: Microbes can adapt to the conditions and can co-metabolize various components in the medium.

Many factors affect the leaching efficiency of microorganisms (Bosecker, 1997). Some of these are as follows:

- **Nutrients**: Mainly obtained from the environment provided for the growth of organism and also from the material to be leached.

- **O₂ and CO₂**: Essential for the optimum growth as well as enhanced leaching efficiency. CO₂ acts as a carbon source.

- **pH**: Decisive factor for metal solubilization apart from favoring organism’s growth.

- **Temperature**: Usually in the range of 25°C - 30°C but higher (50°C-80°C) in the case of thermophilic bacteria.
• Mineral substrate: Mineralogical composition followed by increased total surface area of the substrate is of primary importance as this favours higher yield with no change in total mass of particles

• Heavy metals: High metal tolerance favours increased leaching efficiency

• Surfactants and organic extractants: Usually have inhibitory effect on the organism

The availability of suitable conditions for the growth of organisms responsible, facilitates the process of bioleaching (Bosecker, 1997). The three principles that are responsible for the mobilization and leaching efficiency of microorganisms from solid materials are: (i) the conversion of organic or inorganic acids (protons) (ii) oxidation and reduction reactions and (iii) the release of complexing agents (Brandl et al., 1997). In general, bacteria belonging to the group *Thiobacillus* are most active in acidic environments with pH ranging from 1.5-3 (where most of the metal ions remain in solution) which favors bacterial leaching. Other than acidophilic bacteria, heterotrophic microorganisms like *Bacillus*, *Aspergillus* and *Penicillium* are most effective against Cu, Ni, Al and Zn (Brandl et al., 2001; Yang et al., 2009). A two-step process for bioleaching (Aung & Ting, 2005; Pradhan & Kumar, 2012) usually followed in order to reduce the toxic effects on the microorganisms is as follows:

a. The organism was grown in the media without metal scrap

b. Afterwards, varying metal scrap concentrations were added to the biomass and left for further incubation

Terms like “contact leaching” i.e. bioleaching by attached cells; “non-contact leaching” i.e. bioleaching by planktonic cells; and “cooperative leaching” explain
the dissolution of sulfur colloids, sulfur intermediates, and mineral fragments by planktonic cells and can be used to describe the physical condition of cells in the bioleaching process (Rawlings, 2002; Tributsch, 2001). The thiosulfate and polysulfate mechanisms are the two different chemical pathways that describe oxidation of metal sulfide (Sand et al., 2001; Schippers & Sand, 1999). Microorganisms play a very important role in the oxidation process of intermediate sulfur compounds that are formed by the metal sulfides chemical dissolution. Microorganisms, under different basic and acidic conditions, possess an ability to oxidize Fe (II) to Fe (III) ions. They can also catalyze the oxidation of intermediate sulfur compounds to sulfuric acid (Vera et al., 2013). Here we review the use of bioleaching in the treatment of waste materials containing Zn and Fe.

1.3.1 Bioleaching of Zinc

Zinc is an essential metal for various metallurgical, chemical and textile industrial applications. The disposal of residues from Zn sources like sulphide concentrates, secondary resources such as zinc ash, zinc dross, flue dusts of electric arc furnace, brass smelting, automobile shredder scrap, rayon industry sludge, zinc-carbon batteries etc. (Cheikh et al., 2010; Gega & Walkowiak, 2011; Gouvea & Morais, 2007; Jha et al., 2001) is now becoming expensive and difficult because of increasingly rigid environmental protection regulations. Therefore, there is a rising demand for developing processes for the recovery of Zn from secondaries/wastes. The process should be designed such that the residue produced can be recycled for further processing or safely disposed-off without causing any harm to the environment. The treatment of these resources is usually carried out through pyrometallurgical and hydrometallurgical processes. High energy requirements and the need for dust collecting/gas cleaning system are some of the major drawbacks of
these methods. Salts like chloride and fluoride in the dust can result in severe corrosion problems and thus demands the use of expensive alloys as materials of construction (Jha et al., 2001). Lupa and coworkers have carried out the extraction of zinc from zinc ash produced from thermal zinc coating industry, using chlorihidric acid solution (Lupa et al., 2006). de Souza et al. used H$_2$SO$_4$ as leachant for recovery of Zn from used alkaline batteries (de Souza et al., 2001).

In order to overcome the above problems, Solisio and coworkers worked on bioleaching of Zn (76 %) and Al (78 %) from two types of industrial waste sludges, that is, dust from iron-manganese alloy production in an electric furnace and sludge from the treatment plant of aluminium anodic oxidation, using a strain of acidophilic bacteria *Thiobacillus ferroxidans*. The culture obtained was not a pure culture but mainly constituted by *T. ferroxidans* species (Solisio & Lodi, 2002). Hudec and co-workers have used *Acidiphilium acidophilum* for carrying out the bioleaching of Cu, Ni and Zn from computer printed circuit boards (PCBs). It was observed that this mesophilic bacterial strain was able to leach out Cu and Zn from shredded PCBs, but not Ni. Low PCB concentrations (lower than 20g L$^{-1}$) and low pH was found to be favorable for the bacterial growth and metal bioleaching efficiency (results for higher PCB concentrations were not reported) (Hudec et al., 2005). The results obtained were supported by other studies (Brandl et al., 2001; Ilyas et al., 2007; Yang et al., 2014). Nie et al. have reported that the process of bioleaching of waste PCBs using a home-made bioreactor with cotton gauze as a source for *A. ferroxidans* immobilization was a superior method, over using free cells, as it improved the ferrous ion oxidation ratio of 96.90 % after 12 hrs, and also high cell concentrations were achieved. About 91.68 % Cu, 95.32 % Zn, 90.32 %
Mg, 86.31 % Al and 59.07 % Ni were leached out after 96 hrs of reaction time (Nie et al., 2015).

Brandl and co-workers worked with the objective of developing a two-step process of bioleaching of metals (Al, Ni, Cu, Zn) from e-wastes using a mixed culture of *Thiobacillus thiooxidans* and *T. ferrooxidans* as well as two fungal strains, *Penicillium simplicissimum* and *Aspergillus niger*. These organisms were termed as “Computer-munching microbes”. Bacterial leaching efficiency of about 90 % was observed for all the metals, whereas for both the fungal strains, reduced metal mobilization was experienced, particularly in the case of Al and Cu, but there was still 60 % and 95 % for Ni and Zn, respectively (Brandl et al., 2001). Direct interaction of microorganisms and the metal w.r.t. growth and leaching is poorly suited, thus a two-step process is advisable where first the fungus is grown in the absence of metal and then the released metabolites are used for reaction. Reports are already available on the following advantages of this strategy of bioleaching from fly ash (Bosshard et al., 1996; Brombacher et al., 1998):

- There is no direct interaction of biomass with metal-containing waste
- Contamination of waste material by microbial biomass prevented
- Optimization of acid formation possible in the absence of waste material
- Application of higher concentrations of waste possible which is difficult in the case of one-step process, thus leading to increased yields of metal

Xin and co-workers have carried out the bioleaching of Zn and Mn from spent alkaline and zinc-carbon batteries (ZnO, Mn₂O₃ or Mn₃O₄) using *Alicyclobacillus* sp. as sulfur-oxidizing bacteria and the *Sulfobacillus* sp. as iron-oxidizing bacteria, in the form of individual culture as well as a consortium. Zn leaching rate was
comparatively high (96 %) as compared to Mn leaching, irrespective of the bacterial species, whereas variation was observed in the amount of Mn being leached out by mixed culture (97 %) and *Alicyclobacillus* sp. (56 %). The results obtained indicate that Zn-Mn batteries have the potential for fast bioleaching of Zn, as compared to Mn. Extraction of both the metals decreased with increase in pH (1.5-4.5) (Xin et al., 2012).

Pradhan and Kumar worked on the bioleaching of metals from electronic waste i.e. PCBs (containing metals like Cu, Fe, Se, Zn, Au, Ag, Cr, Co and Ni) from Personal Computers (PCs), using the cynogenic bacteria *Chromobacterium violaceum*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. It was observed that maximum bioleaching efficiency was exhibited by *C. violaceum* followed by *P. aeruginosa* as a single culture for Cu (79.3 % w/w), Au (69.3 % w/w), Zn (46.12 % w/w), Fe (9.86 % w/w) and Ag (7.08 % w/w). Whereas in the case of mixed cultures *P. aeruginosa + C. Violaceum*, maximum leaching efficiency was shown (83.46 %, 73.17 %, 49.11 %, 13.98 % and 8.42 % w/w of total Cu, Au, Zn, Fe and Ag, respectively) of all the three combinations. According to Pradhan and co-workers, this might be due to the higher metal-toxicity tolerance of *P. aeruginosa* and *C. violaceum*. It can also be due to the release of additional secondary metabolites as a defence mechanism (Pradhan & Kumar, 2012). Ilyas et al. have studied the bioleaching ability of *Penicillium chrysogenum* in recovering metals like Zn (65 %), Cu (67 %), Ni (55 %), Co (60 %) and Mg (69 %) from mine tailings. They exploited the culture’s property of releasing a range of organic acids (Ilyas et al., 2013).
The physico-chemical properties of the waste such as ash and combustion slag are related to the metal leachability. Mixed culture of acidophilic bacteria *Acidithiobacillus thiooxidans* and biosurfactant-producing bacteria *Bacillus subtilis* PCM 2012 and *Bacillus cereus* PCM 2019 were found to be suitable for bioleaching of metals from combustion wastes. Among the two different wastes, the power plant slag having high metal content in the exchangeable fractions (bound to carbonates and Fe and Mg oxide fractions as compared to the organic fractions) showed the best bioleaching results with Zn, Ni and Cu recovery exceeding 90% (Karwowska et al., 2015).

**1.3.2 Bioleaching of Iron**

Iron has a wide range of applications in the form of stainless steel, ferrous scrap etc. being used in a variety of industries, such as building materials, kitchenware and fertilizer industry. It is also found in many ores such as haematite (Fe₂O₃), magnetite (Fe₃O₄), limonite (2Fe₂O₃·3H₂O), siderite (FeCO₃), pyrite (FeS₂) and chalcopyrite (CuFeS₂) (Parker, 1997). The steelmaking industry is one of the major sources of iron. It generates huge amount of dust (approximately 7-15 kg) released during the melting process in steelmaking plants. The dust obtained is composed of waste oxide materials with iron oxide being the major component. The presence of Zn prevents recycling and thus the dust is being dumped in landfills. Trung et al. have carried out acid leaching of Fe from the dust obtained from basic oxygen furnace using 1M H₂SO₄ at a high temperature of 80°C (Trung et al., 2011). Various reports are available on the precipitation of Fe from zinc ores or concentrates (Buban et al., 1999; Dutrizac, 1980). Ismael and Carvalho reported the extraction with organophosphorus acid for the precipitation of iron from sulfate leach liquors at a very high temperature of around 97°C. The major disadvantage of this method...
was the precipitation of other metal ions alongside like Cu, Zn, Co, Ni, Mn, In, Ga, Ge, and Al. The application of these acidic extractants is a cumbersome process, which further involves the use of sulphuric acid and complex methods like reductive or hydrolytic stripping (Ismael & Carvalho, 2003). Methods like magnetizing roasting–magnetic separation was also used to recover iron from red mud (Li, 2001; Yang et al., 2011) and iron ore tailings (Li et al., 2010). Zhang and his co-workers have carried out the leaching of iron from cyanide tailings produced during extraction of gold from gold ore. They have used the process of roasting-water leaching followed by magnetic separation (Zhang et al., 2012). Ming Kuo has reported another method of vitrification for recovery of Fe and Zn from Zinc phosphating sludge. This process though can help in immobilization of toxic metals and transformation of hazardous waste into stabilized slag, it requires high energy consumption and subsequently high cost (Kuo, 2012).

Therefore, researchers have shifted to an alternative method of biological extraction of iron from the waste materials. Aung and Ting have carried out the leaching of heavy metals from spent fluid catalytic cracking catalyst using *Aspergillus niger*. Comparative experiments for chemical leaching were set up with organic acids like citric, gluconic and oxalic acids; mineral acids like sulphuric and nitric acids. Reaction with mixture of organic acids at the concentrations similar to those produced by the organism was also performed. The results confirmed that bioleaching increased the metal extraction (Ni-9 %, Fe-23 %, Al-30 %, V-36 % and Sb-64 %) by around 2.7-20 % than leaching through commercially available organic acids (at similar concentrations) at 1 % pulp density (Aung & Ting, 2005). One step and two-step processes of bioleaching of mine tailings have been carried out using *Aspergillus fumigatus* (Seh-Bardan et al., 2012). As per the observations
made by Seh-Bardan and co-workers, one-step process where oxalic acid was more dominant as compared to other organic acids was found to be more efficient in the leaching of Fe (58 %), As (62 %), Mn (100 %) and Zn (54 %) whereas Pb (88 %) was more efficiently removed by the two-step process, where citric acid was highest.

Investigation has been done by Bayat et al. for the bioleaching capability of *Acidithiobacillus ferrooxidans* for Fe and Zn from the dust obtained from a steel making plant (major constituent being Fe around 54.73 %). Bioleaching efficiency of bacteria was highest at pH 1.3 i.e. 37 % for Fe and 35 % for Zn. It was observed that the production of H$_2$SO$_4$ by bacteria was high at this pH value (Bayat et al., 2009). Thus, bioleaching of metals from waste is an important tool for the remediation of land and environmental pollution. The metal-tolerance of some microbes could be further exploited for the nanoparticles synthesis.

1.4 Biosynthesis of Nanoparticles

Nanoparticles, the leading edge of Nanotechnology, are quite versatile with a wide range of applications in the food and biomedical areas, including biosecurity, industrial coatings, remediation of ground water and agriculture.

Various physical and chemical methods have been reported for the synthesis of nanoparticles, but these are complex processes and are not friendly to the environment, whereas biological routes of synthesis are environmental friendly and safe. Biological entities have a unique potential to synthesize molecules with selective properties, thus becoming a potential tool for nanoparticles synthesis (Vinod et al., 2008). Biosynthesis have been carried out by exploiting microbes,
especially bacteria, under either aerobic or anaerobic conditions. Anaerobic conditions pose some limitations of culture conditions and optimization, as a result the biosynthesis scale up process becomes difficult. These limitations can be overcome under aerobic conditions. The nanoparticle synthesis can be categorized into intracellular and extracellular synthesis. The conversion of metal ions in the environment into their elemental form is carried out by the enzymes released during the cellular activities of microorganisms. A wide range of nanoparticles from metals, including Au, Ag, Pt, Hg, Se, CdTe, CdS, Fe₃O₄, Fe₂O₃, ZnO and Mn, have been synthesized biologically (Li et al., 2011; Mohanpuria et al., 2008; Talebi et al., 2010). Out of these, Zn and Fe nanoparticles biosynthesis holds the importance in agriculture w.r.t micronutrients.

1.4.1 Zinc Nanofactories

Zinc nanoparticles have been chemically synthesized mostly in oxide and sulphide forms. The considerable attention received by zinc oxide nanoparticles is due to their unique antibacterial, antifungal and UV-filtering properties, besides high catalytic and photochemical activity. A number of chemical and physical methods have been employed for the synthesis of zinc nanoparticles. These include precipitation (Meruvu et al., 2011), thermal decomposition (Salavati-Niasari et al., 2008), microemulsion-mediated synthesis (Hingorani et al., 1993), hydrothermal (Aneesh et al., 2007), electropolymerization (Moghaddam et al., 2009), laser ablation (Singh & Gopal, 2007) and chemical vapor synthesis (Polarz et al., 2005) for ZnO nanoparticle synthesis, while solid state (Wang & Hong, 2000) and coprecipitation methods (Bahmani et al., 2007) have been reported for ZnS nanoparticle synthesis. In comparison to the above methods, few microbes have been identified as zinc nanofactories (Table 1.4).
Streptomyces sp. HBUM171191 was reported to be a potential candidate for synthesizing Zn nanoparticles in the size range of 100-150 nm and 10-20 nm (Usha et al., 2010; Waghmare et al., 2011). Labrenz et al. have reported a family of aero-tolerant sulfate-reducing bacteria, Desulfobacteriaceae for the formation of spherical aggregates of 2-5 nm diameter sphalerite (ZnS) particles within natural biofilms dominated by these bacteria (Labrenz et al., 2000). Bai and co-workers have carried out the synthesis of ZnS nanoparticles (12 nm) by Rhodobacter sphaeroides, which displayed unique optical properties (Bai et al., 2006). Pseudomonas aeruginosa rhamnolipids (RLs) have been used for the biosynthesis of spherical crystalline ZnO nanoparticles with size range of 35-80 nm (Singh et al., 2014). These nanoparticles showed antioxidant property and inhibition of β-carotene oxidation and lipid peroxidation.

Jain and co-workers have verified that there is a positive correlation between soil fungi metal tolerance and their efficiency for nanoparticle synthesis. Aspergillus aeneus NJP12 isolated from rhizospheric soil near a zinc mine, showed an ability to carry out extracellular synthesis of spherical shaped ZnO nanoparticles in the range of 100-140 nm. These nanoparticles were found to be capped with proteins. Efforts were also made for confirming the role of extracellular proteins in the synthesis (Jain et al., 2013). ZnO nanoparticles (57.72 nm) synthesized by Aeromonas hydrophila were tested for their antimicrobial activity and showed positive results against Pseudomonas aeruginosa and Aspergillus flavus (Jayaseelan et al., 2012). Sarkar et al. have also reported the extracellular mycosynthesis of stable ZnO nanoparticles with an average size of 75 ± 5 nm using Alternaria alternata. They also checked and confirmed that particles could induce cytotoxicity at a concentration of 500 μg mL⁻¹ and above (Sarkar et al., 2014).
Zinc sulfide nanoparticles have found various applications such as in phosphors, IR window and solar cells. Zinc Oxide nanoparticles because of their antimicrobial properties (Jin et al., 2009; Liu et al., 2009; Padmavathy & Vijayaraghavan, 2008; Xie et al., 2011) have reported to be an effective application on cotton fabrics (Rajendra et al., 2010).

1.4.2 Iron Nanofactories

Iron nanoparticles have been synthesized by various physical and chemical methods. Methods like laser pyrolysis (Kalyanaraman et al., 1998), spray pyrolysis (Kalyanaraman et al., 1998), chemical vapour condensation (Tavakoli et al., 2007), thermal decomposition (Huber, 2005), sonochemical decomposition (Huber, 2005) and the usage of ball mills (Rawers & Cook, 1999; Roh et al., 2001) are complex processes having some negatives such as high energy consumption and a requirement for the use of expensive chemicals and surfactants. Synthesis of nanoparticles through ball milling can also lead to contamination by steel balls. In order to overcome the above problems, Fe nanoparticles have been synthesized using a large number of microbes (Table 1.4). Some of the reports on biosynthesis of Fe nanoparticles are as follows: Bharde et al. reported extracellular synthesis of iron based magnetic nanoparticles, namely maghemite ($\gamma$-Fe$_2$O$_3$) and greigite (Fe$_3$S$_4$) by bacterium Actinobacter sp. on reactions with ferric chloride and ferric chloride-ferrous sulfate, respectively. The synthesized nanoparticles possessed supermagnetic properties. Clusters of Fe nanoparticles of an average size of 50 nm were obtained, which were capped with proteins (Bharde et al., 2008). Bharde and group have reported extracellular biosynthesis of magnetite nanoparticles by challenging fungi namely, Fusarium oxysporum (20-50 nm) and Verticillium sp. (100-400 nm) with ferric and ferrous salt mixtures at room temperature (Bharde et
Another fungus, *Pleurotus* sp., has been identified as a potential iron nanofactory by Mazumdar and Haloi. In their study, the fungus was allowed to grow in FeSO₄ solution for 3 days leading to both intracellular and extracellular synthesis of nanoparticles and the involvement of proteins in synthesis was also analysed (Mazumdar & Haloi, 2011). *Thermoanaerobacter ethanolicus* (TOR-39) have been used for the synthesis of magnetic octahedral nanoparticles of sizes less than 12 nm (Roh et al., 2001). Srivastava and Constanti have carried out the biosynthesis of a range of nanoparticles, including Fe, Ag, Pd, Rh, Ni, Ru, Pt, Co and Li by using a single bacterial strain *Pseudomonas aeruginosa* SM1 (Srivastava & Constanti, 2012).

Magnetic nanoparticles (Fe₃O₄ and Fe₂O₃) are biocompatible and are thus potent for targeted cancer treatment, gene therapy etc. (Fan et al., 2009; Xie et al., 2009). Iron reducing bacteria like *Geobacter sulfurreducens* (Byrne et al., 2014) and *Clostridium* sp. (Kim & Roh, 2015) have been reported to synthesize superparamagnetic Zn-substituted magnetite nanoparticles with enhanced magnetic properties, thus giving superior performance in MRI applications. Fe nanoparticles play an important role in the degradation of pesticides like Lindane and Atrazine (Elliott et al., 2009; Joo & Zhao, 2008; Paknikar et al., 2005). Zero-valent Fe nanoparticles have been reported to be an efficient means for degradation of pesticides like alachlor (Bezbaruah et al., 2009).
Table 1.4 Microbial nanofactories for Zn and Fe.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Precursors</th>
<th>Location</th>
<th>Microorganisms</th>
<th>Size (nm)</th>
<th>References</th>
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<tr>
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<td>Zinc sulphate</td>
<td>Intracellular</td>
<td><em>Streptomyces</em> sp. HBUM171191</td>
<td>10-20</td>
<td>Waghmare et al., 2011</td>
</tr>
<tr>
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<td>Zinc nitrate</td>
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<td><em>Streptomyces</em> sp.</td>
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<td>Usha et al., 2010</td>
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<tr>
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<td>Bai et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Zinc acetate</td>
<td>Extracellular</td>
<td><em>Aspergillus aeneus</em> NJP12</td>
<td>100-140</td>
<td>Jain et al., 2012</td>
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<tr>
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<td>Jayaseelan et al., 2012</td>
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<td>Sarkar et al., 2013</td>
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<td>Singh et al., 2014</td>
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<td>Extracellular</td>
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<td>Bharde et al., 2006</td>
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<td>Ferric chloride</td>
<td>Extracellular</td>
<td><em>Actinobacter</em> sp.</td>
<td>50</td>
<td>Bharde et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Ferrous sulphate</td>
<td>Extracellular</td>
<td><em>Pleurotus</em> sp.</td>
<td>NA*</td>
<td>Mazumdar et al., 2011</td>
</tr>
<tr>
<td>Iron nitrate</td>
<td>Extracellular</td>
<td><em>Pseudomonas aeruginosa</em> SM1</td>
<td>20.5</td>
<td>Srivastava and Constanti, 2012</td>
<td></td>
</tr>
<tr>
<td>ZnCl₂, FeCl₃</td>
<td>Extracellular</td>
<td><em>Thermoanaerobacter ethanolicus</em> (TOR-39)</td>
<td>&lt; 12</td>
<td>Roh et al., 2001</td>
<td></td>
</tr>
<tr>
<td>ZnCl₂, FeCl₃</td>
<td>Extracellular</td>
<td><em>Geobacter sulfurreducens</em></td>
<td>-</td>
<td>Byrne et al., 2014</td>
<td></td>
</tr>
<tr>
<td>ZnCl₂, FeCl₃</td>
<td>Extracellular</td>
<td><em>Clostridium</em> sp.</td>
<td>5-10, 3-8 (Zn-substituted)</td>
<td>Kim et al., 2015</td>
<td></td>
</tr>
</tbody>
</table>
Thus, the biological synthesis of nanoparticles (Zn and Fe) is a promising technology which enhances their efficiency, therefore providing a platform for their application in the various fields of medicine, agriculture etc.

1.5 Application of Zn and Fe nanoparticles as nanonutrients

Elements that are essential for plant growth and development are categorized into macronutrients and micronutrients. Micronutrients, although required in very small quantities, play a vital role in plant growth, development and yield, similar to macronutrients. Absence or deficiency of any element negatively affects the growth and yield of plants (Mengel et al., 2001). Micronutrients play very important roles like acting as co-factors in the enzyme systems, and are involved in vital processes like photosynthesis and respiration (Marschner, 2011; Mengel et al., 2001). Rehman et al. have reported Zn-deficiency as a foremost restraining factor of yield in many Asian countries (Rehman et al., 2012). To overcome Zn-deficiency in soils, there is a requirement for extensive application of chemical Zn fertilizers (eg-ZnSO₄ around 25-50 kg ha⁻¹, depending on the crop and the rate of deficiency) (Alloway, 2008), which leads to environmental pollution (eutrophication, water contamination) and soil quality degradation. Thus, in order to combat these problems of chemical fertilizers, the possibility of using nanoparticles in the field of agriculture is emerging as a potential boon to farmers.

Researchers are working on the efficacy of nanoparticles with respect to plant growth and yield. Reduced particle size affects the efficacy of fertilizers. Mortvedt have reported that increase in surface area of a granular fertilizer increases the suspension rate of less soluble fertilizers in water like ZnO. The reduced size also increases the number of particles per unit weight of applied Zn, which indicates that
more soil would be affected, thus preventing repeated application of fertilizer (Mortvedt, 1992).

The usage of nanoparticles as nanonutrients help in increasing the efficiency of fertilizer with comparatively lower dosage (Prasad et al., 2012), which in turn signifies an economical solution. Prasad and his co-workers have tested the efficiency of ZnO nanoparticles on the germination, growth and yield of peanut. Field trials were carried out through foliar application of nano ZnO particles at 15 times lower dosage compared to bulk ZnSO₄. Treatment of nanoscale ZnO (25 nm mean particle size) at 1000 ppm concentration on peanut plant promoted 100 % germination and 34 % higher pod yield as compared to bulk ZnSO₄ whereas higher nanoparticles concentration (2000 ppm) showed inhibitory effect.

Higher concentration of nanoparticles as nanofertilizers can damage plant growth. Vinoth Kumar and Udayasoorian have shown the toxic effects of concentration as high as 2000 mg L⁻¹ of ZnO, TiO₂ and Al₂O₃ nanoparticles on the growth parameters, such as decrease in germination percentage (25.7 %, 21.4 %, 22.9 %), root (75.71 %, 62.15 %, 63.28 %) and shoot length (74 %, 58 %, 68 %) and vigour index (81.94 %, 69.46 %, 74.07 %), respectively, as compared to control in the case of maize plant (Dr. K. Vinoth Kumar, 2014). A similar report was given by Lin and Xing where commercially available Zn (35 nm) and ZnO (15-25 nm) nanoparticles at a higher concentration 2000 mg L⁻¹ have shown seed germination inhibition in the case of ryegrass and corn, respectively (Lin & Xing, 2007). Comparative study indicated that lower concentration of nano Zn (10 ppm) and higher concentration of bulk Zn (100 ppm) have shown similar increase in germination percentage when
applied on *Macrotyloma uniflorum* (Lam.)Verdc (horse gram) (Gokak & Taranath, 2015).

Nualgi Nanobiotech in Bangalore have developed and patented a product called NUALGI. This product contain the micronutrients P, Ca, Mg, Fe, Mn, Cu, Zn, B, S, Co, Mo (in the range of 20-150 nm) absorbed on silica, and is very cost-effective. The product has proven to be useful in the treatment of micronutrient deficiencies through foliar application, the recommended dosage being 100 g L\(^{-1}\) of water to 1 acre of crops (Indian Patent No. 209364, dated 27/08/07; http://www.nualgi.org/foliar-spray-liquid-fertilizer).

Mazaherinia and co-workers have tested the efficiency of nano iron oxide particles of two sizes 25-250 nm, on the concentrations of Cu, Mn, Zn and Fe (0, 0.05, 0.1, 0.5 and 1 %) in wheat plant. For comparison, treatments were set up with normal iron oxide (0.02-0.06 mm). Significant increase of 80.1mg Kg\(^{-1}\) of Fe in plant was observed on treatment with nano-iron oxide (at 1% concentration) due to increased solubility and availability of nano iron oxide particles, whereas reduction was observed in the case of Mn due to negative interaction with iron in soil. On the other hand, Zn and Cu concentration in plant was significantly increased on treatment with normal iron oxide as compared to nano iron oxide due to the increased solubility of Zn and Cu in acidic soil because of acidic properties of iron oxides (Mazaherinia et al., 2010).

The effect of iron nanofertilizer was tested on spinach by Moghadam and coworkers. They reported that 4 kg ha\(^{-1}\) application of nanofertilizer enhanced the leaf weight by 58 % and 47 % of leaf area index was increased as compared to the control (Moghadam et al., 2012a). Mitra et al. tested the effect of spraying time and
concentration of iron nanoparticles on wheat plant. Their report indicated that no significant effect was observed on combination basis of spraying concentration with spraying time, however, on individual basis, 0.04 % of Fe concentration showed a significant increase in the spike weight (24.36 %), 1000 grain weight (15.66 %), biologic yield (6.91 %), grain yield (13.87 %) and protein content (19.39 %), as compared to the control. The first spraying time of nanoparticles also showed significant increase from the second spraying which reduced the spike weight (3.75 %), 1000 grain weight (4.04 %), biologic yield (99.04 %), grain yield (5.36 %) and protein content (4.75 %) (Bakhtiari et al., 2015). Iron and copper nanoparticles have shown stimulatory and inhibitory effect respectively on germination and growth of wheat (Triticum aestivum) (Yasmeen et al., 2015). They also studied and compared the effect of soaking seeds in nanoparticles suspension (NPD), with seeds incubated with nanoparticles suspension (DNP). In the case of iron nanoparticles, NPD seeds have shown reduced root growth and increased shoot growth, whereas deteriorating effects were observed in the case of copper nanoparticles, however, DNP treatments have shown overall retardation effects on wheat.

Among the different methods, plant agar method (i.e. dual agar culture media alongwith varying nanoparticles concentrations) have been reported to inhibit the effect of ZnO nanoparticles on mung (Vigna radiata) and gram (Cicer arietinum) seedlings (Mahajan et al., 2011). Maximum effect was observed at 20 ppm for mung and 1 ppm for gram seedlings whereas the concentrations higher than this showed inhibition. The concentration of nanoparticles exposed to the seeds and seedlings greatly affected the uptake and accumulation. Jayarambabu and co-workers also reported that 20 mg (among 20 mg, 40 mg, 60 mg and 100 mg tests) of ZnO nanoparticles showed 100 % seed germination, 84.21 % increase in root length
and 7.2 % increase in shoot length of Mungbean Seeds (*Vigna radiata* L.) from control (Jayarambabu et al., 2014).

Biosynthesized ZnO nanoparticles were tested as a nanofertilizer by Tarafdar et al. on Pearl millet (*Pennisetum americanum*). Nanoparticles were synthesised using fungus, *Rhizoctonia bataticola* TFR-6 from salt solution of aqueous zinc oxide as a precursor. Foliar application of 16L ha⁻¹ (10mg L⁻¹) of ZnO nanoparticles showed significant increase in the plant dry biomass (12.5 %), grain yield (37.7 %), root length (4.2 %), shoot length (15.1 %), plant zinc concentration (10.4 %), photosynthetic pigment chlorophyll (24.4 %) and total soluble leaf protein (38.7 %), compared to the control (Tarafdar et al., 2014). There are reports on nanoparticles taken up by the plant cell either through the stomata or vascular pathway (Eichert et al., 2008; Raliya & Tarafdar, 2013; Wang et al., 2013), which might boost metabolic activities of plant cells leading to increased crop production.

Thus, nanoparticles due to their efficacy in terms of lower dosage, plant yield, application cost and environment protection, can be a potential substitute to the application of chemical fertilizers, although considerable research still needs to be carried out with respect to their efficiency.

### 1.6 Conclusion

In summary:

- Ever increasing generation of waste worldwide is threatening the environment and human health. Waste contains potentially usable metals and so various methods of extraction for these metals are being developed. The challenge remains to find more environmental friendly and economical
ways of extraction than existing technologies. In this regard, bioleaching of waste for the extraction/recovery of metals is of interest.

- A large body of research and development exists with respect to the microbial-mediated synthesis of nanoparticles. Reports support a relationship between higher metal tolerance ability of microorganisms and synthesis of nanoparticles. Biosynthesis is advantageous as the nanoparticles formed are capped with protein, which enhances the solubility and stability of these particles and their encapsulated metals. This property along with their other properties of high surface area to volume ratio makes them suitable for biomedical and industrial applications.

- Although chemically synthesized nanoparticles (Zinc oxide, Iron oxide etc.) have been reported, further research needs to be carried to study the efficacy of biosynthesized nanoparticles, particularly in the field of agriculture as nanonutrients, pesticides and related products.

1.7 Aims of the study

The Rationale of this project was based on the following hypothesis:

“Wastes dumped in the environment, can potentially be used as a resource to cater to worldwide problem of nutrient deficiencies of zinc and iron”

The research aims were defined as follows:

1. The selection of waste resources

2. Isolation and purification of metal-tolerant organisms from the selected waste
3. Screening of collected isolates for their efficiency to bioleach and biosynthesize nanoparticles. And subsequent characterization of selected isolates

4. Standardization and optimization for scaling up bioleaching and biosynthesis of nanoparticles

5. Functionalize the nanoformulation and studying their assimilation in plants as nanonutrients
Chapter 2

*Isolation, screening, selection and characterization of fungal isolates for the biosynthesis of nanoparticles from jarosite and iron ore tailings*
2.1 Introduction

Ever increasing generation of waste worldwide is negative for the environment and as well as human health. Mining waste includes waste from the extraction and processing of mineral resources. This hazardous waste is dumped in ponds and landfill areas around mines and extraction plants globally. This thus becomes a burden on the environment due to release of toxic metals like arsenic, mercury and lead. Microbial action on these ores can lead to acid drainage. These rejected minerals and rocks can also damage the surrounding soil, vegetation and aquatic life (Franks et al., 2011). Thus managing/treating and utilizing this hazardous waste is an important challenge.

Jarosite and iron ore tailing are two such wastes that are being released in large amounts and are acting as a menace to the environment. The leaching plant’s main aim is to dissolve and recover zinc present in the calcine as a solid free, pre-purified neutral ZnSO₄ solution. Jarosite is released as a solid waste during the hydrometallurgical leaching of concentrates in lead-zinc smelters (Figure 2.1). A conventional hydro-metallurgical process, such as the Roast-leach-electrolysis (RLE) process, produces about 80 % of the world’s zinc (Chen & Dutrizac, 2001; Er. Nitisha Rathore, 2014; Havanagi et al., 2012; Katsioti et al., 2005). Jarosite mainly contain oxides of zinc, iron and sulphur along with calcium, aluminium, silica, lead and magnesium, although in less than 7 % concentration (Asokan et al., 2006). About 3220 MT of iron ore is produced globally, both for domestic use and export markets (http://www.ironorefacts.com/the-facts/iron-ore-global-markets). A major concern is the release of tailings along with other rejects during the extraction process (Figure 2.2) in the tailing dams.
Figure 2.1 Working of Zinc extraction plant.

(http://www.epa.gov/epawaste/nonhaz/industrial/special/mineral/pdfs/part10.pdf)

Figure 2.2 Mining process and generation of waste (Kuranchie et al., 2013; Yellishetty et al., 2008).
The concentration and availability of heavy metals in the polluted environment in and around mines and other polluting industries, along with the nature of metals and temperature, effects the microbial population thriving in those conditions. Some fungi are known to thrive in these stressed conditions, as they can tolerate extremes of temperature, pH and heavy metal concentrations (Baldrian, 2003; Gavrilescu, 2004). They are known to survive due to various biological mechanisms like biosorption to the cell wall, metal transformation, extra and intracellular precipitation (Baldrian, 2003; Congeevaram et al., 2007; Srivastava & Thakur, 2006). These diverse characteristics are involved in microbial leaching processes. Bioleaching refers to the extraction of metals from ores etc. through different mechanisms. There are many reports on fungi being used as bioleaching agents from waste materials (Bosshard et al., 1996; Dacera & Babel, 2008; Khan et al., 2014b; Madrigal-Arias et al., 2015), ores (Biswas et al., 2013; Mishra & Rhee, 2014; Mulligan et al., 2004) and minerals (Amiri et al., 2011; Anjum et al., 2010; Brisson et al., 2016; Brisson et al., 2015; Hosseini et al., 2007). According to these studies, organic acids such as citric acid and gluconic acid, exogenously produced by the fungus, can help in leaching of metals due to their chelating or reducing abilities. This ability of microbes to tolerate high metal ion concentrations, have encouraged scientists to use these microbes as eco-friendly nanofactories for biological synthesis of nanoparticles (Duran et al., 2005; Khan et al., 2014b).

The aim of this study was to firstly identify the mining wastes, which are being released and dumped in the environment at a large scale and have high concentration of zinc and iron content (major soil nutrient deficiencies globally), followed by the isolation of fungi from them. These fungal isolates were then screened for their bioleaching and nanoparticles biosynthesis efficacy and this was
confirmed using Transmission electron microscopy (TEM) and Energy dispersive X-ray spectrum (EDX) analysis. The fungal strains showing promising results were later morphologically characterized through microscopic analysis using Scanning electron microscopy (SEM), along with molecular identification.

2.2 Materials and Methods

2.2.1 Chemicals used

All chemicals used in this study were purchased from Fischer Scientific (Mumbai, India) and were of analytical grade. Potato dextrose agar (PDA) and Mycological peptone used were procured from HiMedia (Mumbai, India) and were sterilized by autoclaving at $120^\circ C$ for 15 min at 15 psi before use.

2.2.2 Sampling site

Jarosite (light yellow in colour) was collected from the jarosite pond secure landfill at the Zinc Smelter, Debari, Hindustan Zinc Limited, Udaipur, in Rajasthan, India (Figure 2.3). This is a Hydrometallurgical zinc smelter with production capacity of 88,000 MT of zinc per annum. It produced around 69,385 MT in the year to March 2015 (http://www.hzlindia.com/operations.aspx?ID=main_6). The smelter is located at Debari, 13 kms from Udaipur. Iron ore tailings (brick red in colour) were collected from Codli mines, Goa, India (Figure 2.4). Codli mine is one of the largest mining sites with an annual production allowance of about 5.5 MT, in India (http://www.vedantalimited.com/our-operations/iron-ore.aspx). The samples collected were crushed and ground to powder and dried.
2.2.3 Elemental analysis and particle size imaging of Jarosite and Iron Ore Tailings

Acid digestion of jarosite and iron ore tailings was carried out to record the total zinc and iron present using Method 3050B (Arsenic et al., 1996). Finely powdered 0.5 g of samples was refluxed for 10 min with 10 mL of 1:1 HNO₃, 5 mL of...
concentrated HNO₃ was added, and the mixture refluxed for 30 min, after which heating was continued until a final volume of 5 mL was attained. The sample was later cooled and 2 mL water and 3 mL of 30 % H₂O₂ were added, followed by addition of 1 mL aliquots of H₂O₂ until bubbling subsided. Volume was reduced to 5 mL. Based on the atomizer available (flame atomizer), 10 mL of concentrated HCl was added for digestion and the samples were again refluxed for 15 min. Finally, they were filtered and analyzed by Flame Atomic Absorption Spectroscopy (FAAS) using an Atomic Absorption Spectrometer, Thermo, iCE 3500. Bioavailability analysis was carried out through DTPA-extraction (Lindsay & Norvell, 1978; Sharma et al., 2006). DTPA (diethylenetriamine penta-acetic acid) extraction solution was prepared \{(0.005 M DTPA, 0.01 M CaCl₂, 0.1 M TEA (triethanolamine)} at pH 7.3 (adjusted using HCl). 40 mL of this solution was added to 20 g of air-dried sample in a conical flask. The flask was then covered with stretchable parafilm and kept on a shaker (120 rpm) for 2 hrs. After 2 hrs, the suspension obtained was filtered using Whatmann no.42 filter paper. DTPA was chosen because it helps in providing the favorable combination of stability constants for simultaneous complexing of Fe, Mn, Zn and Cu. pH 7.3 buffered with TEA prevented excess dissolution of trace metals and CaCl₂ reduced the dissolution of CaCO₃ from calcareous soils.

Elemental analysis was also recorded through Energy dispersive x-ray spectrum (EDX). Samples were prepared for TEM and EDX analysis by drop-casting on a carbon-coated copper grid after sonication for 20 min. The sample was then air-dried prior to analysis. TEM micrographs and EDX spectrum were taken at an accelerated voltage of 200 kV using TECNAI G² T20 TWIN (Netherlands) and EDAX Instruments, respectively.
2.2.4 Isolation and purification of fungal strains

Various fungal strains were isolated from jarosite and iron ore tailings using culture enrichment technique. 21.27 g (10,000 ppm Zn) of jarosite and 17.3 g (20,000 ppm of Fe) of tailings were individually suspended in 100 mL of mycological peptone. After 48 hrs of incubation (140 rpm, 30±2°C), 100 μL of sample was plated on potato dextrose agar (PDA) plates. The concentration of Zn in the case of jarosite and Fe in the case of tailings was successively increased in the suspension from 10,000 ppm to 40,000 ppm using ZnSO₄·7H₂O (Qualigens, Mumbai, India) and 20,000 ppm to 50,000 ppm using FeSO₄·7H₂O (HiMedia, Mumbai, India), respectively, by filter sterilization using 0.22 μmmdi sterile syringe filters (Advanced Microdevices Pvt.Ltd., Ambala Cantt., India). The standard spread plate technique was used to isolate the filamentous fungi on PDA plates after necessary dilution (up to 10⁻⁴) in three replicates. The plates were then incubated at 28±1°C for 72 hrs (Joshi et al., 2011). The colonies obtained were picked and subcultured on PDA plates for further purification. These fungal isolates obtained from jarosite and tailings were then subjected to 40,000 ppm Zn and 50,000 ppm Fe, respectively, to check for metal tolerance. Isolates thus obtained, were further purified on fresh PDA plates and later screened for bioleaching and nanoparticles biosynthesis efficiency.

2.2.5 Bioleaching screening of fungi

The fungal strains isolated from jarosite (J1-J5) and iron ore tailings (T1-T6) were screened for their bioleaching and nanoparticles biosynthesis efficiency.
2.2.5.1 Microorganism and growth

The fungal strains were maintained on PDA plates. Fungus was then inoculated in 100 mL of PDB in 250 mL Erlenmeyer flasks. The inoculated flasks were then incubated at 28°C for 96 hrs on a rotary shaker at 140 rpm. The fungal biomass was harvested by centrifugation (Microcentrifuge, Heraeus Biofuge-stratos) at 12000 rpm for 20 min at 25°C followed by washing thrice with sterile Milli Q (MQ) water under aseptic conditions, so as to remove the entire culture medium. The harvested mycelium was then used for further studies. The same protocol was followed for all the fungal isolates.

2.2.5.2 Bioleaching and biosynthesis of nanoparticles

Approximately, 20 g of washed biomass was re-suspended in 100 mL of autoclaved MQ so as to obtain the cell-free filtrate required for further studies (Raliya, 2013). The inoculum was again subjected to rotary shaking at 140 rpm, 28°C for 96 hrs. After re-suspension, the biomass was filtered using Whatmann filter paper no.1 and the cell-free filtrate containing the extracellular proteins and organic acids was used for further studies. 10 g of jarosite/ 10 g of tailings were then mixed in 100 mL of the filtrate and kept on a shaker at 28°C (140 rpm) for 96 hrs. The cell-free filtrate (without jarosite/tailings) was used as a control. The bioleachate obtained was then characterized as described below.

2.2.5.3 Characterization technique using TEM and EDX

Samples were prepared for TEM and EDX analysis by drop-casting on a carbon-coated copper grid after sonication for 20 min. The sample was then air-dried prior to analysis. TEM micrographs and EDX spectra were taken at an accelerated
voltage of 200 keV using TECNAI G² T20 TWIN and EDAX Instruments (Netherlands), respectively.

2.2.6 Characterization of fungi

Fungal strains (2 from Jarosite and 2 from Iron ore tailings), which were selected on the basis of their leaching efficiency and nanoparticles biosynthesis capability, were identified on the basis of monographs (Nyongesa et al., 2015; Samson et al., 2011) and their macro as well as micro morphological features captured by Scanning Electron Microscopy. The fungal isolates were preserved using 50 % glycerol in sterile MQ and stored at -80°C.

2.2.6.1 Scanning electron microscopy (SEM)

Morphological features of all the fungal isolates were observed through SEM (Carl Zeiss, Oberkochen, Germany). The fungal isolates were subcultured on PDA and incubated at 25°C for 4-5 days. After incubation, fungal discs were taken and then immersed in fixative solution (modified Karnovsky’s fixative containing 2.5 % glutaraldehyde, 2.5 % paraformaldehyde, 0.05 M cacodilate buffer and 0.001 M CaCl₂) at pH 7.2. Cacodilate buffer was then used to wash the discs (thrice for 10 mins each), followed by post-fixation in 1 % osmium tetraoxide solution and water for 1 hr (Silva et al., 2011). The samples were then washed with sterile distilled water three times and subjected to dehydration in acetone (25 %, 50 %, 75 %, 90 % and 100 %) for 10 min, followed by critical point drying (CPD) (Emitech K850, Berkshire, U.K.). The samples were then assembled on double-sided carbon tape placed on aluminium stubs and coated with gold-palladium in a sputter coater (Quorum Technologies SC7620, Berkshire, U.K.) and viewed in SEM at an accelerating voltage of 10 kV.
2.2.6.2 Molecular characterization of fungi

2.2.6.2.1 DNA extraction

Two days old culture (of each isolate) grown in PDB was centrifuged at 8,000xg for 10 min at 4°C. The fungal biomass was washed three times with sterile MQ water. Around 100 mg of washed biomass was crushed in Liquid N2 and then the total genomic DNA was extracted using DNeasy Plant MiniKit (Qiagen, Germany) according to manufacturer’s protocol, which was as follows: 400 μL of Buffer AP1 was added to the crushed cells in 1.5 mL microcentrifuge tube and gently mixed followed by addition of 4 μL of RNaseA. The solution was vortex and incubated for 10 min at 65°C. The tube was inverted two-three times during incubation for a uniform reaction. 130 μL of Buffer P3 was added and mixed gently. The sample was incubated on ice for 5 min. The lysate was centrifuged at 14000xg for 5 min at room temperature (RT). Then the lysate was pipetted into a Q1A shredder spin column placed in a 2 mL collection tube, which was again centrifuged at 14000xg for 2 min at RT. The flow-through was transferred into a new tube without disturbing the pellet, if present. 1.5 volume of Buffer AW1 was added and mixed by pipetting. 650 μL of mixture was transferred into a DNeasy minispin column placed in a 2 mL collection tube. The flow-through obtained was discarded after centrifugation at 8000xg for 1 min at RT. The same was repeated with the remaining sample. The spin column was placed into a new 2 mL collection tube, 500 μL of Buffer AW2 was added and centrifuged for 1 min at 8000xg, RT. The flow-through was discarded. Another 500 μL of Buffer AW2 was added and centrifuged at 8000xg for 2-3 min. The spin column was then transferred to a new 2 mL microcentrifuge tube (column has to be removed very carefully, so that it
doesn’t come in contact with the flow-through). The sample was eluted in 25 μL of elution buffer AE consisting of tris HCl at pH 8.0. The mixture was then incubated for 5 min at RT followed by centrifugation at 8000xg for 1 min. The elution step was repeated. The extracted DNA was stored at -20°C for further use.

2.2.6.2.2 PCR amplification

Amplification of extracted DNA was done using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC TCC TAT TGA TAT GC-3') as the forward and reverse primers, respectively, as described by White and co-workers (White et al., 1990). The final PCR reaction mix was of 25 μL reaction volumes containing 2.5 μL of 10x buffer, 1.5 μL of MgCl2 (25 mM), 0.5 μL of dNTP (10 mM), 1 μL of ITS1 primer (20 pM), 1 μL of ITS4 primer (20 pM), 0.2 μL of Taq Polymerase (2.5 U), 3 μL of DNA sample (5 μg mL⁻¹), and remaining volume was made up using sterile MQ water. The PCR reaction was carried out in Veriti 96-well Thermal Cycler (Applied Biosystems, Massachusetts, U.S.A) with conditions as follows: Denaturation for 5 min at 94°C, 33 elongation cycles (50 s at 95°C, 50 s at 56°C, 1 min at 72°C) final extension for 10 min at 72°C and lastly storage at 4°C. Negative controls were used to confirm the absence of contamination. The final products were analyzed by electrophoresis on 1.2 % Agarose (Sigma-Aldrich, St. Louis, U.S.A). The amplified DNA was cut from the gel and purified using a Gel extraction kit (Qiagen, Germany).

2.2.6.2.3 Ligation, transformation and cloning

The purified PCR product was ligated using 2X Rapid Ligation buffer. The master mix (10 μL) was prepared as follows: 5 μL of 2X Rapid ligation buffer, 1 μL of pGEM® - T Easy Vector (50 ng) (Promega, U.S.A), 2 μL of PCR Product and T4
DNA ligase (3 Weiss units mL⁻¹). The final volume was made up using deionized water. 0.5 mL tubes known to have low DNA-binding capacity was used. The reactions were mixed by pipetting and incubated overnight at 4°C to allow the ligation reaction to stabilize and increase the number of transformants. The next day, transformation was confirmed using the competent cells (E. coli DH5-α). The protocol used was as follows: Cells were kept on ice. 10 μL of ligated sample was added to 100 μL of the competent cells. The reaction vials/eppendorfs were incubated for 30 mins on ice with gentle tapping after 10 and 20 min. The cells were then subjected to heat shock at 420°C for 60 s followed by incubation on ice for 2 min. About 600-800 μL of SOC medium (Super Optimal Broth with glucose for Catabolite repression) (HiMedia, Mumbai, India) was added to the eppendorf tubes. Reactions were incubated at 370°C for 1 hr at 600 rpm, followed by centrifugation at 7000 rpm for 1 min at RT. Supernatant was removed, leaving about 100 μL to dissolve the pellet. The samples were then plated on X-Gal-IPTG-Ampicillin-LA plates, which were then incubated at 370°C overnight. The next day the plates were subjected to a blue - white screening. Half of each white colony obtained was then added to 10 μL of PCR reaction mix [MQ, PCR buffer, MgCl₂, Primers {M13FP (5’- GTA AAA CGA CGG CCA G-3’) + M13RP (5’- CAG GAA ACA GCT ATG AC- 3’)}, dNTPs, Taq polymerase]. The remaining half of the colony was streaked on LA-Amp plates and incubated at 370°C for 24 hrs. Colony-PCR was then carried out with the reaction mix. The final products were analyzed on 1 % Agarose. The streaked colonies corresponding to samples showing bright bands in the gel, were then inoculated in 5 mL Luria broth (LB) (HiMedia, Mumbai, India) for plasmid isolation. LB was incubated at 370°C, 180 rpm overnight.
The plasmid was extracted using a Geneaid High Speed Plasmid Mini Kit (New Taipei City, Taiwan) in 7 major steps: Harvesting, Re-suspension, Lysis, Neutralization, DNA Binding, Wash and DNA Elution, as per manufacturer’s instructions, which were as follows: 1.5 mL of cultured cells were taken to a microcentrifuge tube and then harvested by centrifugation at 14,000xg for 1 min. Supernatant was discarded. About 200 μL of PD1 Buffer with RNase A was added and the pellet was re-suspended by a vortex. Lysis was carried out by adding 200 μL of PD2 buffer. The reaction was mixed by inverting the tube gently 10 times and then was allowed to stand at room temperature for at least 2 min. This was followed by the addition of 300 μL of PD3 buffer, mixed by inverting the tube 10 times gently, and then centrifugation at 14,000xg for 30 s. The vortex was avoided so as to prevent shearing of genomic DNA. The supernatant obtained was added to the PD column placed in a 2 mL collection tube. The flow-through obtained after centrifugation at 14,000xg for 30 s was discarded and the column was placed back. In the 6th step, 400 μL of W1 buffer was added to the PD column and then centrifuged at 14,000xg for 30 s. The column was placed back in the collection tube after discarding the flow-through. 600 μL of wash buffer with ethanol was added in the column and then centrifuged at 14,000xg for 30 s. After discarding the flow through, the PD column was placed back in the collection tube. In order to dry the column matrix, it was centrifuged for 3 min at 14,000xg. Sample was eluted in 30 μL elution buffer and further analysed on 1 % Agarose gel. It was then cloned into an *Escherichia coli* plasmid library (Pitkaranta et al., 2008) using a master mix (0.3 μL of Enzyme *Eco*R1, 2 μL of 10X Cut Smart Buffer, 3 μL of Plasmid DNA of each isolate, remaining volume of 20 μL with sterile MQ water) for restriction
digestion. Plasmid DNA concentration was measured in duplicates using a Synergy H1 Hybrid Reader (Biotek, U.S.).

2.2.6.2.4 Phylogenetic analysis

Nucleotides similarity analysis was carried out by BLASTN against the sequences available in GenBank (Altschul et al., 1990). Construction of a Phylogenetic tree was performed using multiple sequences obtained from GenBank using interactive software Phylogeny.fr (http://phylogeny.lirmm.fr/phylo.cgi/simple_phylogeny.cgi).

2.3 Results and Discussion

2.3.1 Elemental analysis and particle size imaging of jarosite and iron ore tailings

Quantitative analysis of Jarosite and Iron ore tailings was carried out to determine the concentration of different metals present in the two substrates. Jarosite was observed to mainly constitute zinc (~34,000 ppm), iron (~38,000 ppm), sulphur (~11,000 ppm) and lead (~14,000 ppm), along with trace elements like copper and aluminium (Table 2.1). The most abundant metals observed in Iron ore tailings were iron (~42,000 ppm) and aluminium (~34,000 ppm) (Table 2.2). The results are presented as mean ± SD of samples setup in triplicates. Heterogeneity was observed when the data obtained was compared with other reports, both for jarosite and iron ore tailings (Acharya et al., 1992; Ilyas et al., 2013; Pappu et al., 2011). These differences may be due to the origin of substrate and various physical parameters like temperature and climate of the sample collection site. Analytical methods used to study the elemental composition might also contribute to the variation. Although the total concentration of Zn and Fe is quite high, the bioavailable form is low, that
is 740 ppm Zn and 14.512 ppm of Fe in the case of jarosite and tailings, respectively. This is probably due to the type of complexes in which these elements are present in jarosite and tailings (Er. Nitisha Rathore, 2014; Seh-Bardan et al., 2012).

TEM micrographs of the collected particles of jarosite (control) and iron ore tailings (control) showed irregularity in shape with size around 400±100 nm (Figure 2.5 A) and 600±100 nm (Figure 2.5 C), respectively. Jarosite is found in combination of hydrous sulfate of KFe$_3^+$($\text{OH}_6$($\text{SO}_4$)$_2$ which is an insoluble hydrophobic form and a hydrophilic soluble form $2\text{Fe}_2\text{O}_3\text{SO}_3.5\text{H}_2\text{O}$, released as a by-product of zinc purification and refining (Er. Nitisha Rathore, 2014). The presence of iron, sulphur, potassium, sodium and zinc in jarosite was further confirmed by EDX spectrum together with AAS analysis (Figure 2.5 B). In the case of tailings (Figure 2.5 D), iron, aluminium and silicon showed strong signals in the EDX spectrum.

Table 2.1: Chemical analysis of Jarosite for metal content.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Concentration (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td>33,504.55±15.61</td>
</tr>
<tr>
<td>Iron</td>
<td>37,912.79±0.70</td>
</tr>
<tr>
<td>Lead</td>
<td>14,162.74±9.15</td>
</tr>
<tr>
<td>Sulphur</td>
<td>10,042.31±3.27</td>
</tr>
<tr>
<td>Aluminium</td>
<td>5,122.50±7.11</td>
</tr>
<tr>
<td>Copper</td>
<td>284.0427±14.77</td>
</tr>
<tr>
<td>Silica</td>
<td>116.083±5.01</td>
</tr>
</tbody>
</table>
Table 2.2: Chemical analysis of Tailings for metal content.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Concentration (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td>435.50±86.10</td>
</tr>
<tr>
<td>Iron</td>
<td>41,190.93±1.16</td>
</tr>
<tr>
<td>Aluminium</td>
<td>34,530±5.18</td>
</tr>
<tr>
<td>Nickel</td>
<td>7.375167±0.06</td>
</tr>
</tbody>
</table>

Figure 2.5 Transmission electron micrograph and EDX spectrum of controls (A & B) Jarosite particles with an average size of 400 nm & (C & D) Iron ore tailings with an average size of around 500 nm and more respectively.

2.3.2 Isolation of fungi

The fungal strains were obtained based on their metal-tolerance ability. They were subjected to 40,000 ppm Zn (about 34,000 ppm Zn was recorded for Jarosite through AAS) and 50,000 ppm Fe (about 42,000 ppm Fe in tailings was recorded
through AAS). Nearly five strains from jarosite (Figure 2.6) and six from iron ore tailings (Figure 2.7) were isolated and purified through repeated sub culturing on PDA. Fungi are known to tolerate and help with the detoxification of metals through various processes. Reports are available for the isolation of members of this diverse group of fungi from metal-contaminated sites (Akhtar et al., 2013; Ilyas et al., 2013; Iram et al., 2013; Seh-Bardan et al., 2012). However, to the best of our knowledge, there are few reports on the isolation of fungi from jarosite (Oggerin et al., 2014; Oggerin et al., 2016). The fungi were coded as J1-J5 in the case of jarosite and T1-T6 in the case of iron ore tailings.

Figure 2.6 Colony morphology of 5-days old pure fungal isolates from Jarosite on PDA plates at 28±2°C.
2.3.3 Bioleaching and biosynthesis of nanoparticles

Under equivalent experimental conditions different isolated fungal cultures have shown varying percentage of leaching of zinc and iron, along with nanoparticles biosynthesis from jarosite and iron ore tailings. The variation in the inherent capability of leaching and nanoparticle biosynthesis of isolated fungal cultures may be attributed to the exogenously released different organic acids, such as gluconic, citric, oxalic acid, and enzymes such as reductases, that impact nanoparticles formation (Brisson et al., 2016; Brisson et al., 2015; Duran et al., 2005; Seh-Bardan et al., 2012).

2.3.3.1 Jarosite

The percentage of bioleaching and nanoparticle biosynthesis efficacy from jarosite, after 96 hrs of reaction under the same set of experimental conditions, with the
extracellular filtrate of all the five fungal isolates, is shown in Figure 2.8. The graph shows that J2 and J4 have maximum efficacy of about 57.8 and 67.07 %, respectively. J1 and J5 showed 16.5 and 2.16 %, respectively, with J3 showing the lowest efficacy.

Of the fungal isolates J1-J5 that were tested for bioleaching and nanoparticles biosynthesis efficiency in comparison with control jarosite, in terms of zinc, the two isolates J2 and J4 showed the highest efficiency, as observed by TEM and EDX analysis (Figure 2.9 & 2.10). Isolate J1 and J5 produced large agglomerated particles. For J3, there was no leaching observed for zinc. Isolates J2 and J4 showed maximum leaching efficiency, with TEM analyses showing nanoparticles formed in the range 20-50 nm and ±20 nm, respectively.

Figure 2.8 Graph indicating the bioleaching efficiency of the fungal isolates from jarosite.
Figure 2.9 TEM micrographs and their respective EDX spectra of bioleachate indicating the presence or absence of zinc bioleaching and biosynthesis of nanoparticles from Jarosite by J1-J3 fungal isolates.
Figure 2.10 TEM micrographs and their respective EDX spectra of bioleachate indicating the presence or absence of zinc bioleaching and biosynthesis of nanoparticles from Jarosite by J4-J5 fungal isolates.

2.3.3.2 Iron ore tailings

For iron ore tailings, different cultures (T1-T6) showed variation in their leaching and nanoparticles biosynthesis efficiency in terms of iron under the same experimental conditions. This variation in terms of percentage can be seen in Figure 2.11. T4 and T6 showed maximum efficacy of about 33.23 and 77.26 %, respectively. T1 and T5 showed 8.21 and 7.17 %, respectively, and isolates T2 and T3 showed no bioleaching ability.

TEM and EDX analysis of the bioleachates further confirmed the bioleaching efficiency of the fungal isolates (Figure 2.12 & 2.13). Two isolates (T4 and T6) showed to exhibit the highest efficiency. Isolate T1 resulted in the formation of
bigger agglomerates, whereas for the fungal isolates T2 and T3 no leaching was observed. TEM of T5 bioleachate showed formation of agglomerated particles, whereas TEM analyses of the isolates T4 and T6 showed nanoparticles with an average size of ±20 nm and maximum leaching of iron.

Figure 2.11 Graph indicating the bioleaching efficiency of the fungal isolates from tailings.
Figure 2.12 TEM micrographs and their respective EDX spectra of bioleachate (T1-T3 fungal isolates) indicating the presence or absence of iron bioleaching and biosynthesis of nanoparticles from tailings.
Figure 2.13 TEM micrographs and their respective EDX spectra of bioleachate indicating the presence or absence of iron bioleaching and biosynthesis of nanoparticles from tailings by T4-T6 fungal isolates.

2.3.4 Characterization of fungi

2.3.4.1 Morphological characterization through SEM

Preliminary identification of fungal isolates was carried out on the basis of morphological characteristics, which were similar to those described by Thom and Church (Kurtzman et al., 1987; Nyongesa et al., 2015; Thom & Church, 1918). *A. flavus* strain J2 showed velvety yellow to green mat on a PDA plate at 28±2°C (Figure 2.14 A). Culture appears red-brown on the reverse side. The conidiophores
showed variability in length, were rough and spiny in texture as seen in SEM images (Figure 2.14 B & C). They were found to be predominantly uniseriate with some biseriate, covering the vesicle completely with loosely packed phialides. Conidia were seen globose. *A. terreus* strain J4 colonies appear beige to buff to cinnamon on PDA at 28±2°C (Figure 2.15 A). Culture was yellow on the reverse side and there was usually the presence of yellow soluble pigments. The culture showed rapid to moderate growth. SEM images showed that the hyphae were septate. Conidial heads were columnar and biseriate (Figure 2.15 B & C).

*A. nomius* strain T4 showed velvety to floccose white and green mycelium growth on PDA plate (Figure 2.16 A). SEM images showed conidial heads to be uniseriate. Stipes were smooth (Figure 2.16 B & C). *A. aculeatus* strain T6 colonies on PDA (Figure 2.17 A) appeared coffee brown to black with white mycelial mat beneath and cinnamon to brown colour on the reverse. Conidial heads were uniseriate with globose vesicle (Figure 2.17 B & C).

Figure 2.14 (A) Colony morphology on PDA plate; SEM images showing structural morphology of fungal conidiophores (B) and spores (C) of *A. flavus* strain J2.
2.3.4.2 Molecular characterization of fungal isolates

The plasmid digested samples obtained were observed on Agarose gel (Figure 2.18) and consisted of around 1.5 Kb (1 Kb-900 bp plasmid and ~600 bp insert) when analyzed using a 1 Kb ladder. The plasmid DNA concentration obtained from J2, J4, T4 and T6 was 280.7, 346.5, 120.3 and 272.4 ng μL⁻¹ respectively. The
molecular identification of fungal isolates was carried out using 18S rRNA gene sequence analysis. The nucleotide sequences that were obtained were then compared using Basic Local Alignment Search Tool (BLAST) of NCBI followed by phylogenetic tree using the Maximum Likelihood Method (Edgar, 2004; Guindon et al., 2010). This analysis revealed that the isolates obtained in this study exhibited evolutionary closeness to many available sequences. Based on the evolutionary distance between the fungal strains, the phylogenetic search (Figure 2.19 A & B) showed that the isolates J2 exhibited 99 % homology to *A. flavus* strain 176 (GenBank Accession No. KP784374.1) and isolate J4 exhibited 99 % homology to *A. terreus* strain LCF17 (GenBank Accession No.FJ867934.1) respectively. Similarly, isolates T4 showed 98 % homology to *A. nomius* strain UOA/HCPF 12657 (GenBank Accession No. KC253960.1) and T6 showed 100 % match to *A. aculeatus* strain A1.9 (GenBank Accession No. EU833205.1) (Figure 2.20 A & B).

Figure 2.18 Agarose gel electrophoresis of the plasmid digested samples using *Eco*R1. Lane 1 denotes ladder (100 bp to 1000 bp); Lane 2 to 5 – J2; Lane 12 to16 - T4; Lane 19 to 22 – J4 and Lane 24 to 27 – T6.
Figure 2.19 Phylogenetic relationship of the 18S RNA sequences of fungal isolates from Jarosite based on their similarity to closely related sequences.

Figure 2.20 Phylogenetic relationship of the 18S RNA sequences of fungal isolates from tailings based on their similarity to closely related sequences.
2.4 Conclusion

Jarosite and iron ore tailings have been identified as two important waste materials based on their global abundance and negative effect on the environment. These two wastes have high concentrations of our elements of interest for agriculture, in particular zinc (~35,000 ppm) and iron (~42,000 ppm), as determined through AAS analysis. Five isolates collected from jarosite and six collected from tailings were obtained through culture enrichment technique. Out of them, the *A. flavus* strain J2 and *A. terreus* strain J4 from jarosite, and the *A. nomius* strain T4 and *A. aculeatus* strain T6 from tailings showed maximum leaching efficiencies of 57.8 (J2), 67.07 (J4), 33.23 (T4) and 77.26 % (T6), as measured by TEM and EDX analysis. To the best of our knowledge, this is the first study of its kind on bioleaching from Jarosite, till date. These fungal isolates could be further exploited for the eco-friendly biosynthesis of iron or zinc nanoparticles from the waste, which could then be potentially used as nanonutrients in agriculture to enhance plant growth.
Chapter 3

Bioleaching and biosynthesis of nanoparticles from Jarosite and Iron ore tailings
3.1 Introduction

Nanotechnology is the branch of science which deals with the design, fabrication, characterisation and application of structures and devices at the nanoscale (<100 nm) (Cao, 2004; Rao & Cheetham, 2001). Richard Feynman, an eminent physicist, mentioned nanotechnology as controlled miniaturization of matter (< 100 nm) at the molecular level where its properties are significantly different from that of bulk materials (Feynman, 1960). Living cells are recognized to be the best models to operate at nano levels with very high efficiency in order to perform various functions, from energy generation to targeted extraction of materials (Goodsell, 2004). The hybrid of nanobiotechnology provides insight into aspects of biology, ranging from drug delivery to bioremediation to combating nutrient deficiencies, and the production of active ingredients such as pesticides. Hence, the development of a reliable and eco-friendly biological process for the synthesis of nanoparticles is important for the application of nanotechnology in the field of biotechnology.

Some filamentous fungi are known to have high metal tolerance, easy biomass handling, easy to scale-up and intracellular uptake of metal (Shankar et al., 2003; Volesky & Holan, 1995). In addition to these capabilities, the extracellular secretion of metabolites such as reducing enzymes and organic acids by some fungi helps in bioremediation and biosynthesis of metallic nanoparticles through conversion of toxic metal ions into non-toxic metallic nanoparticles (Aung & Ting, 2005; Pradhan & Kumar, 2012; San et al., 2012; Wagner & Kohler, 2005). Furthermore, the extracellular nanoparticles are synthesized outside the cell and are devoid of cellular constituents, simplifying their isolation (Narayanan & Sakthivel, 2010).
A wide range of characterization techniques for nanoparticles are being applied, some of which are as follows:

UV-Vis Spectroscopy helps to study the unique optical properties of the nanomaterials (Burda et al., 2005). Transmission Electron Microscopy (TEM) and Energy-dispersive X-ray spectrum (EDX) are the techniques typically utilized for high resolution imaging of thin films of solid samples, for morphological and compositional analysis, along with High Resolution TEM (HRTEM), which helps to study the crystal structure using Bravais lattice and lattice parameters of nanorods and nanocrystals like XRD (Cullity & Stock, 2001; Wang et al., 2000; Wang, 2000). This also confirms the crystal structure of matters by calculating the crystal lattice constants of the particles using Bragg’s equation (Cullity & Stock, 2001). Fourier Transform Infrared spectroscopy (FTIR) is used to determine the chemical groups based on the vibrational frequencies unique to every specific bond, for example the 1400–1700 cm\(^{-1}\) region contains signals representative of –CO- and –NH- groups (Sarkar et al., 2014). Zeta potential helps to determine the stability of nanoparticles based on the electrostatic repulsion/attraction between particles.

The aim of this study was to optimize the bioleaching and biosynthesis of nanoparticles from jarosite and iron ore tailings using fungal cell-free extracts from *A. flavus* strain J2 and *A. terreus* strain J4 from jarosite, and *A. nomius* strain T4 and *A. aculateus* strain T6 from tailings. Here, we optimized the process by exploring the impact of growth kinetics, time of reaction and varying concentrations of substrate and cell-free extract. The optimized conditions were then applied for the culturing of these fungal isolates and the biosynthesis of nanoparticles from them.
3.2 Materials and Methods

3.2.1 Chemicals used

All chemicals used in this study were purchased from Fischer Scientific (Mumbai, India) and were of analytical grade. Potato dextrose agar and Potato dextrose broth used were procured from HiMedia (Mumbai, India) and were sterilized by autoclaving at 120°C for 15 min at 15 psi before use.

3.2.2 Fungal growth kinetics study through Ergosterol estimation

Growth kinetics studies were carried out for the four fungal strains (A. flavus strain J2, A. terreus strain J4, A. nomius strain T4 and A. aculateus strain T6) in order to determine the exponential phase. Ergosterol is the major sterol present in the cell membranes of filamentous fungi and monitoring its level is a useful method for estimating fungal biomass (Axelsson et al., 1995; Klamer & Baath, 2004; Steudler & Bley, 2015). 50 mg of fungal mycelium was taken at regular time interval of 24 hrs. The mycelium was ground using a mortar and pestle with liquid nitrogen, followed by the addition of 1 mL of absolute ethanol. This mixture was agitated for 30 s, kept in ice for 1 hr and then centrifuged for 5 min at 14,000 rpm. The supernatant was collected and a pellet was suspended in 1 mL of absolute ethanol and treated once again as described above. The two supernatants were pooled together, filtered using 0.22 mm nitrocellulose filters (Millipore, Darmstadt, Germany) and the filtrate was analysed for Ergosterol using the protocol of Lindblom (Mille-Lindblom et al., 2004) with some modification. Analysis was carried out using HPLC (CBM- 20A, Shimadzu, Kyoto, Japan) equipped with a quaternary pump (LC - 20AT), solvent degasser system (DGU - 20 A5),
autosampler (SIL – 20A) and diode array detector (SPDM – 20A). Inbuilt software (Shimadzu, LC solution) was used to control the HPLC pump and acquire data from the diode array. A C18 Phenomenex column (Gemini- NX 250 mm × 4.6 mm × 5 μm particle diameter) was used for the analysis. A series of ergosterol standards of varying range 10-50 ppm were prepared in ethanol. The standard peak was obtained with a UV detector set at 282 nm and a runtime of 20 min. The mobile phase was methanol (97 %) and water (3 %) at a flow rate of 1 mL min⁻¹ and the injected sample volume was 50 μL.

3.2.3 Microorganism and growth

All the fungal strains were maintained on PDA plates. Fungus was then inoculated in 100 mL of PDB in 250 mL Erlenmeyer flasks. The inoculated flasks were then incubated at 28±1°C on a rotary shaker at 140 rpm for 96 hrs-144 hrs, depending on the results, that is, based on variation in the time when exponential phase growth is achieved for different fungi, as measured using Ergosterol. The fungal biomass was then harvested by centrifugation (Microcentrifuge, Heraeus Biofuge-Stratos, U.S.A.) at 12000 rpm for 20 min at 25°C, followed by three subsequent washings with sterile MQ under aseptic conditions, so as to remove the culture medium. The harvested mycelium was then used for further studies. The same protocol was followed for all fungal isolates.

3.2.4 Optimization of bioleaching and biosynthesis of nanoparticles using fungal cell-free extract

Approximately, twenty grams of washed biomass of A. terreus strain J4 was re-suspended in 100 mL of autoclaved MQ so as to obtain the cell-free extract required for further studies (Raliya, 2013). The inoculum was again subjected to rotary
shaking at 140 rpm, 28±1°C for 96 hrs. pH of the filtrate was recorded at regular time intervals up until 144 hrs. After re-suspension, the biomass was harvested and the cell-free extract containing the extracellular enzymes and organic acids was used for further studies.

10 g of jarosite/10 g of tailings were then mixed in 100 mL of the filtrate and kept on a shaker at 28±1°C (140 rpm) for 96 hrs. The cell-free extract (without jarosite) was used as a control. The bioleachate was then filtered and subjected to further characterization for optimization of the following parameters:

3.2.4.1 Effect of reaction time on the bioleaching and subsequent biosynthesis of nanoparticles

Bioleachate obtained from the reaction between the fungal cell-free extract and jarosite/iron ore tailings was taken at regular time interval of 24 hrs up to 196 hrs to determine the effective time of bioleaching. This was confirmed through TEM and EDX analysis.

3.2.4.2 Effect of different concentrations of cell-free extract and substrate i.e. jarosite/iron ore tailings along with change in shaker speed

50 mL and 100 mL concentrations of cell-free extract of the fungal cultures (A. flavus strain J2, A. terreus strain J4, A. nomius strain T4 and A. aculeatus strain T6) were subjected to varying concentrations of substrate (1g, 5g and 10 g) and incubated at 28±1°C at 140 and 160 rpm shaking speed. The results were recorded after 96 hrs of reaction, since, after this time effective bioleaching of zinc and iron was observed from jarosite and tailings, respectively. All the samples were
observed for bioleaching and formation of nanoparticles through TEM and EDX analysis.

3.2.5 Bioleaching and biosynthesis of nanoparticles using fungal cell-free extract from jarosite and iron ore tailings

Optimization of bioleaching and biosynthesis of nanoparticles indicated that when 100 mL of fungal cell-free extract was subjected to 10 g of jarosite (for *A. flavus* strain J2 and *A. terreus* strain J4) and 10 g of iron ore tailings (for *A. nomius* strain T4 and *A. aculeatus* strain T6) for 96 hrs reaction time, resulted in the maximum amount of nanoparticles. Therefore, further optimization was carried out, as below:

100 mL of fungal cell-free extract was incubated with 10 g of jarosite and iron ore tailings at 28±1°C (140 rpm) for 24 hrs on a rotary shaker. The bioleachate from the reaction mixture was collected into new flask and stirred at 35±2°C for 3 hrs for the complete nucleation of metals with *A. terreus* strain J4 filtrate. Afterwards, the nucleated bioleachate was subjected to metal nanoparticles biosynthesis by increasing the pH (~8.0) of the reaction mixture using aqueous ammonia. The biosynthesized metal nanoparticles were harvested using centrifugation at 5,000 rpm for 10 min followed by washing three times with sterile MQ water. The dried biosynthesized nanoparticles powder was stored in amber coloured vials at room temperature under dry and dark conditions until used for further characterization.

3.2.5.1 UV-Vis Spectroscopy

The bioleachate containing nanoparticles was then subjected to UV-Vis spectrophotometric measurements using UV-Visible spectrophotometer (UV-2450,
Shimadzu, Japan) at a resolution of 1 nm. The scans were recorded in the range of 200-800 nm.

3.2.5.2 Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the samples were recorded using a Nicolet 6700 FT-IR, Thermo Fischer Scientific, U.S.A. This spectrum helps in identification of the capping agents responsible for biosynthesized nanoparticles stabilization. The spectrum was obtained by an average scan of 64 in the range 400–4000 cm$^{-1}$.

3.2.5.3 Determination of zeta-potential

50 $\mu$L of each sample (nanoparticles from jarosite and iron ore tailings) was diluted with 1 mL of MQ water for measurement of zeta potential. The samples were then transferred to a polycarbonate zeta cell (having gold plated electrode) and the potential was then measured at 25$^0$C (25 subruns) using Zeta Sizer Nano ZS90, Malvern, U.K. This was done to further confirm the presence of surface-bound proteins on nanoparticles indicated by the negative charge.

3.2.5.4 TEM, HRTEM, EDX and XRD analysis

Samples were prepared for TEM, HRTEM and EDX analysis by drop-casting on a carbon-coated nickel/copper grid after sonication for 20 min. The sample was then air-dried prior to analysis. TEM as well as HRTEM micrographs and EDX spectrum were taken at an accelerated voltage of 200 kV using TECNAI G$^2$ T20 TWIN (Netherlands) and EDAX Instruments, respectively. The X-ray diffraction (XRD) patterns of powder sample was recorded on MiniFlex™ II benchtop XRD system (Rigaku Corporation, Tokyo, Japan) operating at 40 kV and a current of 30
mA with Cu Kα radiation (λ = 1.54 Å). The diffracted intensities were recorded from 20° to 80° 2Θ angles.

### 3.3 Results and Discussion

#### 3.3.1 Fungal growth kinetics study

The HPLC responses when checked for linearity with Ergosterol standards gave good correlation coefficients (R²) of 0.9936 for jarosite (*A. flavus* strain J2 & *A. terreus* strain J4) and 0.9973 for tailings (*A. nomius* strain T4 & *A. aculateus* strain T6) (Figure 3.1 A & 3.2 A). On the basis of growth kinetic studies (Figure 3.1 B & C), 4-days (3rd-5th day exponential phase) and 5-days (4th-7th day exponential phase) old cultures of *A. flavus* strain J2 and *A. terreus* strain J4, respectively, were used for the bioleaching studies. Similarly, as exponential phase was observed between 4th-7th day and 4th-6th day for *A. nomius* strain T4 and *A. aculeatus* strain T6, respectively (Figure 3.2 B & C) thus, 6 and 5 days old cultures for T4 and T6 were targeted for bioleaching studies, respectively. The results are presented as mean±SD of samples set up in triplicates.
Figure 3.1 (A) Standard ergosterol graph; (B & C) Growth kinetics study of *A. flavus* strain J2 and *A. terreus* strain J4, respectively, by Ergosterol assay.

\[ y = 18685x \]
\[ R^2 = 0.9936 \]
Figure 3.2 (A) Standard ergosterol graph; (B & C) Growth kinetics study of *A. nomius* strain T4 and *A. aculeatus* strain T6, respectively, by Ergosterol assay.
3.3.2 Optimization of bioleaching and biosynthesis of nanoparticles using fungal cell-free extracts

Table 3.1 summarized the work carried out on the study of different concentrations of fungal cell-free extract and varying concentrations of substrate (jarosite and iron ore tailings). 5 g and 10 g of jarosite/tailings, when treated with 50 mL and 100 mL of cell-free extract \([A. \text{ flavus} \text{ strain } J2 \ (30 \%, \ 36 \%) \text{ and } A. \text{ terreus} \text{ strain } J4 \ (41 \%, \ 46 \%)]\) in case of jarosite; \([A. \text{ nomius} \text{ strain } T4 \ (20 \%, \ 25 \%) \text{ and } A. \text{ aculateus} \text{ strain } T6 \ (50 \%, \ 46 \%)]\) in case of tailings], respectively, showed good bioleaching and subsequent biosynthesis as compared to other combinations. This data demonstrated that concentrations of substrate as low as 1 gm showed negligible bioleaching and nanoparticles biosynthesis, followed by a lower percentage of leaching efficacy for 5 g/100 mL (too diluted) and 10 g/50 mL (highly concentrated) reactions ranging from 6-11 %. This could be due to the insufficient interaction of available substrate in the case of 1 g and 5 g/100 mL of jarosite and tailings, respectively, in the reaction mixture. Variation in speed of shaking (140 rpm or 160 rpm) didn’t impact the bioleaching and biosynthesis of nanoparticles. A drop in pH from 7.05 to 5.21 after 96 hrs of reaction time was observed (Figure 3.3), with pH remaining almost constant thereafter. The results are presented as mean ± SD of samples set up in triplicates. A similar drop in pH was observed for all four fungal isolates \([A. \text{ flavus} \text{ strain } J2 \text{ and } A. \text{ terreus} \text{ strain } J4 \text{ from Jarosite}; A. \text{ nomius} \text{ strain } T4 \text{ and } A. \text{ aculeatus} \text{ strain } T6 \text{ from iron ore tailings}].\) The leaching efficiency of these fungi is probably related to organic acids released by the fungal biomass, which contribute to the fall in pH. Oxalic acid and citric acid have been reported to be involved in the leaching process (Ambreen et al., 2002; Aung & Ting, 2005; Raliya, 2013; Ren et al., 2009; Santhiya & Ting, 2005).
Table 3.1 Effect of different parameters on the bioleaching and biosynthesis of nanoparticles from jarosite and iron ore tailings using fungal cell-free extract.

<table>
<thead>
<tr>
<th>Substrate Concentration/ Concentration of cell-free extract</th>
<th><em>A. flavus</em> strain J2 (Jarosite)</th>
<th><em>A. terreus</em> strain J4 (Jarosite)</th>
<th><em>A. nomius</em> strain T4 (Iron ore tailings)</th>
<th><em>A. aculateus</em> strain T6 (Iron ore tailings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1g/50mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1g/100mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5g/50mL</td>
<td>~30 %</td>
<td>~41 %</td>
<td>~20 %</td>
<td>~50 %</td>
</tr>
<tr>
<td>5g/100mL</td>
<td>~10 %</td>
<td>~9.3 %</td>
<td>~8 %</td>
<td>~11 %</td>
</tr>
<tr>
<td>10g/50mL</td>
<td>~8 %</td>
<td>~10 %</td>
<td>~6 %</td>
<td>~10 %</td>
</tr>
<tr>
<td>10g/100mL</td>
<td>~36 %</td>
<td>~46 %</td>
<td>~25 %</td>
<td>~46 %</td>
</tr>
<tr>
<td>Shaker speed (rpm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>~36 %</td>
<td>~46 %</td>
<td>~20 %</td>
<td>~22 %</td>
</tr>
<tr>
<td>160</td>
<td>~36 %</td>
<td>~49 %</td>
<td>~21 %</td>
<td>~27 %</td>
</tr>
</tbody>
</table>

Figure 3.3 pH of MQ with washed biomass recorded at various time intervals to confirm the release of organic acids.
3.3.3 Bioleaching and biosynthesis of nanoparticles from jarosite and iron ore tailings

3.3.3.1 Visual Observations

Figure 3.4 shows the process for bioleaching and subsequent biosynthesis of nanoparticles from jarosite using a cell-free extract of *A. terreus* strain J4. A similar protocol was followed for other fungal strains for biosynthesis from jarosite and iron ore tailings. The colour change from yellow to brick red for jarosite and brick red to darker shade for iron ore tailings indicated the biosynthesis of nanoparticles. Figure 3.5 (A & B) shows the dried biosynthesized nanoparticles from jarosite and tailings respectively.

Figure 3.4 Protocol for bioleaching and biosynthesis of nanoparticles from Jarosite.
Figure 3.5 Biosynthesized nanoparticles from (A) Jarosite-colour changed from white yellow to brick red, and (B) Iron ore tailings- colour shifted to a darker tone.

3.3.3.2 UV-Vis Spectroscopy

The UV-Vis spectra (Figure 3.6) showed a well-defined surface plasmon band centered at around 310-370 nm, characteristic of oxide nanoparticles. Absorption between 270-280 nm suggested the presence of aromatic amino acids like tryptophan, tyrosine and phenylalanine in the extracellular cell-free extract. Proteins and peptides containing these residues might be released under stress-conditions by the fungus in sterile MQ water and some of these proteins are probably bound on the surface of the nanoparticles (Khan et al., 2014a; Mazumdar & Haloi, 2011; Zak et al., 2011).
Figure 3.6 UV-spectra of biosynthesized nanoparticles using cell-free extract of (A, B) *A. flavus* strain J2 and *A. terreus* strain J4; (C, D) *A. nomius* strain T4 and *A. aculateus* strain T6 from jarosite and iron ore tailings, respectively.

### 3.3.3.3 FTIR

The samples kept in -80°C were analyzed to identify the molecules that may be responsible for the observed bioleaching activity by the fungal cell-free extract. Figure 3.7 (A-D) shows the FTIR spectrum with an absorption peak at ~1640 cm⁻¹, corresponding to the amide I functional group from the carbonyl stretch of proteins (Sarkar et al., 2014; Sathyavathi et al., 2010) and ~3270-3310 cm⁻¹ the –NH group of amines (Ninganagouda et al., 2014; Sundaram et al., 2012). The absorption bands observed around ~588 cm⁻¹ correspond to the R-CH (Singh et al., 2010), and around ~432 cm⁻¹ are attributed to metal-oxide stretching vibrations (Becheri et al., 2008; Jain et al., 2013). Thus, the presence of these functional groups indicate that
proteins form a capping layer on the nanoparticles, preventing them from agglomeration and thus stabilizing them in a solution.

Figure 3.7 FTIR spectrum of bio-synthesized nanoparticles using fungal cell-free extract (A) *A. flavus* strain J2; (B) *A. terreus* strain J4; (C) *A. nomius* strain T4 and (D) *A. aculateus* strain T6, showing different peaks representing a range of functional groups.

3.3.3.4 Zeta potential

The negatively charged zeta potential of all four samples further indicates the protein-capping of biosynthesized nanoparticles, and contributes to particle stability. Figure 3.8 showed zeta potential of -5.99 and -10.2 mV for the nanoparticles synthesized from jarosite using the cell-free extract of *A. flavus* strain J2 and *A. terreus* strain J4, respectively. Zeta potential values of -22.7 and -15.2 mV of nanoparticles synthesized from iron ore tailings using extract of *A. nomius*
strain T4 and *A. aculateus* strain T6, shown in Figure 3.9, indicated that these particles are highly stable.

Figure 3.8 Zeta potential of nanoparticles biosynthesized from jarosite using cell-free extract of (A) *A. flavus* strain J2; (B) *A. terreus* strain J4.
3.3.3.5 TEM, HRTEM, EDX and XRD analysis

3.3.3.5.1 Jarosite

Figure 3.10 (A & D) shows TEM micrographs of bio-synthesized nanoparticles from jarosite using cell-free extract of *A. flavus* strain J2 and *A. terreus* strain J4 respectively. The particles were observed to be of semi-quasi spherical morphology and approximately 10-50 nm in size, with an average size range of 45 ± 5 nm for J2 and 15 ± 5 nm for J4. The lattice structure of particles is shown in the HRTEM micrograph, indicating their crystalline structure, although the fringes were not very
clear and distinct, probably due to the consortia of different metals present (Figure 3.10 C & F). Figure 3.10 (B &E) displayed the EDX spectrum of bio-synthesized nanoparticles. The spectrum was recorded from one of the densely populated nanoparticles area and confirms the leaching of zinc and iron, along with other metals, by the fungal cell-filtrate, showing strong signal for zinc, iron and oxygen, along with calcium and magnesium. The sharp optical absorption peak in the range of 5-10 keV signified the presence of zinc and 0–1 keV confirmed the presence of oxygen in the nanoparticles. The P, S, Cl, and K signals were probably due to X-ray emission from biological macromolecules (carbohydrates/proteins/enzymes) present in the cell wall of the fungal mycelium. The strong peaks of Ni and C are due to the carbon-coated nickel grid. The usual carbon-coated copper grid was not used to minimize interference from the grid, since jarosite already has copper present in it. The XRD was run but the data had a high noise level and so was inconclusive (data not shown).
Figure 3.10 (A, D) TEM images; (B, E) EDAX based elemental composition; and HRTEM images (C, F) at scale of 5 nm and 10 nm showing lattice fringes indicating crystalline nature of biosynthesized nanoparticles from jarosite using cell-free extract of *A. flavus* strain J2 and *A. terreus* strain J4, respectively.

### 3.3.3.5.2 Iron ore tailings

TEM micrographs Figure 3.11 (A & D) of bio-synthesized nanoparticles from iron ore tailings using cell-free extract of *A. nomius* strain T4 and *A. aculateus* strain T6 showed that the particles were mostly spherical in morphology and approximately 10-30 nm in size with an average size range of 15 ± 5 nm. The lattice structure of particles can be observed in HRTEM micrograph, indicating their crystalline structure. However, the fringes were not very clear and distinct, perhaps due to the consortia of different metals present (Figure 3.11 C & F). The EDX spectrum shown in Figure 3.11 (B &E) of biosynthesized nanoparticles was recorded from one of the densely populated nanoparticles areas and confirms the leaching of iron along with other metals by the fungal cell-filtrate, showing strong signals of iron and oxygen, along-with aluminium and silicon. The sharp optical absorption peak in
the range of 0-10 keV signified the presence of iron and 0–1 keV confirmed the presence of oxygen in the nanoparticles. The strong peaks for Cu and C are due to the carbon-coated copper grid. The XRD was run but the data had a high noise level and so was inconclusive (data not shown).

Figure 3.11 (A, D) TEM images; (B, E) EDAX based elemental composition; and HRTEM images (C, F) at scale of 5 nm and 10 nm showing lattice fringes indicating crystalline nature of biosynthesized nanoparticles from iron ore tailings using cell-free extract of *A. nomius* strain T4 and *A. aculateus* strain T6, respectively.

### 3.4 Conclusion

The present study showed the bioleaching followed by biosynthesis of nanoparticles from Jarosite and Iron ore tailings waste using cell-free extract of *A. flavus* strain J2 and *A. terreus* strain J4 (from Jarosite) and *A. nomius* strain T4 and *A. aculateus* strain T6 (from Iron ore tailings), where nanoparticles with a robust layer of protein capping were observed. Here, a novel biomethodology was developed for
bioleaching and subsequently nanoconversion of waste materials into nanostructured form. The fall in pH of cell-free extract indicated that release of organic acids is at least partially responsible for the bioleaching from these wastes. However, the enhanced biosynthesis of nanoparticles was observed at pH 8.0. The nanoparticles synthesized range from 10-50 nm with a varying average size range of 45 ± 5 nm for J2, 15±5 nm for J4 and 15±5 nm for T4 and T6. The advantage of biological synthesis of nanoparticles over other existing methods was that there was minimal use of chemicals, surfactants and energy and significant stabilization in solution due to protein matrix, which was confirmed by FTIR spectroscopy. Thus, the fungus-mediated two-step approach could be very useful in the cost-effective, environmental-friendly synthesis of agriculturally important nanoparticles from cheap raw material, in this case industrial and mining wastes. The utilization of waste would hence serve the dual purpose of reducing waste and producing bioavailable zinc and iron nanoparticles for agricultural use.
Chapter 4

*Application of biosynthesized nanoparticles as plant nanonutrients*
4.1 Introduction

In today’s world, nanotechnology has been established as a major technology that has proven to contribute to solutions to a range of industrial problems, including the agricultural sector. The wide range of applications include food packaging, production, transport and storage of agricultural products (Chen & Yada, 2011). Research is being carried out on nanoscale carriers for smart delivery of pesticides, herbicides, coloring agents and fertilisers (Sun et al., 2014; Zhang et al., 2007). Mortvedt have reported that increase in surface area of a granular fertilizer increases the suspension rate of less soluble fertilizers like ZnO in water. The reduced size also increases the number of particles per unit weight of applied Zn, which indicates that more soil would be affected, thus preventing repeated application of fertilizer (Mortvedt, 1992). The usage of nanoparticles as nanonutrients helps in increasing the efficiency of fertilizer with comparatively lower dosage (Prasad et al., 2012); which in turn signifies an economical solution. Various researchers are studying the effect of these engineered nanoparticles (ENP) such as nano TiO₂ and carbon nanotubes (CNT) as nanofertilizers. Work has been carried out to study their effect on seed germination, plant growth and plant physiology by enhancing factors like photosynthesis and nitrogen-metabolism (Hong et al., 2005; Khot et al., 2012; Lin et al., 2009; Moghadam et al., 2012b; Yang et al., 2006; Zheng et al., 2005).

A comparative study on the application of nano and bulk Zn indicated that low concentrations of nano Zn (10 ppm) and higher concentrations of bulk Zn (100 ppm) have shown similar increase in germination percentage when applied on Macrotyloma uniflorum (Lam.)Verdc (horse gram) (Gokak & Taranath, 2015). Iron nanoparticles have been tested as nanofertilizer for the growth of spinach and it was
shown to promote growth at 4 kg ha\(^{-1}\) application (Moghadam et al., 2012b). Iron nanoparticles have also been reported to enhance the grain yield by 20 % for wheat by increasing the protein content (Balali & Malakouti, 2002).

There are few reports on biosynthesized nanoparticles being used as nanofertilizer. Biosynthesized ZnO nanoparticles have been tested as a nanofertilizer by Tarafdar and co-workers (Tarafdar et al., 2014) on Pearl millet (\textit{Pennisetum americanum}). They have synthesized nanoparticles from a salt solution of aqueous zinc oxide as a precursor using fungus, \textit{Rhizoctonia bataticola} TFR-6. Foliar application of 16 L ha\(^{-1}\) (10 mg L\(^{-1}\)) of ZnO nanoparticles have shown significant increase in the plant dry biomass (12.5 %), grain yield (37.7 %), root length (4.2 %), shoot length (15.1 %), photosynthetic pigment chlorophyll (24.4 %) and total soluble leaf protein (38.7 %) over the control. There are reports on nanoparticles taken up by the plant cell either through stomata or vascular pathway (Eichert et al., 2008; Raliya & Tarafdar, 2013) which might boost metabolic activities of plant cells leading to increased crop production.

The aim of this study was to apply biosynthesized nanoparticles as nanonutrients, where these particles were synthesized using a cell-free extract of \textit{A. terreus} strain J4 from jarosite. Here, the first step was to make the negatively charged biosynthesized nanoparticles to positively charged through surface modification using polymers which are non-toxic and environmental friendly. Thereafter, these nanonutrients were tested under \textit{in vitro} conditions for effect on seed germination, uptake and nutrient assimilation studies with respect to zinc and iron. These preliminary studies were carried out on wheat (\textit{Triticum aestivum}), which is a major cereal crop grown worldwide and in the northern parts of India (U.P., Haryana,
Punjab etc.) as it is adaptable to varying climatic conditions and is one of the most susceptible crop to zinc and iron deficiency (Alloway, 2008; Frossard et al., 2000).

4. 2 Materials and method

4.2.1 Chemicals used

Poly L-Lysine (PLL) and Agar extra pure were procured from Sigma-Aldrich, St. Louis, U.S.A. All other chemicals used were purchased from Fischer Scientific (Mumbai, India) and were analytical grade. Wheat (*Triticum aestivum*) seeds (Raj 3765 variety from Rajasthan, India) was purchased locally.

4.2.2 Surface modification of biosynthesized nanoparticles (synthesized by *A. terreus* strain J4 cell-free extract) using polymers

Nutrients are taken up by the plant roots through cation exchange from the soil using proton pumps. It is at this site that $H^+$ displace the cations attached to negatively charged soil particles (Epstein, 1972; Morgan & Connolly, 2013). Using Zeta-sizing, we confirmed that the nanoparticles synthesized from jarosite using cell-free extract of *A. terreus* strain J4 were negatively charged due to protein capping. Thus, in order to apply them as nanonutrients, surface modification of the nanoparticles was carried out to modify them to positively charged forms. The major aim of this study was to select a cationic polymer which was cost-effective and non-toxic/ non-hazarduous to the environment. On this basis, two polymers Atlox Semkote E-135 (Croda Care, U.S.A.) and PLL (natural homopolymer of cationic poly amino acid group), were selected and tested for efficient capping of biosynthesized nanoparticles. Atlox Semkote range polymers have been widely used in seed coating with active ingredients like pesticides and pigment colorant.
(Arthur et al., 2009; Herbert, 2003; Tang et al., 2012; Volgas et al., 2003). PLL has also been widely studied as a stable capping agent for nanoparticles (Marsich et al., 2012; Mondragon et al., 2014).

5 mL of Atlox polymer (0.5 % w/v) was taken and added dropwise to 50 mL of biosynthesized nanoparticles suspension with continuous stirring at RT for 4-5 hrs. From the reaction solution, 1 mL was taken after every 1 hr and subjected to Zeta analysis to check the change in surface charge. A similar protocol was followed with PLL. However, there was hardly any change in surface charge even after 24 hrs of reaction (-0.31 mV) in the case of Atlox (Figure 4.1), whereas PLL showed efficient capping after only 1 hr of incubation. Thus, PLL was selected and capping of biosynthesized nanoparticles was optimized with this polymer.

Here, 100 mg of biosynthesized nanoparticles were added to 5 mL of sterile MilliQ with continuous sonication at RT (Ultrasonic cleaner, 3510-DTH, Branson, Danbury, U.S.A.) for 15 mins for homogenous mixing followed by slow addition of the remaining 4.95 mL of sterile MQ. 50 μL of PLL (0.1 % w/v in H$_2$O) was added dropwise to make up the final volume of 10 mL. The entire reaction was allowed to sonicate for 5 mins and then kept on static for 30 mins before analysing for zeta potential.
Figure 4.1 Zeta potential graph showing the surface modification of negatively charged biosynthesized nanoparticles using Atlox Semkote E-135.

4.2.2.1 Determination of Zeta potential

50 μL of each sample (polymers and nanoparticles as control and polymer–capped nanoparticles) was diluted with 1 mL of MQ water for measurement of zeta potential. The samples were then transferred to a polycarbonate zeta cell (having gold plated electrode) and the potential was then measured at 25°C (25 subruns) using Zeta Sizer Nano ZS90 (Malvern, U.K.). This was done to further confirm the change in surface charge of biosynthesized nanoparticles.

4.2.2.2 TEM and EDX analysis

Samples were prepared for TEM and EDX analysis by drop-casting on a carbon-coated nickel grid after sonication for 20 min. The sample was then air-dried prior to analysis. TEM micrographs and EDX spectrum were taken at an accelerated voltage of 200 kV using TECNAI G² T20 TWIN (Netherlands) and EDAX Instruments, respectively.
4.2.3 Application of nanoparticles as nanonutrients

4.2.3.1 Surface sterilization of seeds

The seeds of wheat (*Triticum aestivum*) (Raj 3765 variety from Rajasthan, India) were purchased locally and stored dry. The seeds were surface sterilized by immersion in 0.01 % HgCl₂ twice for 2 mins each and then subjected to washing with sterile distilled water three times. The entire study was carried out under sterile condition in a laminar flow hood.

4.2.3.2 Preparation of seeds with treatments

Based on the AAS analysis of biosynthesized nanoparticles from jarosite waste using cell-free extract of *A. terreus* strain J4, the stock solution of PLL-capped nanoparticles (361.11 ppm Zn/ 445.19 ppm Fe) was prepared. 100 mg of biosynthesized nanoparticles was dissolved in 10 mL sterile MQ water and capped with 50 μL of PLL using the above procedure. Using this stock solution, the working solutions of 10-50 ppm concentration of biosynthesized nanoparticles were prepared as given in Table 4.1.

Table 4.1 Preparation of working solutions of surface modified biosynthesized nanoparticles for seed treatments.

<table>
<thead>
<tr>
<th>Treatments (Concentration)</th>
<th>x mL of stock in MQ (10 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ppm</td>
<td>225 μL</td>
</tr>
<tr>
<td>20 ppm</td>
<td>449 μL</td>
</tr>
<tr>
<td>30 ppm</td>
<td>674 μL</td>
</tr>
<tr>
<td>40 ppm</td>
<td>898 μL</td>
</tr>
<tr>
<td>50 ppm</td>
<td>1.123 mL</td>
</tr>
</tbody>
</table>
Controls included full-strength Hoagland solution (without Zn and Fe), raw jarosite (100 mg in 10 mL), PLL (50 μL of PLL in 10 mL sterile MQ) and bulk Zn-Fe (ZnSO₄·7H₂O + FeSO₄·7H₂O – 40 ppm each in 10 mL sterile MQ). The surface-sterilized seeds were then soaked in the respective treatments and kept on shaker at RT for 2 hrs.

4.2.3.3 Seed germination study of biosynthesized nanoparticles

This was carried out to study the effect of surface modified biosynthesized nanoparticles on seed-germination. The treated seeds were placed into a petridish (90 mm) containing 0.8 % water agar supplemented with full-strength Hoagland solution (without Zn and Fe). The petridishes were then sealed with parafilm, covered with aluminium foil and set for seed-germination at 25±2⁰C in a temperature controlled plant growth room. Each treatment was set up in 5 replicates. Following 3 to 7 days, seed germination was recorded. Seeds that had coleoptile longer than 2 mm were considered germinated; and the others were considered non-germinated.

4.2.3.4 In vitro nutrient assimilation studies

Here, sterile jars containing 0.8 % water agar (40 mL in each jar) supplemented with full-strength Hoagland solution (without Zn and Fe) were used as plant growth substrate. A hole was made using sterile hot forceps in the lid, to enable plant growth. The treated seeds were placed on agar media from the hole using sterile forceps. The sterile cotton plug was replaced back in the hole and the jar was further sealed with parafilm in order to avoid any chance of contamination (Figure 4.2). The plants were then allowed to grow under controlled conditions at 25±2⁰C. The jars were then supplemented with either 2 mL of nanoparticles treatment or
Hoagland solution after 5 days followed by 2 mL of sterile water every 5\textsuperscript{th} day to maintain moisture. After 30 days, the following observations were recorded and results are presented as mean ± SD of samples set up in 5 replicates.

![Image of treated seeds on agar](image)

Figure 4.2 Treated seeds kept on 0.8 % water agar supplemented with Hoagland solution.

**4.2.3.4.1 Growth parameters**

Root and shoot length of the plants were measured in cms. Fresh weight in grams of roots and shoots was recorded. They were then kept in oven at 60\textdegree C for 4 days and dry weight (g) was taken after 48 hrs until it became constant.
4.2.3.4.2 Confocal microscopy

Confocal microscopy was carried out to confirm the uptake of surface modified biosynthesized Zn and Fe nanoparticles by plants. In order to see the fluorescence tracking of nanoparticles in the shoots and roots, thin sections were cut with safety razor and subjected to different protocols, with respect to Zn and Fe nanoparticles, as described below.

4.2.3.4.2.1 Fluorescence tracking of Iron nanoparticles

For the study of Fe nanoparticles (Brumbarova & Ivanov; Roschztardtz et al., 2013; Stacey et al., 2008), the sections were fixed in 1-2 mL of fixing solution that was methanol: chloroform: glacial acetic acid (6:3:1) for 2 hrs under vaccum (500 mbar) using vaccum desicator. Application of vaccum speeds up the penetration of fixative. Meanwhile, the staining solution (Perls Stain) was prepared as follows: 4 % (v/v) HCl and 4 % (w/v) K₄Fe(Cn)₆ were mixed together in equal proportions and incubated in the dark. The staining solution was pre-warmed at 37°C prior to application. The fixative solution was removed and the sections were washed 3 times with sterile MQ water for 1-2 mins. The pre-warmed staining solution was added and incubated for 1 hr under vaccum (500 mbar). After incubation, the solution was removed and washing was done three times for 1-2 mins along with slight shaking. The stained samples were then subjected to dehydration with ethanol dilution series 10 %, 30 %, 50 % and 70 % for 10 mins each. Dehydration was done to intensify the stain. After carrying out different time incubations and staining without dehydration, the above optimized protocol was designed. The laser microscope imaging was performed with a confocal microscope (LSM710, Carl Zeiss Microimaging, Germany). An Argon laser at 561 nm provided excitation for
the Alexa Fluor 568. The fluorescence emission signals were detected in Multi Track using a band-pass filter of 568–712 nm. The sections were observed with a x20 and x40 Zeiss objective.

4.2.3.4.2. Fluorescence tracking of Zinc nanoparticles

For the study of Zn nanoparticles uptake, fluorescent indicator Zinpyr-1 (Sigma-Aldrich, St. Louis, U.S.A.) was used. It can readily penetrate through biological membranes and is highly Zn-specific (Seregin et al., 2011; Seregin et al.). 1 mM of Zinpyr-1 stock solution was prepared by dissolving 1 mg of Zinpyr-1 in 500 μL of dimethyl sulfoxide (DMSO) using a vortex mixer and later on adding 714 μL of DMSO. The 50 μL aliquots of stock solution were stored in dark at -20°C. Prior to the analysis, 5 mL of working solution of 10 μM was prepared by dissolving 50 μL of stock solution in 4.95 mL of sterile MQ. Thin section of roots and shoots were kept on a drop of stain taken on a glass slides. They were then incubated for 2 hrs in dark before examination. Distribution of the fluorescent complex of Zn-Zinpyr1 were studied using confocal microscope (LSM710, Carl Zeiss Microimaging, Germany). An Argon laser at 488 nm provided excitation for the Alexa Fluor 488. The fluorescence emission signals were detected in Multi Track using a band-pass filter of 493-695 nm. The sections were observed with a x20 and x40 Zeiss objective.

4.3 Results and Discussion

4.3.1 Surface modification of biosynthesized nanoparticles

PLL as control showed +30.0 mV (Figure 4.3 A) and biosynthesized nanoparticles using cell-free extract of A. terreus strain J4 were negatively charged showing zeta
potential of -10.2 mV (Figure 4.3 B). These biosynthesized nanoparticles when capped with PLL showed surface charge of +24.9 mV as can be seen in Figure 4.3 C. This surface modification was further confirmed by the TEM and EDX analysis of the sample with EDX spectrum showing the presence of Br (from PLL) alongwith other components of biosynthesized nanoparticles. The low contrast of particles might be because of this capping which prevents penetration of electron beam (Figure 4.4).

Figure 4.3 Zeta potential of (A) nanoparticles biosynthesized from jarosite using cell-free extract of *A. terreus* strain J4, (B) PLL as control; (C) PLL-capped nanoparticles showing surface modification.
Figure 4.4 (A) TEM image; (B) EDAX based elemental composition of PLL-capped biosynthesized nanoparticles from jarosite using cell-free extract of *A. terreus* strain J4, respectively.

### 4.3.2 Evaluation of seed germination using varying treatments

Table 4.2 depicted the effect of different treatments on the germination of wheat seeds. The increase in percentage of seed germination was observed on treatment with biosynthesized nanoparticles. Maximum germination was recorded in the control (100 %), 10 ppm (100 %) and 20 ppm (100 %) treated seeds, which decreased with higher concentrations (30 ppm- 90 %, 40 ppm- 87 % and 50 ppm- 80 %). Least germination was observed in the case of treatments with only PLL (43.33 %) and bulk Zn-Fe (40.00 %). The increase in seed germination depends on the adsorption, uptake and penetration of surface modified biosynthesized nanoparticles. There are reports which indicate penetration in the seed by nanoparticles (Khodakovskaya et al., 2009). This report suggested multi-walled carbon nanotubes (MWCNTs) could penetrate in tomato seed and enhance germination by increasing water uptake.
Figure 4.5 (A) Different treatments of seeds; (B) effect as observed on seed germination.

Table 4.2 Effect of surface modified biosynthesized nanoparticles on wheat seed germination.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Germination %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Hoagland)</td>
<td>100.00</td>
</tr>
<tr>
<td>Control (Jarosite)</td>
<td>60.00</td>
</tr>
<tr>
<td>Control (PLL)</td>
<td>43.33</td>
</tr>
<tr>
<td>Bulk Zn-Fe</td>
<td>40.00</td>
</tr>
<tr>
<td>10 ppm</td>
<td>100.00</td>
</tr>
<tr>
<td>20 ppm</td>
<td>100.00</td>
</tr>
<tr>
<td>30 ppm</td>
<td>90.00</td>
</tr>
<tr>
<td>40 ppm</td>
<td>86.67</td>
</tr>
<tr>
<td>50 ppm</td>
<td>80.00</td>
</tr>
</tbody>
</table>
4.3.3 In vitro nutrient use efficiency of nanostructured jarosite

4.3.3.1 Growth parameters

Length of root and shoot was recorded in cms after 30 days of treatment and it was found to be inversely proportional to the concentration. Similar trends were observed in the cases of fresh and dry weight. The results might be due to the compounding effects of PLL-capped nanoparticles. Lower efficacy of bulk Zn-Fe treatment could be due to the large particle size effecting the uptake by plants. At lower concentrations, surface modified biosynthesized nanoparticles enhanced the growth of seedlings, which decreased thereafter. This reduction in growth measurements might be due to the toxicity of nanoparticles at higher concentrations (Azimi et al., 2013; Boonyanitipong et al., 2011; Lee et al., 2008; Zhu et al., 2008). These results are in accordance to studies that reported lower concentrations of 10 ppm or 20 ppm promoted plant growth (Liu et al., 2005). The application of nanoparticles affected the plant growth hormones and plant physiology, which in turn effects the growth parameters (Koizumi et al., 2008; Raskar & Laware, 2014). The effect of different treatments on in vitro grown wheat after 30 days is depicted in Figure 4.6.
Figure 4.6 Effect of respective treatments on wheat plant growth (A) Control; (B) Raw jarosite as control; (C) PLL as control; (D) Bulk Zn-Fe as control; (E) 10 ppm nanoparticles; (F) 20 ppm nanoparticles; (G) 30 ppm nanoparticles; (H) 40 ppm nanoparticles and (I) 50 ppm nanoparticles.
4.3.3.1.1 Root growth characteristics

The results were recorded in terms of root length, fresh weight and dry weight (Figure 4.7-4.8). The lower concentration of 20 ppm nanoparticles showed maximum enhanced growth (average root length- 19.320 cm, fresh weight- 0.398 g, dry weight- 0.180 g), which decreased thereafter (30 ppm-17.80 cm, 0.310 g, 0.138 g; 40 ppm-15.080 cm, 0.248 g, 0.103 g; 50 ppm-14.040 cm, 0.229 g, 0.092 g). A similar growth pattern was observed in the case of 10 ppm treatment (15.900 cm, 0.304 g, 0.135 g) and seeds treated with just Hoagland solution (without Zn and Fe) taken as control (16.220 cm, 0.286 g, 0.114 g) followed by raw jarosite treatment (13.600 cm, 0.185 g, 0.080 g). On the other hand, lowest efficacy was observed when seeds were treated with PLL (10.400 cm, 0.157 g, 0.041 g) and bulk Zn-Fe (9.320 cm, 0.166 g, 0.052 g) alone.

Figure 4.7 Effect of surface modified biosynthesized nanoparticles on root and shoot length of wheat.
Figure 4.8 Effect of surface modified biosynthesized nanoparticles on fresh and dry weight of roots.

4.3.3.1.2 Shoot growth characteristics

The comparative results were recorded in terms of shoot length (Figure 4.7), fresh weight and dry weight (Figure 4.9) with respect to different treatments. Maximum enhancement was seen in the case of 20 ppm nanoparticles (average shoot length- 29.20 cm, fresh weight- 0.390 g, dry weight- 0.168 g), which decreased thereafter (30 ppm- 25.94 cm, 0.305 g, 0.105 g; 40 ppm- 24.24 cm, 0.279 g, 0.108 g; 50 ppm- 22.74 cm, 0.250 g, 0.093 g). A similar growth pattern was observed in the case of 10 ppm treatment (27.04 cm, 0.297 g, 0.112 g) and seeds treated with just Hoagland solution (without Zn and Fe) taken as control (23.80 cm, 0.272 g, 0.106 g) followed by raw jarosite treatment (20.5 cm, 0.166 g, 0.062 g). Lowest efficacy was observed when seeds were treated with PLL (18.64 cm, 0.129 g, 0.038 g) and bulk Zn-Fe (17.64 cm, 0.145 g, 0.054 g) alone.
4.3.3.2 Confocal Microscopy

Confocal microscopy confirmed the uptake of nanoparticles in the roots and shoots of different treatments. The intensity of fluorescence was found to be low in the case of control root and shoot, with respect to both iron (Figure 4.10) and zinc (Figure 4.16). On the other hand, fluorescence intensity was high, confirming the movement of iron (Figure 4.11-4.15) and zinc (Figure 4.17-4.21) nanoparticles in the plant after the application of jarosite nanoparticles. 3-D images show the location of nanoparticles in the plant tissue. In terms of zinc nanoparticles, fewer particles were visible, which might be due to the lower bioavailability and P-interaction of \( \text{KH}_2\text{PO}_4 \) present in Hoagland solution that might have reduced its mobility as Zn-P antagonistic interaction is well-established (Das et al., 2005; Zhu et al., 2001).

Figure 4.9 Effect of surface modified biosynthesized nanoparticles on fresh and dry weight of shoots.

![Graph showing the effect of treatments on fresh and dry weight of shoots.](image-url)
Figure 4.10 Control stained roots (A) and leaves (B) respectively.

Figure 4.11 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 10ppm nanoparticles.

Figure 4.12 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 20ppm nanoparticles.

Figure 4.13 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 30ppm nanoparticles.
Figure 4.14 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 40ppm nanoparticles.

Figure 4.15 Iron nanoparticles in roots (A) and leaves (B) respectively treated with 50ppm nanoparticles.

Figure 4.16 Control stained roots (A) and leaves (B) respectively.

Figure 4.17 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 10ppm nanoparticles.
Figure 4.18 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 20ppm nanoparticles.

Figure 4.19 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 30ppm nanoparticles.

Figure 4.20 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 40ppm nanoparticles.

Figure 4.21 Zinc nanoparticles in roots (A) and leaves (B) respectively treated with 50ppm nanoparticles.
4.4 Conclusion

In this study, surface modification of the jarosite nanoparticles (biosynthesized by \textit{A. terreus} strain J4) using PLL resulted in efficient capping with a surface charge of +24.9 mV. These surface modified positively charged nanoparticles when tested as Zn-Fe nanomicronutrient fertilizer for wheat (\textit{Triticum aestivum}), showed enhanced plant growth after the application of a 20 ppm dose (average root length- 19.320 cm, average shoot length- 29.20 cm, plant fresh weight- 0.788 g, dry weight- 0.348 g) in comparison with a control (average root length- 16.220 cm, average shoot length-23.80 cm, plant fresh weight- 0.558 g, dry weight- 0.22 g). A decreasing trend in growth and germination was observed with increasing concentration of nanoparticles, possibly due to toxicity of particles at higher concentrations. Fluorescence tracking of nanoparticles using confocal microscopy confirmed their uptake by the plants and indicated their efficiency of use.
Chapter 5

*Summary and Future Directions*
Increase in waste generation is correlated with worldwide population increases. Large amounts of industrial and mining waste are being generated and dumped globally, which poses a serious threat to the environment. Jarosite and Iron ore tailings are two such waste materials which are disposed off in large quantities, and when dumped, toxic metals are leached out into the surrounding water and soil. Researchers are working towards removing these metals and utilizing the treated waste for construction materials and other uses. But the different physical and chemical methods adopted have their own advantages and disadvantages. Keeping this in mind, our major objective for the work described in this thesis was based on the following: “Mining waste contaminates the earth’s surface. If we can bioremediate this waste and convert it to nanonutrients we can contribute to solving two problems at once. That is, bioremediation and the problem of plant nutrient deficiencies of zinc and iron.” Jarosite and Iron ore tailings were selected for this thesis study, since these two waste materials have Zn and Fe in substantial amounts.

Chapter 2 describes the collection of jarosite from the jarosite pond secure landfill at the Debari Zinc Smelter, Hindustan Zinc Limited, Udaipur, in Rajasthan, India and iron ore tailings from Codli mines, Goa, India, followed by their complete elemental and structural analysis using AAS, TEM and EDX. Jarosite was found to contain zinc (~34,000 ppm), iron (~38,000 ppm), sulphur (~11,000 ppm) and lead (~14,000 ppm), along with trace elements like copper and aluminium etc. Iron ore tailings contained primarily iron (~42,000 ppm) and aluminium (~34,000 ppm). Different fungi were isolated using culture enrichment techniques, based on their metal-tolerant abilities. The fungi were coded as J1-J5 in the case of jarosite and T1-T6 in the case of iron ore tailings. These fungal strains were then screened for their bioleaching and subsequent nanoparticles biosynthesis efficacy with respect to
Zn and Fe. After screening, the best four isolates were selected. They were J2 and J4 from jarosite and T4 and T6 from iron ore tailings, and these showed maximal leaching efficiencies of 57.8% (J2), 67.07% (J4), 33.23% (T4) and 77.26% (T6), as measured by TEM and EDX analysis. These four selected fungal isolates were then morphologically characterized using SEM. Molecular characterisation using 18S rRNA gene sequence analysis indicated that J2 exhibited 99% homology to *A. flavus* strain 176 (GenBank Accession No. KP784374.1) and isolate J4 exhibited 99% homology to *A. terreus* strain LCF17 (GenBank Accession No. FJ867934.1). Similarly, isolates T4 showed 98% homology to *A. nomius* strain UOA/HCPF 12657 (GenBank Accession No. KC253960.1) and T6 showed 100% match to *A. aculeatus* strain A1.9 (GenBank Accession No. EU833205.1).

In Chapter 3, the main aim was optimization of protocols for enhanced bioleaching and biosynthesis of nanoparticles from these two waste materials using fungal cell-free extract (extracellular proteins and organic acids). Based on a growth kinetics study using an Ergosterol assay, 4-5 days old cultures were taken for further process development. It was observed that a fall in pH of the cell-free extract indicated enhanced bioleaching efficiency. This bioleachate was then subjected to metal nanoparticles biosynthesis by increasing the pH (~8.0) of the reaction mixture using aqueous ammonia. The biosynthesized nanoparticles were characterized using FTIR and Zeta analysis (confirmed the protein-capping which resulted in stabilization) and TEM and EDX (45 ± 5 nm for J2, 15±5 nm for J4 and 15±5 nm for T4 and T6). Thus, a novel biomethodology was developed using fungal cell-free extract for bioleaching and subsequently nanoconversion of the waste materials into nanostructured form. These promising strains could be further exploited for bioremediation and production of metal nanoparticles from waste materials.
Chapter 4 describes the preliminary application of these biosynthesized nanoparticles (jarosite nanoparticles synthesized using *A. terreus* strain J4 cell-free extract) as nanonutrients, with respect to Zn and Fe. Here, the first target was to carry out surface modification of negatively charged biosynthesized nanoparticles to convert them to positively charged nanoparticles. Efficient capping was achieved using PLL (50 μL for 10 mL nanoparticles), as confirmed by Zeta analyser (zeta potential changed from -10.2 mV to +24.9 mV). These surface modified biosynthesized nanoparticles were then tested for their efficacy in terms of seed germination and nutrient uptake by wheat (*Triticum aestivum*). It was observed that lower concentration of nanoparticles (10 and 20 ppm) showed 100% germination and promoted plant growth in terms of root and shoot length and weight, whereas a decreasing trend was seen at higher concentrations of 30-50 ppm. These nanoparticles also masked the deteriorating effect of PLL. The fluorescence tracking of Zn and Fe was carried out using confocal microscopy to confirm the uptake of these nanoparticles in treated plants. Clearly, optimization of nanoparticle concentration and the correct selection of parts of the plant for uptake and localization (using confocal and TEM) are required for a detailed study of nanonutrient uptake, adsorption and assimilation by plants.

The present study shows that bioleaching from jarosite and iron ore tailings wastes can be achieved using cell-free extracts of metal-tolerant fungi. The fall in pH of the cell-free extracts indicates that the release of organic acids is an important part of the process that is responsible for the bioleaching from these wastes. The advantage of the biological synthesis of nanoparticles over existing methods is that there is minimal use of chemicals, surfactants and energy, but also significant stabilization in solution due to the protein matrix, the presence of which was confirmed by FTIR
spectroscopy in our study. Thus, a fungi-mediated two step approach could be a cost-effective, environmental-friendly method for the synthesis of useful metal nanoparticles from inexpensive raw waste materials.

This is the first report on bioleaching and the biosynthesis of nanoparticles from jarosite and also the first report to use converted jarosite waste materials as nanonutrients/nanofertilizer. However, there are a some previous reports on the application of biosynthesized nanoparticles using metal salt precursors as nanonutrients.

This ability of microorganisms to leach out metals from the wastes is being exploited in the field of bioremediation. The reducing ability of some enzymes from these microbes could be utilized for the synthesis of metal nanoparticles from these waste materials. Metal nanoparticles have found applications in various fields including agricultural, but these are produced primarily from metal salt precursors. The use of myconanomining for the bioleaching and nanoparticles biosynthesis from waste offers several potential advantages over other processes: (i) Higher biomass production; (ii) fungal secretome contains large amounts of extracellular proteins with diverse functions; (iii) more absorption of metallic elements/compounds at low pH; (iv) high metal reducing activity of secretome; and (v) bioremediation. The utilization of waste could thus serve the dual purpose of reducing waste and building new avenues for waste utilisation in agriculture to maximize the plant productivity in a cost-effective and environmental-friendly manner.
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123

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