Microbial synthesis of nanoparticles and their effect on the growth of nitrogen fixing cyanobacteria

A

DISSERTATION REPORT

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BIOTECHNOLOGY

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DECLARATION

I hereby declare that the work which is being presented in this thesis "**Microbial synthesis of nanoparticles and their effect on the growth of nitrogen fixing cyanobacteria**" submitted by me for the award of the degree of **Masters in Science** in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. Dinesh Goyal, Professor & Head, Department of Biotechnology, Thapar University, Patiala, Punjab, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Place: Patiala

Date: July 10, 2015

(Amrit Kaur)

CERTIFICATE

Certified that the thesis "**Microbial synthesis of nanoparticles and their effect on the growth of nitrogen fixing cyanobacteria**" submitted by Ms. Amrit Kaur, in fulfillment of the requirement for the award of the degree of **Masters in Science** in the Department of Biotechnology, Thapar University, Patiala, Punjab, India is the record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

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Place: Patiala
Date: 10-July-2015

AMRIT KAUR

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LIST OF ABBREVIATIONS

Al_2O_3	Aluminium oxide
ALS	Atomic force microscopy
ATP	Adenosine triphosphate
BG11	Blue Green Medium
C_3O_2	Carbon suboxide
CdS	Cadmium sulphide
CeO_2	Cerium(IV) oxide
Cm	Centimetre
CNT	Carbon nano-tubes
CO_2	Carbon dioxide
CuO	Copper oxide
CVD	Chemical vapour deposition
DAN	Diaminonapthotriazole
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
e.g.	Example
EDX	Energy-dispersive X-ray spectroscopy
et al	et alii
Etc	et cetera
FDA	Food and Drug Administration
Fe_2O_3	Iron(III) oxide
FRAP	Fluorescence recovery after photobleaching
FTIR	Fourier transform infrared spectroscopy
G	Gram
GSH	Glutathione
H_2O	Dihydrogen oxide
H_2SO_4	Sulfuric acid
H_3BO_3	Boric acid
HCl	Hydrochloric acid
Hr	Hour
HR-FE-SEM	High-Resolution Field Emission Scanning Electron Microscopy
HRTEM	High-resolution transmission electron microscopy
i.e.	that is
IAA	Indole acetic acid
IARI	Indian Agricultural Research Institute
ICP-AES-	Inductively coupled plasma atomic emission spectroscopy
JCPDS	Joint Committee on Powder Diffraction Standard
KNO_3	Potassium nitrate

LDV	Laser Doppler Velocimetry
M	Molar
Mg	Magnesium
Mg	Milligram
Ml	Millilitre
Mm	Milimolar
MS	Mass spectrometry
N	Normality
α-NADPH	alpha-Nicotinamide adenine dinucleotide phosphate
Na_2CO_3	Sodium carbonate
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NEDD	N-1-naphthyl ethylene diamine dihydrochoride
Nm	Nanometer
Nps	Nanoparticles
NR	Nitrate reductase
OD	Optical density
PC	Phytochelatins
PDB	Potato dextrose medium
Ph	Potential Of Hydrogen
Ppm	part per million
PVC	Polyvinyl chloride
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
RPM	Rotations per minute
SEM	Scanning electron microscope
SiO_2	Silicon dioxide
Sp	Species
TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
UV	Ultra-violet
UV-VISis	Ultraviolet-visible spectrophotometry
XRD	X-ray powder diffraction
μ–XANES	Micro X-ray absorption near edge structure
μ-XRF	Micro X-ray fluorescence
v/v	Volume by volume
$Zn(CH_3COO^-)_2$	Zinc acetate
$ZnCl_2$	Zinc chloride
$ZnNO_3$	Zinc nitrate
ZnO	Zinc oxide
ZnS	Zinc sulphide
$ZnSO_4$	Zinc sulphate

LIST OF SYMBOLS

%	Percentage
&	And
°C	degree Celsius
µ	Micro
Å	Angstrom
Ag	Silver
Au	Gold
Ca	Calcium
K	Potassium
Mg	Magnesium
Mn	Manganese
Ɵ	Bragg diffraction angle
P	Phosphorus
S	Sulfur
α	Alpha
β	Width of the XRD peak at half height
λ	X-ray wavelength

Dedicated to...

The Sacred feet of my beloved Parents and my Respected Advisor

ABSTRACT

Zinc oxide nanoparticles are of great interest due to their versatile applications and noble properties such as high refractive index, high thermal conductivity, binding energy, antibacterial UV-protection etc. They can be synthesized by various physical and chemical methods which are generally toxic, need very high temperatures and release hazardous chemicals. Therefore, we report green approach for the synthesis of Zinc oxide (ZnO) nanoparticles (NPs) using zinc acetate as metal precursor from *Aspergillus niger*, *Anabaena variabilis* and *Trichoderma reesei* which were further characterised by XRD, FTIR and SEM. Sharper and stronger diffraction peaks of XRD analysis confirmed synthesized ZnO NPs. SEM images confirmed the size of nZnO synthesized from various sources. The size of nanoparticles was 64-126 nm from *A. niger*, 72-74 nm from *A. variabilis* and the nano-structures in *T. reesei* remained so compactly arranged that their average size could not be estimated. FTIR analysis confirmed the polysaccharides present in the aqueous extract of *A. variabilis* that are involved in the formation of nZnO. Whereas, functional groups present in the ZnO nanoparticles synthesized from *A.niger* and *T. reesei* was confirmed through the peaks in the range of 3075 to 859 cm^{-1} and 1615.05 to 1099.99 cm^{-1}. In order to assess the role of these synthesized nanoparticles in the environment, effect of ZnO NPs and other sources of Zn (0 to 100 ppm) was studied on the growth of *A. variabilis* and *N. muscorum.* Biomass and chlorophyll content were studied as growth parameters, whereas NR activity and IAA production were studied as physiological parameters. It was found that 2 ppm of ZnO NPs are stimulatory and above 2 ppm were inhibitory for the growth of *A. variabilis*, whereas in *N. muscorum* even 2 ppm of ZnO NPs were inhibitory for growth. Effect of different zinc sources such as $ZnCl_2$, $ZnNO_3$, ZnO, $Zn(CH_3COO^-)_2$ and $ZnSO_4$ was studied on the growth of *A. variabilis* and *Calothrix* sp. The biosynthesized zinc oxide nanoparticles are expected to be clean, non-toxic and environmentally acceptable and can have applications in diverse fields.

CHAPTER 1

INTRODUCTION

Nanotechnology is rapidly growing interdisciplinary science that connects knowledge of biology, chemistry, physics, material science, engineering, pharmacology, and medicine. The products of nanotechnology are nanoparticles, nanotubes, nanorods, nanospheres that have a size below 100 nm, and this is the reason they are able to gain considerable attraction because of their unusual and fascinating properties, with various applications, over their bulk counterparts. These nano size particles have attracted researchers due to their ability to withstand under harsh condition and out of harm's way to mankind (Fu et al., 2005).

There are four basic categories of nanoscale materials that are available as commercial products.

1. **Metal oxides**—These are the ceramics from oxides of zinc, iron, cerium, silicon and zirconium which are used as chemical polishing agents from semi-conductor wafers; scratch resistant coatings for glass and in cosmetics and sunscreens which form the largest part of commercial nanomaterials (Shanmugapriya et al., 2013).
2. **Nanoclays**—These are naturally-occurring plate-like clay particles which are used to improve strength, hardness, heat resistance and flame resistivity of materials. Also they are used to produce barrier films in plastic beverage bottles, paper juice cartons, and tennis balls (Espitia et al., 2012).
3. **Nanotubes and spheres**—These are particularly used in coatings to dissipate and minimize static electricity in fuel lines and hard disk handling trays (Sherman, 2007). It can also be found in electrostatically paintable car exterior components, flame-retardant fillers for plastics, and field emitter sources in flat panel displays.
4. **Quantum dots**—These are used in exploratory medical diagnostics and therapeutics and self assembly of nanoelectronic structures.

Metal oxide nanoparticles are of great importance mainly because of their distinctive optical, electronic and magnetic properties. These nanoparticles of transition metals are the class of semiconductors, which have applications in magnetic storage media, solar energy

transformation, electronics, gas sensors and catalysis (Ramgir et al., 2013; Jani et al., 2013; Shalana et al., 2013; Montferrand et al., 2013; Ahmadi et al., 2011). Although various physical and chemical methods have been extensively used to produce zinc oxide nanoparticles like mechanochemical process (Tsuzuki et al., 2001), precipitation process (Kumar et al., 2013), precipitation in the presence of surfactants (Li et al 2005), sol-gel (Sikora et al., 2012), solvothermal hydrothermal and microwave techniques (Bai et al., 2015) electrochemical Synthesis (Chandrappa and Venkatesha., 2012), by laser ablation in aqueous environment (Singh et al., 2009) etc. During their production several toxic chemicals or non-polar solvents attached on the surface of nanoparticles limits their applications in clinical fields. Therefore, there is an immediate need to develop clean, biocompatible, non-toxic and eco-friendly methods for the synthesis of nanoparticles. Thus the attention in this field has shifted toward 'green' chemistry and bioprocess approach where the bio-organisms are used as environment-friendly, cost effective and biocompatible reducing agents for synthesis of zinc oxide nanoparticles.

Zinc oxide nanoparticles are non-toxic, II-VI semiconductor with wide band gap (3.37eV) and have natural n-type electrical conductivity (Wellings et al., 2007). It has many interesting properties such as high refractive index, high thermal conductivity, binding energy, antibacterial and UV-protection; thus it is widely used as an additive in numerous materials and products. Although it is non toxic to human beings with high biocompatibility, in fact it is taken as ZnO in the form of the daily supplement for zinc because it is a mineral element required to human health. But it is definitely lethal to the microorganisms (Jena et al., 2012, Rajeshkumar et al., 2014). Zinc oxide nanoparticles can be effectively synthesized from various biological agents who secrete a large amount of enzymes, which are capable of hydrolyzing metals that bring about enzymatic reduction of metal ions leading to the formation of nanoparticles (Rai et al., 2009b). They have many applications in various fields such as in agriculture where Zinc is reported to be required for chlorophyll production, pollen function, fertilization, biomass production and germination by various researchers (Pandey et al., 2010, Milani et al., 2012, Khan et al., 2003, Genc et al., 2006, Prasad et al., 2012). Moreover their toxicity to the environment can be tested out with cyanobacteria which had been previously used as a model alga for determining the environmental stresses (Apte et al., 1998), due to their phylogenetic relationship with plants (Rai et al., 1999). Thus by studying the physical and chemical factors that control the nanoparticle toxicity to algae will help in evaluating their ecological risk. In the

present work attempt has been made towards green synthesis of Zinc oxide (ZnO) nanoparticles (NPs) using zinc acetate as metal precursor from *Aspergillus niger*, *Anabaena variabilis* and *Trichoderma reesei* which were further characterised by XRD, FTIR and SEM. The effect of ZnO nano particles was studied on the growth of nitrogen fixing cyanobacteria.

CHAPTER 2

REVIEW OF LITERATURE

Nanoscience is the study of materials on the scale of 1-100 nm (nanometers). Nano-science is currently a rapidly evolving area that describes technology and science involving nano scale that increases the scope of investigating and regulating the interaction at cell level both for synthetic material and biological system (Du et al., 2007). These nanoparticles are the elemental building blocks of nanotechnology. They display completely new and improved properties with special characteristics such as grain size, distribution and morphology, when compared with the bulk material. The reason for this is that they possess a higher surface to volume ratio with their decreasing size (Willems et al., 2005). As surface area is relevant for catalytic reactivity and other related properties, so when their specific surface area is increased, their biological efficacy would increase, mainly due to the amplification in surface energy (Willems et al., 2005).

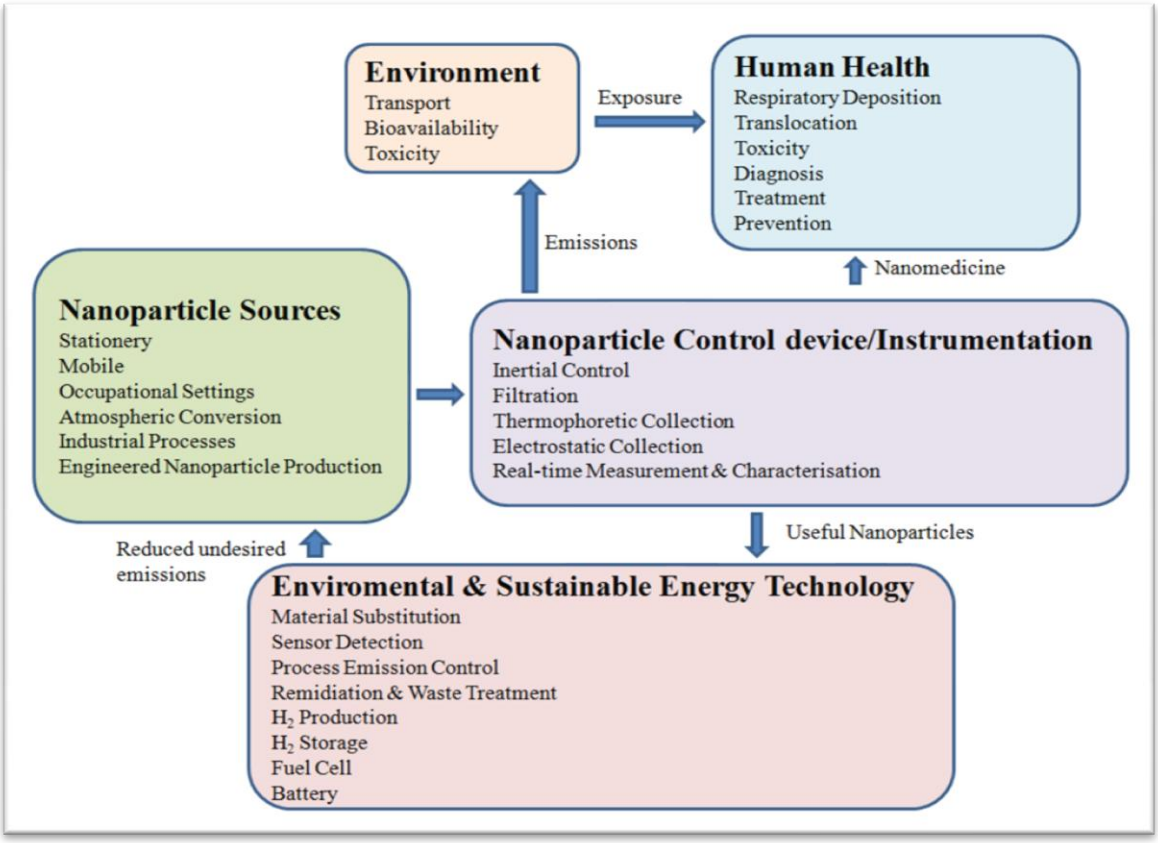


Fig. 1. Potential health implications of Nanoparticles

Nanoparticles are classified into 3 broad catagories (Luther et al., 2004), which are:

1) According to dimensions

a) Zero dimension – e.g. uniform particles arrays (quantum dots), heterogeneous particles arrays, core–shell quantum dots, onions, hollow spheres and nanolenses (Kim et al., 2010, Zhang et al., 2008, Wang et al., 2009, Lee et al., 2009).

b) 1D <100 nm- e.g. films, coatings, multilayer etc.

c) 2D <100 nm- e.g. tubes, fibres, wires, platelets etc.

d) 3D <100 nm- e.g. particles, quantum dots, hollow spheres etc.

2) According to phase composition

a) Single-phase solids- e.g. crystalline, amorphous particles and layers etc.

b) Multi-phase solids- e.g. matrix composites, coated particles etc.

c) Multi-phase systems- e.g. colloids, aerogels, ferrofluids etc.

3) According to manufacturing processes

a) Gas phase reaction- e.g. flame synthesis, condensation, CVD etc.

b) Liquid phase reaction- e.g. sol-gel, precipitation, hydrothermal processing etc.

c) Mechanical phase reaction- - e.g. ball milling, plastic deformation etc.

There are different types of nanoparticles according to their chemistry which are listed in the following Fig. 2.

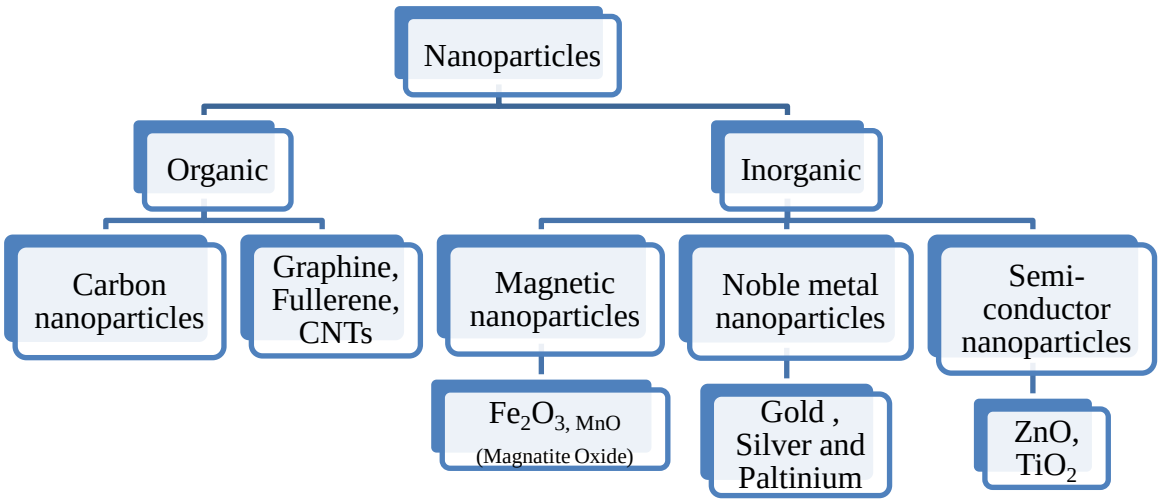


Fig. 2. Different type of nanoparticles

2.1. Mode of synthesis of nanoparticles

There are two broad approaches for the synthesis of nanoparticles (Fig. 3).

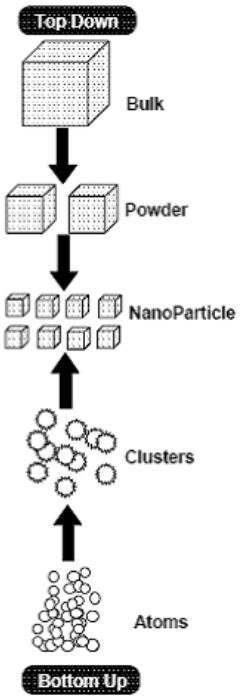


Fig. 3. Mode of synthesis of nanoparticles

2.1.1. Top-down approach

It refers to slicing or successive cutting of a bulk material to get nano-sized particles. Here single bulk crystals are broken down in aqueous solutions to get nano sized particles. It involves several methods, which are discussed below.

2.1.1.1. Mechanical milling- In this method mixtures of powders are placed in a high energy mill and subjected to grinding under protective environment. The main purpose of milling is to reduce the particle size and blending of particles in the new phase. For this purpose, a variety of balls like tumber mills, attrition mills, shaker mills etc have been developed to produce aluminium oxide nanoparticles (Koch et al., 1996).

2.1.1.2. Etchings (chemical) – In this method, regular arrays of nano-sized structures are produced on the planer substrate. So silicon nano-wires are fabricated through non-uniform etching on silicon substrates in aqueous acid solutions, which is catalyzed by electroless deposition of metal nanoparticles on the substrate surfaces (Peng et al., 2002)**.**

2.1.1.3. Electro-explosion (thermal/chemical) - In this method, a very high current is provided in a short time through thin metallic wires, in inert or reactive gas to achieve very high temperatures making the resistivity of the metal infinite, terminating the flow of the current. At that point the electromagnetic field disappears and superheated metal plasma expands with supersonic velocity creating a shock wave in an ionised gas surrounding the wire. Thus metallic powders of approximately 100 nm (Luther et al., 2004).

2.1.1.4. Sputtering (kinetic) – In this method, the impact of the atom or ion on the surface produces sputtering from the surface that results in the transfer of the momentum from the incoming particle. It is generally done on the low pressure on the cold substrate, thus there is no melting of the material occurs like other vapour phase techniques (Luther et al., 2004). For example, there are many effects of sputtering conditions on the formation of gold nanoparticles (Hatakeyama et al., 2011).

2.1.1.5. Laser ablation (thermal) - In this process, pulsed light from the sharp laser beam is focussed onto the solid target placed inside a vacuum chamber to boil off a plume of energetic atoms of the target material e.g. layered zinc hydroxide/ surfactant nanocomposite are prepared by pulsed-laser ablation in a liquid medium (Ullamann et al., 2002). A substrate positioned to intercept the plume will receive a thin film deposit of the target material.

2.1.2. Bottom-up approach

It refers to the make up a material from the bottom i.e. by joining atom by atom, molecule by molecule or cluster by cluster. It involves several methods described as follows.

2.1.2.1. Supercritical Fluid synthesis- In this method the properties of supercritical CO_2 and H_2O are used in the preparation of a great variety of metal oxide nanomaterials (Byrappa et al., 2008). This method is used to produce ZnS nanocrystallites with a different morphology by the use zinc acetate and sodium diethyldithiocarbamite with an experimental temperature of 150–200 °C for 12–72 hr produces (Sapra et al., 2005).

2.1.2.2. Spinning- It is used to produce very fine fibres by the principle of spinning dilute polymer solutions in a high voltage electric field from a liquid which are usually in micro or nano scale (Luther et al., 2004).

2.1.2.3. Use of templates- In this methodology, a membrane with micro or nanometer pore acts as template for succeeding material deposition that would adopt its shape. Several natural

and synthetic materials with pores of nanoscale have been used as templates, from which Al_2O_3 and TiO_2 templates are excellent examples (Macak et al., 2007, Azevedo et al., 2014).

2.1.2.4. Plasma or frame spraying synthesis- Here the suspension that uses water as solvent, is pumped into the plasma plume through an atomization probe to create micron-sized droplets comprising raw material particles. The droplets are flash dried, and the remaining particles are melted, vaporized, and further condensed into nanoparticles (Bouyer et al., 1997). For example plasma spraying was used to process atomized liquid droplets of precursor solutions to produce alumina, zirconia and yttria stabilized zirconia nanoparticles (Karthikeyan et al., 1997).

2.1.2.5. Sol process & Sol-gel process- This method is used for the production of colloidal nanoparticles from liquid phase. The method is well adopted for the fabrication of metal oxides, especially the oxides of zinc (Sekine et al., 2009).

2.1.2.6. Laser pyrolysis- In this method nanoparticle formation starts abruptly when a sufficient degree of supersaturation of condensable products is reached in the vapour phase. Once nucleation occurs, rapid synthesis of nanoparticle takes place by coalescence/coagulation. Ceramic nanoparticles synthesized by this method were tuned for the preparation of coolant nanofluids (Amato et al., 2013).

2.1.2.7. Aerosol based process- Aerosols are the liquid or solid particles suspended in a gas. In this method spraying of the precursor chemical onto the heated surface results in the precursor pyrolysis and subsequently production of the nanoparticles (Luther et al., 2004).

2.1.2.8. Chemical vapour deposition (CVD) -CVD technique can be achieved by taking a carbon source in the gas phase and using an energy source, such as plasma to transfer energy to a gaseous carbon source at a high temperature (~ 720° C) to break their hydrogen carbon bond, producing pure carbon molecules. It is commonly used for the synthesis of carbon nano-tubes (CNTs) and fullerenes (Endo et al., 1993, Dai et al., 1996).

2.1.2.9. Atomic or molecular condensation- In this method a bulk material is heated in vacuum to produce a stream of atomised and vaporised matter, which is further connected to the chamber containing reactive gas (like oxygen for the making of metal oxide

nanoparticles). This is followed by rapid cooling of the metal atoms due to their collisions with the gas molecules results in the condensation and formation of nanoparticles (Luther et al., 2004). For example, Formation of carbon nanoparticles by the condensation of supersaturated atomic vapour obtained by the laser photolysis of C_3O_2 (Gurentsov et al., 2007).

2.1.2.10. Green synthesis – It involve the synthesis of different metallic nanoparticles like gold, silver, zinc oxide, palladium, iron oxide, copper oxide from natural biomass of different organisms by using them as capping and reducing agent.

2.2. Disadvantages of physical and chemical methods

Synthesis of metal nanoparticles has been mainly confirmed by physical and chemical methods as they successfully produce pure and well-defined nanoparticles, (Gao et al., 2008, Rodriguez et al., 2000, Sailaja et al., 2011). But these methods need great expenditure and are toxic, need very high temperatures, release hazardous chemicals, non eco-friendly and have very low productivity. Moreover the nanoparticles produced are of poor morphology. These methods require both strong and weak chemical reducing agent and protective agents like sodium borohydride alcohols, phenyl hydrazine, sodium citrate etc which are highly toxic, flammable and cannot be directly exposed out in the environment (Rai et al., 2008). So there is a need of to adopt green routes for synthesis of nanoparticles.

2.3. Green synthesis of nanoparticles

"Green Nanotechnology" is the new term arisen with a lot of consideration that reduces or eradicates toxic substances to be released into the environment (Moghaddam et al., 2010). The assembly of nanoparticle developed from the simple methods of green synthesis are clean, nontoxic, environmentally acceptable, doesn't need high temperature or dangerous chemicals. Also they have high productivity as compared to physical and chemical methods (Rai et al., 2008). Due to their compatibility to biological functionalization, these biosynthesized nanoparticles have important applications in the field of medicine, particularly related to the antimicrobial activity (Dykman et al., 2012).

For the green synthesis some of the points which are need to be considered for producing highly stable and well-characterized nanoparticles are as follows (Iravani., 2011):

1. **Selection of the best organisms**: Intrinsic properties of the organisms such as enzyme activities and biochemical pathways are to be focused for metal nanoparticle production.
2. **Optimal conditions for cell growth and enzyme activity**: Growth conditions like nutrients, inoculum size, light, temperature, pH, mixing speed, and buffer strength should be optimized. Also the presence of the substrates or related compounds in the subtoxic levels from the beginning of the growth would increase the activity of the enzymes.
3. **Optimal reaction conditions**: The bioreduction conditions for the synthesis for nanoparticles in the reaction mixture are needed to be optimized for better yield and quality (Ahmad et al 2003, Korbekandi et al 2009). The substrate concentration, the biocatalyst concentration, the electron donor and its concentration, pH, exposure time, temperature, buffer strength, mixing speed, and light need to be controlled and optimized which could affect the size, morphology, and rate of reaction.

Applying the in-vivo methods for the synthesis of nanoparticles through green nanotechnology, the cell itself act as living and self-reproducible bioreactor (Iravani, 2011). There are two means for the reduction of the metal ions to metal nanoparticles in order to convert the harmful toxic ions into the harmless substances that can be freely released into the environment.

Intracellular synthesis – In this type of synthesis, the nanoparticles are formed from the enzymes that are present inside the cell and remain inside until they are extracted out from the cell. The nanoparticles produced by this method are stabilized by the cell components (mainly enzymes) and show characteristic morphologies at the same time. Here nanoparticles cannot exceed a certain size range depending on the organism, due to the spatial delimitation of the cell wall and the existing cell components (Deplanche et al., 2012).

Extracellular synthesis –In this type of synthesis, the nanoparticles are formed from the enzymes that are present out of the cell i.e. mainly the enzymes secreted by the cell wall. Here the synthesis of nanoparticles involves trapping the metal ions on the surface of the cells and reducing ions in the presence of enzymes (Zhang et al., 2011).

2.3.1. Green synthesis of nanoparticles from algae

Algae are the wide group of eukaryotic organisms that have autotrophic mode of nutrition. They lack many secondary cells and tissue types found in higher plants such as xylem, phloem, and stomata but have chloroplasts containing circular DNA and chlorophyll as their photosynthetic pigment. Algae perform approximately 50% of the photosynthesis on earth and thus are influential in supporting the biosphere. They use their chlorophyll to capture light energy to fuel the manufacture of sugars, but they are primarily aquatic unlike plants (Elumalai et al., 2013). They are chiefly rich in biologically active substances which can be used for the biosynthesis of nanoparticles (Ganesan et al., 2013). Dry biomass of algae is used for the synthesis of various nanoparticles. Also algal cell disruption is very important for reduction processes in nanoparticle synthesis. Some of the cell disruption methods that can be followed are shown below in the Fig. 4.

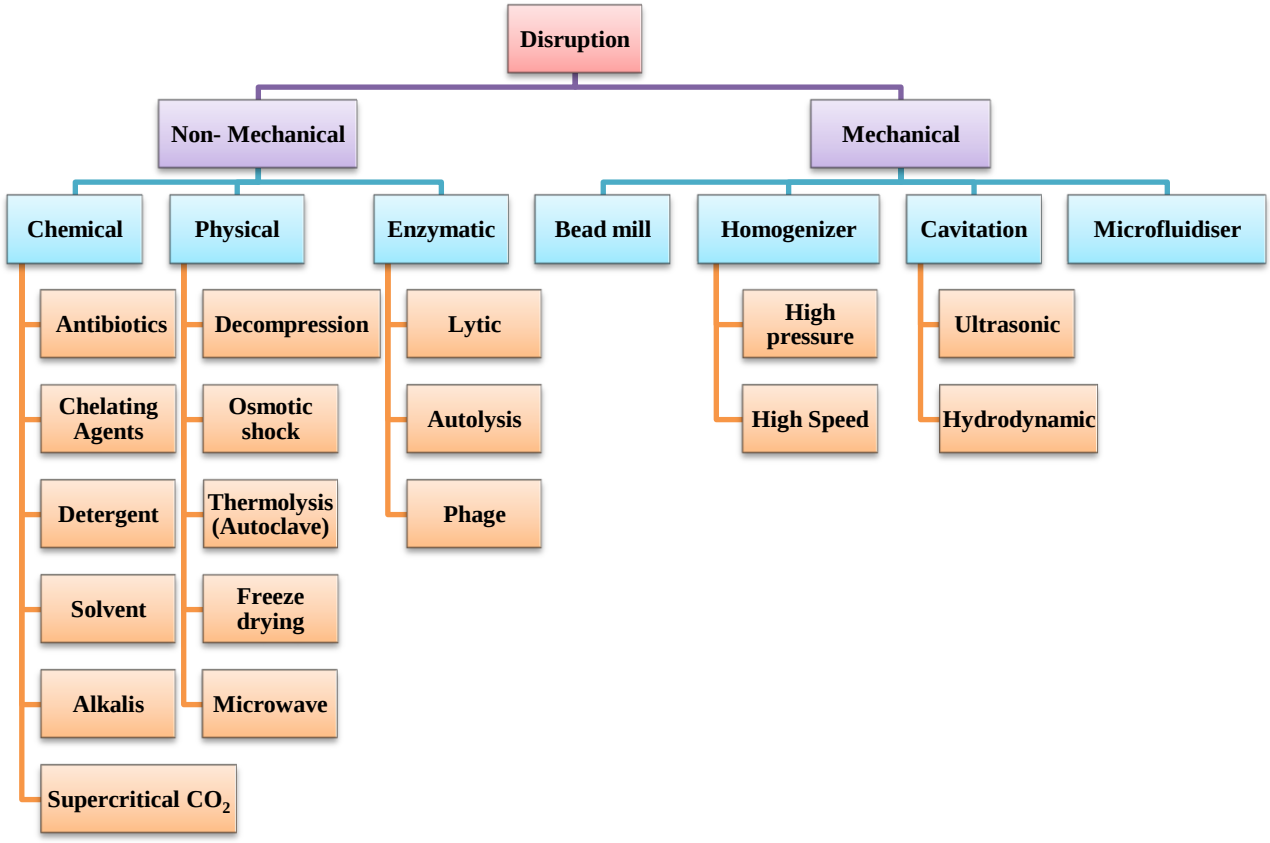


Fig. 4. Algal cell disruption methods

These are categorized according to their pigmentation into green (*Chlorophytes*), brown (*Phaeophytes*) and red (*Rhodophytes*) which live either in marine or brackish water. Some of the algae used as biofactories for the production of metallic nanoparticles are listed in Table 1.

Table 1. Synthesis of metallic nanoparticles from different species of algae

Green algae							
S.No.	**Source**	**Nanoparticle**	**Size**	**Shape**	**Use**	**Characterisation**	**Reference**
1.	*Ulva lactuca*	Ag	20-56 nm	Spherical	Anticancer	UV-VISis, XRD, FTIR, SEM,TEM, EDAX	Devi et al., 2012
2.	*Chlorococcum humicola*	Ag	16 nm	Spherical	Antibacterial	UV-VISis, XRD, FTIR, SEM,TEM	Jenaa et al., 2012
3.	*Spyrogira insignis*	Au	30-50 nm	Sphere-shaped, Polygonal, Triangular, Hexagonal	-		Castro et al., 2013
4.	*Chlorella pyrenoidusa*	Au	25-30 nm	Spherical	-	UV-VISis, XRD, TEM	Oza et al., 2012
5.	*Pithophora oedogonia*	Ag	300-700 nm	Cubical and hexagonal-shaped	Antibacterial	UV-VISis, SEM, XRD, FTIR, SEM, EDS, DLS	Sinha et al., 2014
6.	*Caulerpa racemosa*	Ag	10 nm	Face-centered cubic geometry	Antibacterial	UV-VISis, FTIR, TEM, XRD	Kathiraven et al., 2014
7.	*Spirogyra submaxima*	Au	2-50 nm	Spherical, Triangular, Hexagonal	Biomedical applications	TEM, XRD, DLS, UV-VISis	Roychoudhury and Pal, 2014
Red algae							
1.	*Gracilaria edulis*	Ag	12.5-100 nm	Spherical	-	TEM, SEM, Electron diffraction	Murugesan et al., 2011
2.	*Gelidiella acerosa*	Ag	22 nm	Spherical	Antifungal	UV-VISis, XRD, TEM, SEM	Vivek et al., 2011
3.	*Kappaphycus alvarezii*	Au	10-40 nm	Polydisperse	Antimicrobial and anti-oxidant	XRD, TEM	Rajasulochana et al., 2012
4.	*Kappaphycus alverazii*	Ag	74 nm	Face centered cubic silver	-	UV-VISis, XRD, FTIR, SEM, EDX	Ganesan et al, 2013
5.	*Kappa phycus* sps	Ag	52-104 nm	-	-	UV-VISIBLE spectroscopy, FTIR,AFM	Baskar, 2013
6.	*Gracilaria corticata*	Au	45-57 nm	-	Antimicrobial and antioxidant	UV-VISIBLE spectrophotometry, SEM, FRAP assay	Naveena and Prakash, 2013

7.	*Galaxaura elongata*	Au	3.85–77.13 nm	Rod, Triangular, Truncated triangular, Hexagonal	Antibacterial	UV-VISis, FTIR, TEM, MS	Raouf et al., 2013
Brown algae							
1.	*Turbinaria conoides*	Ag	96 nm	Spherical	Antibacterial	UV-VISis, FTIR, SEM, XRD	Rajeshkumar et al., 2012)
2.	*Stoechospermum marginatum*	Au	18.7 to 93.7 nm	Spherical, Triangular, Hexagonal	-	photoluminescence spectra, FTIR, SEM, TEM, XRD, Wavelength Dispersive X-ray Fluorescence Spectrophotometer	Rajathi et al., 2012
3.	*Padina pavonica*	Ag	54 nm	Spherical	Antifungal and Antibacterial		Sahayaraj et al., 2012
4.	*Sargassum longifolium*	Ag	40-85 nm	Spherical	Antifungal	SEM, XRD, TEM, FTIR	Rajeshkumar et al., 2013
5.	*Sargassum muticum*	Ag	5-15 nm	Spherical	Antifungal, anti-inflammatory, antiviral, anti-angiogenesis, and antiplatelet		Azizi et al., 2013
6.	*Ecklonia cava*	Au	20-50 nm	Spherical	Antimicrobial	UV-VISis, XRD, FTIR, SEM,TEM	Venkatesan et al., 2014
7.	*Sargassum muticum*	ZnO	3-57 nm	Hexagonal wurtzite structures	-	X-Ray, FTIR, FSSEM, UVVis	Azizi et al., 2014

2.3.2. Green synthesis of nanoparticles from Bacteria

Bacteria are the prokaryotic microorganisms which are preferred for the synthesis of metallic nanoparticles mainly due to their ease of multiplication. They have the tendency to produce nanoparticles intracellularly and are able to preserve their viability even after the crystal growth (Nair et al., 2002). Different species of bacteria have the potency to synthesize metal nanoparticles intracellularly (Table 2) and their alloys respectively (Shankar et al., 2004).

Table 2. Synthesis of metallic nanoparticles from bacteria

S.No.	Microorganism	Type of NP	Location	Size range (nm)	Reference
Intracellular					
1.	*Pseudomonas stutzeri*	Ag	Intracellular	200	Klaus et al., 1999
2.	*Lactobacillus strains*	Ag and Au	Intracellular	20–30	Nair et al., 2002
3.	*Rhodococcus* sp	Au	Intracellular	5–15	Ahmad et al., 2003
4.	*Escherichia coli*	CdS	Intracellular	2–5	Sweeney et al., 2004
5.	*Plectonema boryanum*	Ag	Intracellular	1–10	Lengke et al., 2006
6.	*Escherichia coli* DH5α	Au	Intracellular pH = 4	25–33	Liangwei et al., 2007
Extracellular					
1.	*Thermomonospora* sp	Au	Extracellular	8	Ahmad et al., 2003
2.	*Actinobacter* sp	Magnetite	Extracellular	10–40	Bharde et al., 2005
3.	*Rhodopseudomonas capsulate*	Au	Extracellular, pH = 7	10–20	Shiying et al., 2007
4.	*Klebsiella pneumonia*	Ag	Extracellular	5–32	Shahverdi et al.,2007
5.	*Pseudomonas aeruginosa*	Au	Extracellular	15–30	Husseiney et al., 2007
6.	*Shewanella oneidensis*	Uranium (IV)	Extracellular	-	Marshall et al., 2007
7.	*Morganella* sp	Ag	Extracellular	20-30	Parikh et al., 2008
Intracellular and Extracellular					
1.	*Clostridium thermoaceticum*	CdS	Intracellular and extracellular	1–100	Cunningham et al., 1993
2.	*Shewanella algae*	Au	Intracellular, pH = 7, Extracellular, pH = 1	10–20, 50–500	Konishi et al., 2004

(Source- Thakkar et al., 2010)

2.3.3. Green synthesis of nanoparticles from Yeast

Yeast is a unicellular fungus which had been rarely used as a reducing agent for nanoparticle synthesis. Yeast cells increase the cellular pools of glutathione and glutathione-like compounds called phytochelatins in the stress of heavy metals (Mehra et al., 1991, Vido et al., 2001). The resulting metal thio-late complex formation neutralizes the toxicity of heavy metal ions and traps them inside the cell (Li et al., 1996, 1997). Some of the yeast species used for the biosynthesis of nanoparticles is listed in the Table 3.

Table 3. Synthesis of metallic nanoparticles from yeast

S.No.	Yeast	Type of NPs	Location	Size range (nm)	Reference
1.	*Candida glabrata* and *Schizosaccharomyces pombe*	CdS	Intracellular	200	Dameron et al., 1989
2.	MKY3	Ag	Extracellular	2–5	Kowshik et al., 2003

(Source- Thakkar et al., 2010)

2.3.4. Green synthesis of nanoparticles from fungi

Fungi are a large group of eukayotic organisms that can be used for nanoparticles synthesis with an advantage of their ease in the scale-up by thin solid substrate fermentation method, economic viability, simplicity in handling biomass, its potency as a eukaryote to overexpress specific enzymes (Kowshik et al., 2003). They can be used as a reducing agent for the conversion of metals ions into their corresponding nanometals either in intra-cellular or extracellular depending of the location of their reduction enzymes. In fact when the reduction enzymes responsible for the conversion of metals ions into their corresponding nanometals are formed extra-cellularly (usually secreted by the cell walls) then the synthesis of the nanoparticles takes place extracellularly (Mukherjee et al., 2001a, Mukherjee et al., 2001b, Mukherjee et al., 2002). Some of the fungi used for the biosynthesis of nanoparticles are listed in Table 4.

Table 4. Synthesis of metallic nanoparticles from fungi

S.No.	Fungi	Type of NPs	Location	Size range (nm)	Reference
1.	*Verticillium*	Ag	Intracellular	25 ± 12	Mukherjee et al., 2001a
2.	*Phoma* sp. 3.2883	Ag	Extracellular	71.06–74.46	Chen et al., 2003
3.	*Fusarium oxysporum*	Au	Extracellular	20–40	Ahmad et al., 2003
4.	*Aspergillus fumigates*	Ag	Extracellular	5–25	Bhainsa et al., 2006
5.	*Phaenerochaete chrysosporium*	Ag	Extracellular	50–200	Vigneshwaran et al., 2006
6.	*Fusarium oxysporum and Verticillium* sp.	Magnetite	Extracellular	20–50	Bharde et al., 2006
7.	*Trichoderma asperellum*	Ag	Extracellular	13–18	Mukherjee et al., 2008

(Source- Thakkar et al., 2010)

2.4. Mechanism of biomimetic synthesis

The biological agents secrete a large amount of enzymes, which are capable of hydrolyzing metals that bring about enzymatic reduction of metals ions leading to the formation of nanoparticles (Rai et al., 2009b). Extracts from bio-organisms may act both as reducing and capping agents where the reduction of metal ions is by biomolecules found in their extracts such as enzymes/proteins,amino acids, polysaccharides and vitamins is environmentally benign, yet chemically complex. As different biological agents respond differently with metal ions, thus exact mechanism for the synthesis of nanoparticles using biological agents has not been discovered yet. Also some biological agents have the tendency of producing nanoparticles extracellularly, while others produce intracellularly. As in case of intracellular synthesis, cell walls of the microorganisms play a major role for the production of nanoparticles. As cell wall is negatively charged so it interacts electrostatically with the positively charged metal ions. The enzymes present within the cell wall bioreduce the metal ions to nanoparticles, and then these nanoparticles get diffused of through the cell wall due to small size.

Mukherjee et al., (2001) reported stepwise mechanism for intracellular synthesis of nanoparticles using *Verticillium* sp. Here the fungal cell wall when comes in contact with metal ions interacts

electrostatically and traps the metal ions. Then the enzymes present in the cell wall bioreduce these metal ions. Ultimately, aggregation of particles and synthesis of nanoparticles take place.

Howover, Nair and Pradeep (2002) reported that in case of bacteria *Lactobacillus* sp., the initial step of synthesis of nanoparticles is the nucleation of clusters of metal ions which develops an electrostatic interaction between the bacterial cell and metal clusters which leads to the formation of nanoclusters which get diffused through the bacterial cell wall due to small size.

A number of researchers supported the enzyme nitrate reductase for extracellular synthesis of nanoparticles (Duran et al., 2005; Kumar et al., 2007a, b; He et al., 2007; Gade et al,. 2008; Ingle et al., 2008). This specific enzyme α-NADPH dependent nitrate reductase is important for the in the in-vitro synthesis of nanoparticles, as it would also do away with the downstream processing required for the use of these nanoparticles in homogeneous catalysis and other applications (Rai and Duran, 2011). According to them when pH increases, more competition occurs between protons and metal ions for negatively charged binding sites. The enzyme nitrate reductase secreted by the cell wall of microorganisms helps in the bioreduction of metal ions leading to the synthesis of nanoparticles. Duran et al., (2005) conducted the nitrate reductase assay test through the reaction of nitrite with 2,3-diaminophthalene. The emission spectrum demonstrated two major peaks of fluorescence intensity at 405 and 490 nm relating to the maximum emission of nitrite and 2,3-diaminonapthotriazole (DAN), respectively. The intensity of these two bands was increased with the addition of a 0.1% KNO_3 solution, confirming the presence of nitrate reductase which leads to the conclusion that the enzyme reductase is responsible for the reduction of Ag^+ ions and in the formation of silver nanoparticles. Moreover Ingle et al., (2008) reported the reduction of Ag^+ to Ag^0 by nitrate reductase enzyme in the case of fungi. He used commercially available nitrate reductase disks where the color of the disk turned reddish from white when it comes in contact with fungal filtrate demonstrating that C¼O bonds of carboxylate ions and Ag–N bonds from the free amine groups lie perpendicular to the nano silver surface and get directly allied with the capping of silver nanoparticles. Peptide linkages from amino acids undergo hydrolysis to synthesize free carboxylate ions and free amino groups which are involved in the encapsulation of silver nanoparticles.

He et al., (2007) reported similar mechanism of bioreduction of gold ions using *Rhodopseudomonas capsulata* that secrete cofactor for NADH and NADH-dependent enzymes

which leads to the initiation of electron transfer from the NADH by NADH-dependent reductase. Then, the gold ions obtain electrons and are reduced to Au (0) and hence result in the formation of gold nanoparticles.

Nangia et al., (2009) proposed biosynthesis of gold nanoparticles and their stabilization via charge capping by bacterium *Stenotrophomonas maltophilia* which is involved in NADPH-dependent reductase enzyme which converts Au^{3+} to Au^{0} through electron shuttle enzymatic metal reduction process.

Kowshik et al., (2002) reported the synthesis of CdS nanoparticles using *Schizosaccharomyces pombe* dependent on a stress protein response where the enzyme phytochelatin gets activated on exposure of *S. pombe* to cadmium that chelate the cytoplasmic cadmium to phytochelatin–Cd complex. Next, an ATP-binding cassette-type vacuolar protein transports phytochelatin–Cd complex across the vacuolar membrane. Inside the vacuole sulfide gets added to the complex to form a high-molecular-weight phytochelatin–CdS _2 complex or CdS nanocrystal.

Yeast cell are used as bio-factories for the production and stabilization of nanoparticles where the major molecules GSH with the structure g-Glu-Cys-Gly and two groups of metal-binding ligands: metallothioneins and phytochelatins (PC) contribute to the detoxification mechanisms in yeast cells (Rai and Duran, 2011).

2.5. Importance of Zinc

India is ranked 6 for common zinc deficiency problems including Afghanistan, Australia, Bangladesh, Brazil, China, Iran, Iraq, Pakistan, Philippines, Sudan, Syria, Turkey, many states in the USA and parts of Africa and Europe. Zinc is one of the most important minor nutrient sources required by the plants after N, P, K, S, Ca and Mg. It also controls the synthesis of indole acetic acid (IAA) which is a phytohormone to regulate the plant growth. Zinc-dependent enzymes are found in all known classes of enzymes which may play a vital role as a structural constituent or regulatory co-factor for

a) For Carbohydrate metabolism, for both photosynthesis and in the conversion of sugars to starch.
b) For Protein metabolism.
c) For Auxin (growth regulator) metabolism.

d) For Pollen formation.
e) For the maintenance of the reliability of biological membranes.
f) For the resistance to infection by certain pathogens (Alloway, 2008).

Raliya et al., (2014) reported that zinc is essential for the plants to produce chlorophyll. Leaves start losing their green colour when the soil is deficient in zinc and results in stunted growth of the plant. This discoloration caused by zinc deficiency is called chlorosis, which causes the tissue between the veins to turn into yellow colour which were initially green. Chlorosis first appears on the lower leaves and slowly moves up the plant. In stern cases lower leaves turn brown or purple and die. Thus insufficiency of zinc causes reduced crop yields and quality of crop products is frequently impaired. Loss of zinc from biological membranes increases their susceptibility to oxidative damage and impairs their function. It enables the plants to tolerate the lower air temperatures and helps in the biosynthesis of cytochrome which is a pigment to maintain the plasma membrane and also helps in the synthesis of leaf cuticle (Raliya et al., 2013).

2.5.1. Zinc oxide nanoparticle

It is a non-toxic, II-VI semiconductor with wide band gap (3.37eV) and natural n-type electrical conductivity (Wellings et al., 2007). It is called as II-VI semiconductor in materials because it has several distinguishing properties such as good transparency, high electron mobility, wide band gap, and luminescence. It looks like a white powder and is nearly insoluble in water. Due to noble properties such as high refractive index, high thermal conductivity, binding energy, antibacterial and UV-protection of ZnO, it is widely used as an additive in numerous materials and products such as ceramics, glass, cement, lubricants, paints, ointments, adhesives, plastics, sealants, pigments, foods (source of Zn nutrient), batteries, ferrites, and fire retardants. ZnO has best durability, greater selectivity and heat resistance than organic and inorganic materials (Padmavathy et al., 2008, He et al., 2011b). ZnO has high biocompatibility and fast electron transfer kinetics which advocate the use of this material as a bio-mimic membrane to immobilize and modify the bio-molecules (Kumar et al., 2008). Among the other semiconducting materials, ZnO is rich in nanostructures and has the ability to be converted into a number of morphologies (Wahab et al., 2008) such as nanowires (He et al., 2011a), nanorods (Huang et al., 2011), nanocombs (Fan et al., 2010), nanoflowers and nanosheet (Kou et al., 2011). Although it is non

toxic to human beings, in fact it is taken as ZnO in the form of the daily supplement for zinc because it is a mineral element required to human health. But it is definitely lethal to the microorganisms (Jena et al., 2012, Rajeshkumar et al., 2014). ZnO powder coated PVC films showed the antibacterial activity against food borne pathogen (Li et al., 2009).

2.5.2. Application of zinc oxide nanoparticles

Zinc oxide nanoparticles is a wide band gap semiconductor that is of great interest due to their versatile applications in diverse fields such as antibacterial action is much stronger than that of ZnO powder due to the larger surface to volume ratio (Jena et al., 2012), antifungal (Rajeshkumar et al., 2014), UV filtering properties (Basca et al., 2009), high catalytic and photochemical activity (Chandrappa et al., 2012), in biosensors (Singh et al., 2011), nanoelectronics (Pareek et al., 2013), solar cells (Seow et al., 2009), drug delivery and bioimaging probes (Espitia et al., 2012, Xiong et al., 2014), onto cotton fabrics to confer antimicrobial function and finally appraise the finished fabrics in terms of antibacterial, wash durability and topographical function (Rajendran et al., 2008), anticorrosion properties (Heinlaan et al., 2008), cosmetics (Smijs et al., 2011) and sunscreen industry (Shanmungapriya et al., 2013) due to their transparency and ability to reflect, scatter, and absorb UV radiation, and as food additives (Yu et al., 2011). ZnO nanoparticles also have good biocompatibility to human cells (Padmavathy et al., 2008, He et al., 2011b) and documented as safe material by FDA (Food and Drug Administration, USA) (Emamifar et al., 2010). These compounds have been shown to have high Electron resonance measurements so aqueous suspension of ZnO nanoparticles generate amplified level of reactive oxygen species, i.e. hydroxyl radicals which lead to the oxidative stress which in turn increase the cellular internalization of the nanoparticles in the bacteria leading to cell damage (Applerot et al., 2009, Thati et al., 2010).

2.5.3. Application of Zinc oxide nanoparticles in Agricultural crops

Zinc is required for chlorophyll production, pollen function, fertilization, biomass production and germination (Pandey et al., 2010). Among the other micronutrients required by the plants, Zn and Mn can affect the vulnerability of plants to drought stress (Khan et al., 2003). Zn utilization in tissues is called internal efficiency and Zn uptake is called external efficiency (Genc et al., 2006). Treatment of nanoscale zinc (25 nm mean particle size) at 1000 ppm concentarion promoted both

seed germination and seedling vigor and in turn showed early establishment in soil manifested by early flowering and higher leaf chlorophyll content. These particles proved effective in increasing stem and root growth (Prasad et al., 2012). ZnO-NPs have been proposed as an effective Zn fertilizer to alleviate Zn deficiency in soils (Milani et al., 2012). These nano compounds would be quickly and completely absorbed by the plant and would fix all nutritional needs and deficiencies. Some of the effects seen in the plants after the supply of nano zinc is listed in the following Table 5.

Table 5. Effect of Zinc oxide nanoparticles on different plant crops

S. No.	Plant	Size	Effect	Characterisation	Reference
1.	*Cicer Arietinum*	20–30 nm	Increase in the level of IAA in roots (sprouts) i.e. increase in the growth rate	XRD, FE-SEM	Pandey et al., 2010
2.	*Cucumis sativus*	50 nm	Increase in Biomass and root length, also there was less microbial activity in the soil	DLS, TEM	Kim et al., 2011
3.	Tomato	<100 nm	Improved growth and biomass	HR-FE-SEM, EDX	Panwar et al., 2012
4.	*Peanuts*	*25 nm*	Improvement in the germination, root growth, shoot growth, dry weight and pod yield of the treated seeds	HRTEM, SEM-EDAX. AAS	Prasad et al., 2012
5.	*Zea mays*	17.4 ± 4.9 nm	Tunneling effect in the primary roots of the Maize	Zeta potential unit, TEM, UV-Vis spectrophotometer	Pokhrel and Dubey., 2012
6.	*Vigna radiate*	20 nm	Increase in root and shoot length as well as accumulation of biomass	ICP-AES	Dhoke et al., 2013
7.	*Allium cepa*	≤35 and 50 nm	Dose-dependent increment in DNA damage in cells	TEM, DLS, LDV	Demir et al., 2014
8.	Wheat	-	Increase in number of seeds per plant + increased yield	-	Afshar et al., 2014
9.	Groundnut	35-45nm	Improving germination by increased auxin production, induced maximum shoot length/ inhibitory effect at high concentrations on root and shoot growth	SEM, TEM, XRD	Shyla, Natarajan, 2014
10.	Banana	35 nm	Excellent shooting, rooting and regenerated plantlets + accumulated more proline, chlorophyll, antioxidant enzymes activity and developed more dry weight accumulation + elimination of microbial contaminants	-	Helaly et al ., 2014

2.6. Dose dependent toxicity of Zinc oxide nanoparticles on algae

Since nowadays nanoparticles are found in many commercial products, making it more likely that they will also be unrestricted into the aquatic environment that may possibly occur during their production, use or disposal, either directly (e. g. as a result of accidents) or via wastewater. Although the number of engineered nanoparticles in the environment remains low when compared with natural particles (Kaegi et al., 2009) but still they remain harmful to the environment. Thus there is a need for analytical system to authorize quantification of these nanoparticles to understand how these nanomaterials act on organisms and the mechanisms of toxicity. More complications are arising due to the broad spectrum of engineered nanoparticles that differ in their chemical, physical and morphological properties (Behra et al., 2009).

In assessing the risks of nanoparticles in the environment, the emerging research challenges are to find out if nanomaterials are more toxic than the bulk forms of the same material, and up to what extent its toxicity is related by particle size and reactivity. It is not necessary that nanoparticles entering in the aquatic environments will have the equivalent impacts on aquatic biota as their bulk material. Similarly, nanoparticle morphology, surface area, surface charge, coating, purity, and its material solubility are expected to play very important roles in the fate and toxicity of the nanoparticles in aquatic systems.

Algae present at the bottom of the food chain are likely to be a sensitive indicator of toxic effects. It plays an integral role in the ecological system, producing the biomass for basic nourishment for food webs and oxygen for breathing. Thus if large algal population goes on changing due to nanoparticle toxicity then it would lead to the negative effects on the entire environment. Cyanobacteria perform oxygenic photosynthesis and play crucial role in primary production and nitrogen cycling with their ability to fix atmospheric dinitrogen into ammonia, a bioavailable form of nitrogen source for various organisms like plants (Tamagnini et al., 2002). Cyanobacteria have been previously used as a model alga for determining the environmental stresses (Apte et al., 1998), due to their phylogenetic relationship with plants' chloroplasts and their past ecological tolerance that contributed to their survival in a wide range of unreceptive environments (Rai et al., 1999). The metabolic strategies used by cyanobacteria to tolerate unfavourable and fluctuating conditions through physiological adaptation are exceptional and

widely reported (Rachlin et al., 1985, Surosz et al., 2005); these strategies involve physiological, morphological, and ecological modifications (Mackerras et al., 1990, Kangatharalingam et al., 2002). In addition, the accumulation and degradation of cyanobacterial intracellular inclusions with reserve functions has been previously reported under conditions of starvation or in exposure of stressors. Thus by studying the physical and chemical factors that control the nanoparticle toxicity to algae will help in evaluating their ecological risk (Rogers et al., 2013).

ZnO nanoparticle has the highest toxicity followed by aluminum oxide, titanium dioxide and cesium oxide nanoparticles, respectively. Therefore, disposal of ZnO nanoparticles needs more attention and precaution and more strict laws must be regulated for disposal of ZnO nanoparticles in aquatic environments (Pendashte et al., 2013).

ZnO nanoparticles as well as bulk ZnO were toxic to *Pseudokirchneriella subcapitata* already at very low concentrations (0.1 mg/l) and the total inhibition of algal growth was observed already at 0.16 mg Zn/l for both types of particles. Zinc oxides were equally toxic in bulk and nano formulations. The most toxic of the nano metal oxides was nano ZnO followed by nano CuO and nano TiO_2 (Arnoja et al., 2009).

Natasha et al., (2007) have studied the toxicity of ZnO nanoparticles to *P. subcapitata* while also determined the concentration of dissolved Zn ions derived from ZnO. The toxicity of ZnO particles as well as $ZnCl_2$ was found due to dissolved Zn.

Ji et al., (2011) reported that both nano- and bulk-ZnO significantly inhibited the algal growth with a percent survival compared to the control of 48.8±8.7% and 59.0±11.4% at 1000mgL^{-1}, respectively. However, the final Zn^{2+} ion concentrations released from nano-ZnO were significantly lower than that from bulk-ZnO in the culture media, which could possibly be due to the considerable aggregation of nano- ZnO as shown by the greater hydrodynamic size of nano-ZnO than bulk-ZnO.

Studies of the toxicity of ZnO NPs to different organisms have several mechanisms of action. Overproduction of reactive oxygen species (ROS) can be the major mechanism of the toxicity of NPs (Sharifi et al., 2012). During this process, chemical reactions occur and result in overproduction of the superoxide radical (O_2^-), which leads to ROS accumulation leading to the development of holes in the membrane and oxidative stress (De Berardis et al., 2010). Nano ZnO

particles are absorbed onto the cell membrane and there it dissolves into ionic zinc, or to be internalized as whole. This dissolution could carry on inside the cell provoking the so called Trojan-horse effect (Limbach et al., 2007). Algae play an important role in the aquatic ecosystem, not only producing biomass that forms the basic nourishment for food webs, but also contributing to the self-purification of polluted water. Therefore, alga is one of the normally used model organism for toxicity testing of toxicants and nanoparticles as well. Effect of different nanoparticles on growth of algae is listed in the Table 6.

Table 6. Effect of different nanoparticles on algae

S.No.	Microalgae	Nanoparticle	Size	Mode of Action	Reference
1.	*Chlamydomonas reinhardtii*	ZnO & Carbon fullerenes	-	Unknown	Luo, 2007
2.	*Scenedesmus subspicatus*	Au	10 nm	Strongly adsorbed by the cell walls of algae leading to intracellular and wall distruptions	Renault et al., 2008
3.	*Chlamydomonas reinhardtii*	Ag	10 to 200 nm	Inhibitory effects to photosynthesis	Naverro et al., 2008
4.	*Nitellopsis obtuse*	ZnO	10-30 nm	Disappearance of turgor pressure	Manusadzianas et al., 2009
5.	*Pseudokirchneriella subcapitata*	ZnO, TiO_2 and CuO	50–70 nm, 25–70 nm, 30 nm	ZnO- Dose dependent toxicity causing inhibition of algal growth; TiO_2- reduced the light available to the entrapped algal cells and thus inhibited their growth; CuO- dose dependent inhibition of growth	Arnoja et al., 2009
6.	*Desmodesmus subspicatus*	TiO_2	4.5 nm	Unknown	Lang et al., 2010
7.	*Chlamydomonas reinhardtii*	Carbon nanotubes	-	Nanotubes affected photosynthetic membranes and thus decreased the electrochemical proton gradient on the membrane involved in the process of ATP synthesis.	Matorin et al., 2010
8.	*Pseudokirchneriella subcapitata*	CeO_2	25 nm	-	Manier et al., 2011
9.	*Microcystis aeruginosa*	CuO	68 to 36 nm	Enlarge pore size of cell wall, ROS formation and DNA damage	Wang et al., 2011
10.	*Chlorella sp.*	Al_2O_3, SiO_2, ZnO, and TiO_2	5–50 nm	Nano-Al_2O_3-suspension did not inhibit the algal growth, but showed growth promotion by 18.9%, No significant toxicity was observed for nano-SiO_2, Nano-ZnO was toxic to the algae Nano-TiO_2 inhibited the algal growth by 70.5%	Ji et al., 2011
11.	*Chlamydomonas reinhardtii*	Cd^{2+} adsorption on polyacrylate-	46.6 nm	Bioaccumulation of the Cd^{2+} in the algal cells	Yang et al., 2012

		coated TiO_2			
12.	*Ceramium Tenuicorne*	Ag	5 – 10 nm	Alteration of water content and concentration of inorganic ions	Macken et al., 2012
13.	*Chattonella marina*	Ag	300-500 nm	Increase in membrane permeability resulting in cell death	He et al., 2012
14.	*Ceramium tenuicorne*	PVP coated Ag	>5 nm, 5-10 nm, 100 nm, 16.0 (>5.8) nm	No toxicity, Growth rate was observed to increase with increasing salinity	Macken et al., 2012
15.	*Chlorella vulgaris*	Fe_2O_3	195.9, 176.5, and 347.2 nm	Deterioration of photochemical activities of photosynthesis, the induction of oxidative stress, and the inhibition of cell division rate	Barhoumi and Dewez., 2013
16.	*Pseudokirchneriella subcapitata*	ZnO, TiO_3	<100 nm, 21 nm	Destabilization of the cell membranes.	Lee and An., 2013
17.	*Dunaliella tertiolecta*	ZnO	100 nm	Toxicity W.R.T. peculiar physicochemical properties	Manzo et al., 2013
18.	*Chlorella vulgaris*	ZnO	-	Damage of chloroplasts and membranes in algal cells; also cause oxidative stress	Zhou et al., 2014
19.	*Desmodesmus subspicatus*	Au	5 and 58 nm	Unknown	Dedkova et al., 2014
20.	*Pseudokirchneriella subcapitata*	Ag	15 nm	Limited time for algal proliferation, thus minimizing the amount of exudates and oxygen produced	Sorensen and Baun., 2014
21.	*Pseudokirchneriella subcapitata*	Ag	2- 18 nm	Agglomerations on cell membranes	Becaro, 2014

Chapter 3

Materials and Methods

3.1. Synthesis of zinc oxide nanoparticles from *Aspergillus niger* (Baskar et al., 2013, Jacob et al., 2014)

a. *A. niger* was inoculated in potato dextrose broth (PDB) and incubated on orbital shaker at 25±2° C at 120 rpm for 4 days.
b. Biomass was harvested after complete incubation and centrifuged at 4000 rpm for 10 minutes at 4° C.
c. The supernatant after the centrifugation was collected.
d. 50 ml of the supernatant was taken and mixed with 40 ml of distilled water in Erlenmeyer flask and kept on the hot plate at the fixed temperature of 80° C.
e. When the temperature would rise and reach 70° C, then 10 ml of 100 mM zinc acetate was added dropwise.
f. Reaction was carried on for 8-9 hrs.
g. After the completion of reaction, the reaction mixture was centrifuged at 4000 rpm for 10 minutes at 4° C.
h. Nanoparticles were washed with distilled water thrice.
i. Then dried in oven at 90° C for 8 hrs.
j. Characterisation of the nanoparticles is done by the following.
    i. XRD- Phase purity and particle size were determined by X-ray diffraction (XRD) analysis recorded by diffractometer (XPERT- PRO).
    ii. SEM- The shape and size of the sample were determined by Scanning electron microscope (Model: JEOL, JSM-6510 LV, USA).
    iii. FTIR- The chemical structure was examined by using FTIR spectro-meter (Perkin Elmer model RZX spectrometer).

3.2. Synthesis of zinc oxide nanoparticles from *Anabaena variabilis* (Azizi et al., 2014)

a. The algal sample ARM441 was procured from National centre for conservation and utilization of blue green algae, Division of microbiology, IARI, New Delhi.
b. The alga was inoculated in BG11 medium prepared from distilled water with final pH 7.0-7.3 for 30 days at 2500-3000 lux of light conditions at 28° C.

c. After 30 days, alga was harvested by filtering through whatmann filter paper no. 1 and allowed to dry for 24 hrs.
d. After 24 hrs, dried biomass of algae was collected.
e. 100 ml of the distilled water was added to the dried biomass and it was heated to 100° C for 2 hrs by keeping on hot plate at shaking conditions.
f. Then it was filtered using whatmann filter paper no. 1, the filterate was collected to synthesise ZnO NPs
g. Then 50 ml of the algal solution was added to Erlenmeyer flask and mixed with 40 ml of the distilled water and kept on the hot plate at the fixed temperature of 80° C.
h. When the temperature would rise and reach 70° C, then 10 ml of 50 mM Zinc acetate was added dropwise.
i. Reaction was carried on for 6 hrs.
j. After the completion of reaction, the reaction mixture was centrifuged at 4000 rpm for 10 minutes at 4° C.
k. Nanoparticles were washed with distilled water thrice.
l. Then dried in oven at 90° C for 8 hrs.
m. Characterisation of the nanoparticles is done by the following.
    i. XRD- Phase purity and particle size were determined by X-ray diffraction (XRD) analysis recorded by diffractometer (XPERT- PRO).
    ii. SEM- The shape and size of the sample were determined by Scanning electron microscope (Model: JEOL, JSM-6510 LV, USA).
    iii. FTIR- The chemical structure was examined by using FTIR spectro-meter (Perkin Elmer model RZX spectrometer).

3.3. Synthesis of zinc oxide nanoparticles from *Trichoderma reesei* (Baskar et al., 2013, Jacob et al., 2014)

a. *T. reesei* was inoculated in potato dextrose medium (PDB) and incubated on orbital shaker at 25±2° C at 120 rpm for 4 days.
b. Biomass was harvested after complete incubation and centrifuged at 4000 rpm for 10 minutes at 4° C.
c. The supernatant after the centrifugation was collected.
d. 50 ml of the supernatant was taken and mixed with 40 ml of distilled water in Erlenmeyer flask and kept on the hot plate at the fixed temperature of 80° C.

e. When the temperature would rise and reach 70° C, then 10 ml of 100 mM Zinc acetate was added dropwise.
f. Reaction was carried on for 8-9 hrs.
g. After the completion of reaction, the reaction mixture was centrifuged at 4000 rpm for 10 minutes at 4° C.
h. Nanoparticles were washed with distilled water thrice.
i. Then dried in oven at 90° C for 8 hrs.
j. Characterisation of the nanoparticles is done by the following.
   i. XRD- Phase purity and particle size were determined by X-ray diffraction (XRD) analysis recorded by diffractometer (XPERT- PRO).
   ii. SEM- The shape and size of the sample were determined by Scanning electron microscope (Model: JEOL, JSM-6510 LV, USA).
   iii. FTIR- The chemical structure was examined by using FTIR spectro-meter (Perkin Elmer model RZX spectrometer).

3.4. Effect of ZnO nanoparticles on the growth of *Anabaena variabilis* and *Nostoc muscorum*

a. The algal sample ARM441 *Anabaena variabilis* and ARM442 *Nostoc muscorum* were procured from National centre for conservation and utilization of blue green algae, Division of microbiology, IARI, New Delhi.
b. Algae were inoculated in triplicates in the BG11 medium without adding $ZnSO_4$ but replace it with different concentrations of ZnO nanoparticles i.e. 2, 5, 10, 20, 30, 50, 100 ppm.
c. Alga was incubated for 21 days at 7000-8000 lux of light conditions at 28° ±2° C.
d. After 21 days, following tests are done for the estimation of the effects of ZnO nanoparticles on algae.
   i. Wet and dried biomass estimation
   ii. Chorophyll estimation
   iii. Indole acetic acid test
   iv. Nitrate reductase test

3.4.1. Wet and dry biomass emtimation (Richmond and Gobbelaar, 1986)

a. The weight of dried whatmann filter paper 42 was noted as initial reading.
b. BG11 medium was sprinkled on the whatmann filter paper 42 to make it wet.
c. The weight of the wet filter paper was noted.

d. The difference between initial and final reading of the wet filter paper is the wet weight of the algae.
e. The algal cultures were homogenised by vigorous shaking by adding glass beads to it.
f. 10 ml of the culture was taken and filtered through previously dried filter paper and noted its wet weight.
g. Filter paper was kept for drying and transferred to hot air oven maintained at 40° C overnight.
h. The difference between initial and final reading of the dry filter paper is the dry weight of the algae.

3.4.2. Chlorophyll estimation (Mckinney, 1941)

a. 10 ml of algal suspension was taken and washed with sterile distilled water twice.
b. 10 ml of 96% of the methanol was added and kept on the water bath for 60℃ for 30 minutes.
c. Then allowed to cool at room temperature and centrifuged at 8000 rpm for 10 minutes.
d. OD was taken was 650 nm and 665 nm against methanol as blank.

$$\text{Total chlorophyll} = 2.55\times10^{-2}\times E_{650} + 0.4\times10^{-2}\times E_{665} \text{ mg/ml}$$

E_{650} is OD of the sample at 650 nm

E_{665} is the OD of the sample at 665 nm

3.4.3. Estimation of IAA (Glickmann and Dessaux, 1995)

Reagents required- IAA for stock solution and salkoswski reagent

Stock solution of IAA-Dissolve 20 mg of IAA in 20 ml of acetone i.e. concentration of stock solution was 1 mg/ml.

Salkoswski reagent-Mix 2 ml of 0.5 M $FeCl_3$ + 49 ml of water + 49 ml of 70% perchloric acid.

Method-

a. After 21 days, cyanobacterial cells were removed by centrifugation at 4000 rpm for 20 minutes at 4° C.
b. Then salkoswoski reagent was added to the supernetent in the ratio of 1:2 (v/v) and incubated for 30 minutes in dark conditions at room temperature.

c. Concentarion of IAA was estimated by taking the absorbance at 535 nm against a control of 1 ml culture medium and 2 ml of salkoswski reagent.

3.4.4. Nitrate reductase estimation (Lowe and Evans, 1964)

Reagents required-Reagent A: 1% sulphanilamide in 100 ml of 1 N HCl; Reagent B: 0.2% of NEDD (N-1-naphthyl ethylene diamine dihydrochoride)

Procedure-

a. 10 ml of cyanobacterial suspension was taken.
b. Centrifuged at 4000 rpm for 10 minutes and pellet was washed with sterile water twice.
c. Pellet was incubated in basal medium 47 containing 10 mM of $NaNO_3$ to induce NR.
d. After overnight incubation; 1 ml of sample was taken and to this added 2 ml of reagent A.
e. Mixed them well and after 15 minutes added 2 ml of reagent B.
f. Pink colour was allowed to develop for 15 minutes.
g. Absorbance was recorded at 540 nm and values were calibrated against the standard curve using $NaNO_3$.

3.5. Effect of different zinc sources on the growth of *Anabaena variabilis* and *Calothrix* sp

a. The algal sample ARM441 *Anabaena variabilis* was procured from IARI, New Delhi.
b. Algae was inoculated in triplicates in the BG11 medium with different sources of Zinc i.e. $ZnSO_4$, $ZnCl_2$, ZnO and nZnO at same concentrations of 0.222 g per litre.
c. Alga was incubated for 21 days at 7000-8000 lux of light conditions at 28° C.
d. After 21 days, following tests are done for the estimation of the effects of ZnO nanoparticles on algae.
    i. Wet and dried biomass estimation
    ii. Chorophyll estimation
    iii. Nitrate reductase test
    iv. Indole acetic acid test
    v. Available nitrogen test

3.5.1. Total nitrogen estimation (Piper, 1960)

Reagents required

a. Catalyst mixture: for algae- 10 g anhydrous sodium sulphate + 1 g copper sulphate pentahydrate in the ratio 10:1
b. Mixed indicator: Dissolve 0.5 g of bromophenol green and 0.1 g of methyl red indicator in 100 mL of ethanol.
c. Boric acid solution: Dissolve 40 g H_3BO_3 in 1 L of distilled water; add 5 mL of indicator per litre of boric acid solution.
d. NaOH solution - 40% of NaOH solution. This solution is allowed to stand for 24-48 hrs as to precipitate out Na_2CO_3 impurities.
e. Conc. H_2SO_4- Specific gravity 1.84
f. Sodium thiosulphate
g. 0.01 N H_2SO_4

Procedure

a. Algal sample (25 mL) was placed in 500 mL kjeldahl digestion tube.
b. Added 25 mL conc. H_2SO_4 and allowed to stand for 30 minutes.
c. To the above mixture 5 g of sodium thiosuphate was added and allowed to stand for 30 minutes.
d. After that 2-3 g catalyst mixture was added into the flask and glass beads were added to prevent bumping. Digestion was continued for 1 hour until the digest get clear.
e. Placed the flask with material in kjeldahl unit for automatic distillation.
f. To this digest 150 mL of distilled water was added, cooled and again added 120 mL of 40% NaOH along the sides of kjeldahl flask and connected to the distillation system.
g. Ammonia was collected in 25 mL boric acid in 250 mL flask (to which mixed indicator has already been added) and continued till 150 mL distillate was collected.
h. This was followed by the titration against 0.01 N sulphuric acid.

Calculations

Amount (g) of N_2 in samples = (mL of acid for sample – mL of acid for blank) × Normality of acid × 14 × 10^{-3}

% of N_2 in samples = Amount of N_2 in samples × 100/ sample

Chapter 4

Results and Discussion

4.1. Synthesis of zinc oxide nanoparticles from *Aspergillus niger*

A. niger was inoculated in potato dextrose broth and incubated at 25±2° C on orbital shaker at 120 rpm for 4 days. Biomass was harvested by centrifugation and the supernatant was used for the synthesis of zinc oxide nanoparticles. Zinc acetate was used as a metal precursor and the formation of ZnO NPs was confirmed through visual judgment as the colour of the mixture reaction changes from pale yellow to turbid white colour after 8-9 hrs indicating the synthesis of zinc oxide nanoparticles (Baskar et al., 2013). This colour change is an indication of reduction of zinc acetate ions by the proteins present in culture filtrate and ZnO nanoparticle were synthesised (Baskar et al., 2013) and baked at 90° C for 8 hrs to obtain pale brown powder as shown in Fig. 5.

Fig. 5. ZnO NPs synthesized from *A. niger*

4.1.1. Characterisation of nanoparticles

4.1.1.1. XRD

The X-ray diffraction patterns of ZnO nanoparticles are shown in Fig. 6. Sharper and stronger diffraction peaks were observed from Fig. 4 at 31.84°, 32.71°, 34.47°, 36.33°, 47.59°, 56.67°, 59.80°, 62.92°, 67.96°, 68,96°, 76.82°, 79.66°. The appearance of signals was likely due to X-ray emission from carbohydrates/proteins/enzymes present in the cell wall of the biomass (Chauhan et al., 2014). The shift in the 2Θ peak values of ZnO nanoparticles may be due to the presence of protein molecule from fungal culture filtrate (Baskar et al., 2013). The average crystallite size of the nZno synthesised by *A. niger* was 30 nm as calculated by debye sherrer formula (West, 1974).

Debye Sherrer formula $D = K\lambda / \beta_{½} \cos\Theta$

Where, K is the Sherrer constant (K=0.89 for spherical particle)

λ is the X-ray wavelength (λ=1.54060 Å)

$\beta_{1/2}$ is the width of the XRD peak at half height

Θ is the Bragg diffraction angle

After substituting these values,

$D = 0.9\ (1.54060)/\ (0.451)(\cos 0.3170) = 30.76$ nm

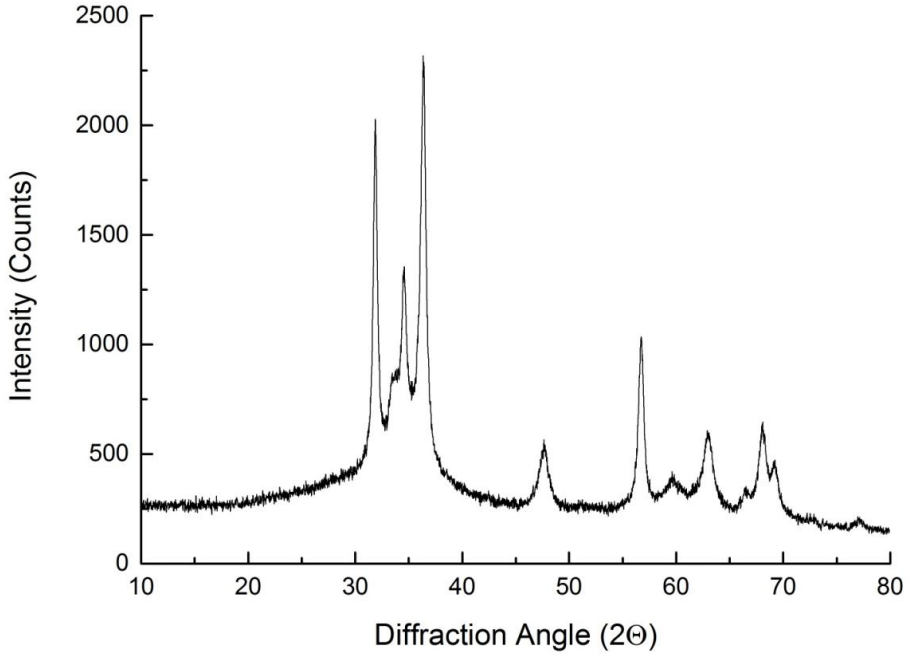


Fig. 6. X-ray diffraction patterns of ZnO nanoparticles synthesized from *A.niger*

4.1.1.2. SEM

Scanning Electron Microscopy was used to assess the particle size and morphology of the synthesized zinc oxide nanoparticles. It was concluded from Fig. 7 that the particles in the samples were compactly arranged and were almost spherical in shape. The size of the synthesized zinc oxide nanoparticles were found in range between 64-126 nm.

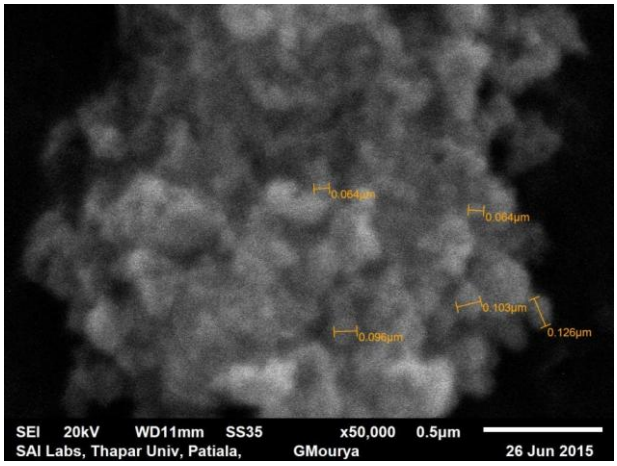


Fig. 7. SEM image of ZnO nanoparticles synthesised from *A. niger*

4.1.1.3. FTIR

The synthesized zinc oxide nanoparticles were subjected to FTIR analysis to detect the various characteristic functional group associated with the synthesized nanoparticles. The peaks indicate the characteristic functional group present in the synthesized zinc oxide nanoparticles. It is inferred from Fig. 8 that the samples have absorption peaks in the range of 3075.71 cm^{-1}, 1675.05 cm^{-1}, 1538.30 cm^{-1}, 1385.57 cm^{-1}, 1051.99 cm^{-1} and 859 cm^{-1}. The absorption peak at 568 cm^{-1} corresponds to metal-oxygen (ZnO stretching vibrations) vibrational mode. The peak at 1052 cm^{-1} is credited to the stretching vibration of C-N bond of the primary amine or to the stretching vibration of the C-O bond of the primary alcohol. The peak at 1269 $cm^{-1}$and 1385 cm^{-1} are ascribed to primary, secondary alcohol in-plane bend or vibrational modes of aromatic primary amine and trimethyl, tertiary alcohol, organic sulphate. The peaks at 1538 $cm^{-1}$and 1675 cm^{-1} are attributed to the vibrational modes of aromatic nitro compounds and alkyl C=C stretch, amide, open chain imino group (Baskar et al., 2013). The FTIR spectrum of ZnO nanoparticles showed distinct peaks at 1615 cm^{-1}, which represent the involvement of C=N in plane vibrations of amino acids, the bands from 1099 – 1142 cm^{-1} represent the involvement of C-N in plane vibrations of aliphatic amines. The above bonds commonly occur in fungal proteins indicating the presence of proteins as ligands for ZnO nanoparticles, which increase the stability of zinc oxide nanoparticles (Jacob et al., 2014).

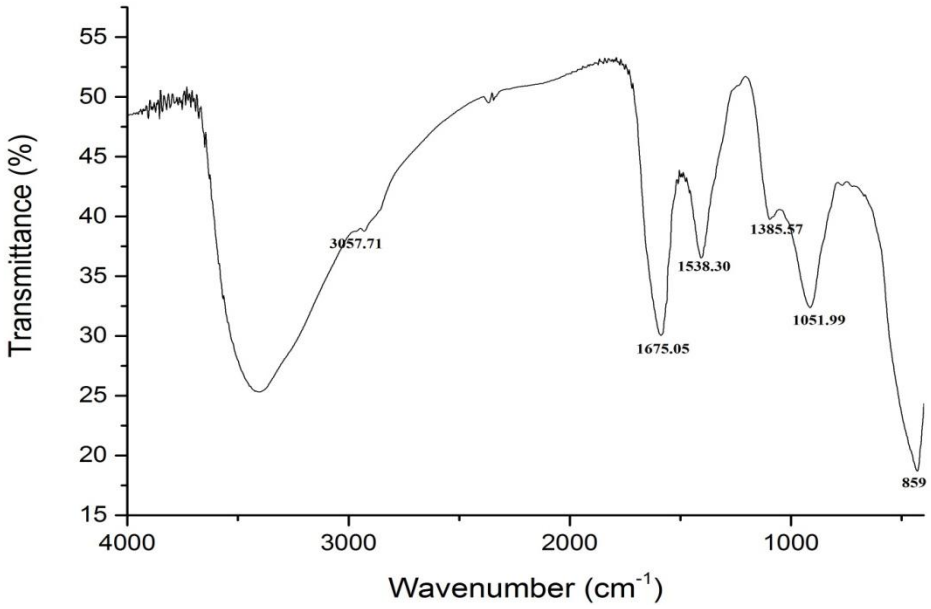


Fig. 8. FTIR spectra of ZnO nanoparticles synthesized from *A. niger*

4.2. Synthesis of zinc oxide nanoparticles from *Anabaena variabilis*

A. variabilis was inoculated in BG11 medium for 30 days at 2500-3000 lux of light intensity at 28° C. After 30 days, alga was harvested by filtering through whatmann filter paper no. 1 and allowed to dry for 24 hrs. Then dried biomass of algae was mixed with distilled water and heated to 100° C for 2 hrs and after filtering through whatmann filter paper no. 1, the filterate was collected to synthesize ZnO NPs. Zinc acetate was used as metal precursor and formation of ZnO NPs was confirmed through visual judgment as the color of reaction mixture was changed from pale green to turbid white color after 6 hrs, which indicates the synthesis of ZnO NPs. Several functional groups exist in bioactive compounds of alga such as fatty acids, alkaloids, amino acids, polyketide and non-ribosomal peptide biosynthetic origins (Jones et al., 2009), which play an important role in the formation and stabilization of ZnO NPs were synthesised and baked at 90° C for 8 hrs as pale green powder (Fig. 9).

Fig. 9. ZnO NPs synthesized from *A. variabilis*

4.2.1. Characterization of zinc oxide nanoparticles

4.2.1.1. XRD

Fig. 10 shows the XRD pattern of the biosynthesized ZnO NPs from *A. variabilis* biomass. Positions and intensities of all relative peaks of the ZnO NPs matched the Joint Committee on Powder Diffraction Standard (JCPDS) card number 36-1451. Sharper and stronger diffraction peaks are observed at 26.70°, 31.83°, 34.00°, 34.52°, 36.32°, 47.60°, 56.64°, 62.92°, 67.99°, 69.11°, 72.60°, 76.98°. All peak intensitities were characteristic of the nanoparticles spherical structure. In addition, no diffraction peaks from other types could be detected, which illustrates that all the precursors have been completely decomposed and no other crystal materials have been maintained (Azizi et al., 2014). The distinct diffraction peaks give confirmation of well-crystallized structure of bio-synthesized ZnO NPs. The average crystallite size of the nZno synthesised by *A. variabilis* was 50 nm as calculated by debye sherrer formula (West, 1974).

$$\text{Debye Sherrer formula } D = K\lambda / \beta_{1/2} \cos\Theta$$

Where, K is the Sherrer constant (K=0.89 for spherical particle)

λ is the X-ray wavelength (λ=1.54060 Å)

$\beta_{1/2}$ is the width of the XRD peak at half height

Θ is the Bragg diffraction angle

After substituting these values,

$$D = 0.9\ (1.54060)/\ (0.277)(\cos 0.3170) = 50.09 \text{ nm}$$

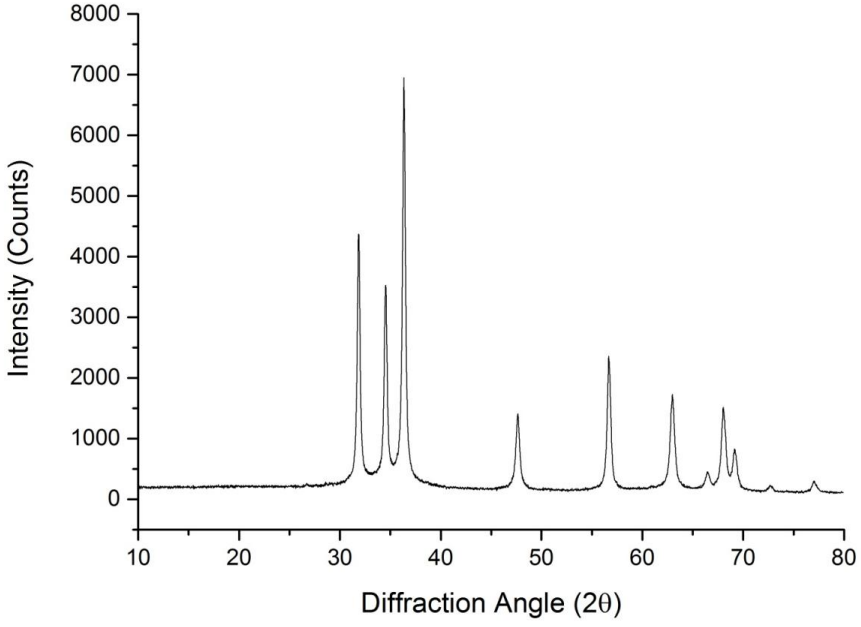


Fig. 10. XRD patterns of ZnO nanoparticles synthesised from *A. variabilis*

4.2.1.2. SEM

Fig. 11 shows the SEM image showing the morphology and particle size of pure ZnO NPs which are closely agglomerated with a spherical structures and particle size ranging from 72-74 nm with some deviations. This agglomeration is due to polarity and electrostatic attraction of ZnO nanoparticles (Azizi et al., 2014).

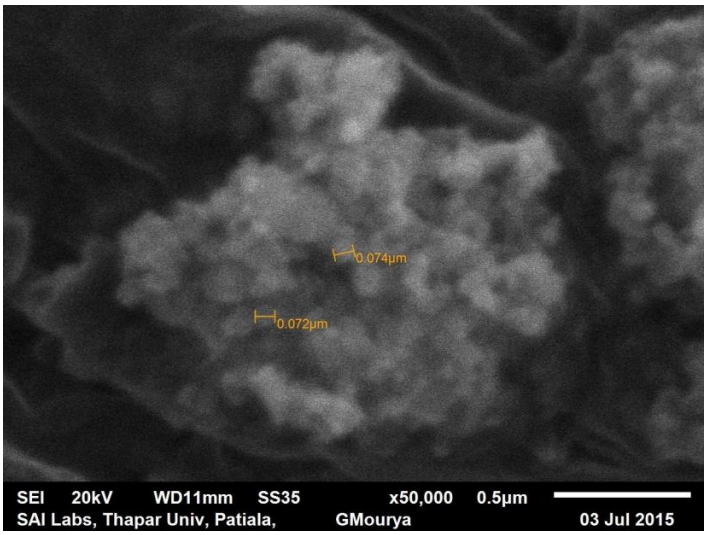


Fig. 11. SEM image of ZnO nanoparticles synthesised from *A. variabilis*

4.2.1.3. FTIR

FTIR spectrum shown in the Fig. 12 confirmed the structure of zinc oxide nanoparticles synthesised from *Anabaena variabilis* with bands at 1610, 1420, 1235 and 1037 cm^{-1} (Azizi et al., 2014). Signal at 1235 cm^{-1} relates to the asymmetric stretching vibration of a sulphate group, that is now disappeared and the bands intensity around 1037–3340 cm^{-1} ascribed to the symmetric C–O vibration associated with a C–O–SO_3 and hydroxyl groups, respectively which are decreased after synthesis of ZnO NPs indicated the involvement of sulphate and hydroxyl groups in the formation of nanoparticles. The above bonds are commonly occuring polysaccharide in algae indicating the participation of sulphated polysaccharides in the synthesis of ZnO NPs. Another band observed at 1610 cm^{-1} is attributed to the stretching vibration of (NH)C¼O group that are characteristic of proteins slightly shifted from 1630 cm^{-1} and became broader, indicating a member of (NH)C¼O group within the cage of cyclic peptide is involved in stabilizing the nanoparticles. The signal at 441 cm^{-1} corresponded to the stretch band of zinc and oxygen (Azizi et al., 2014).

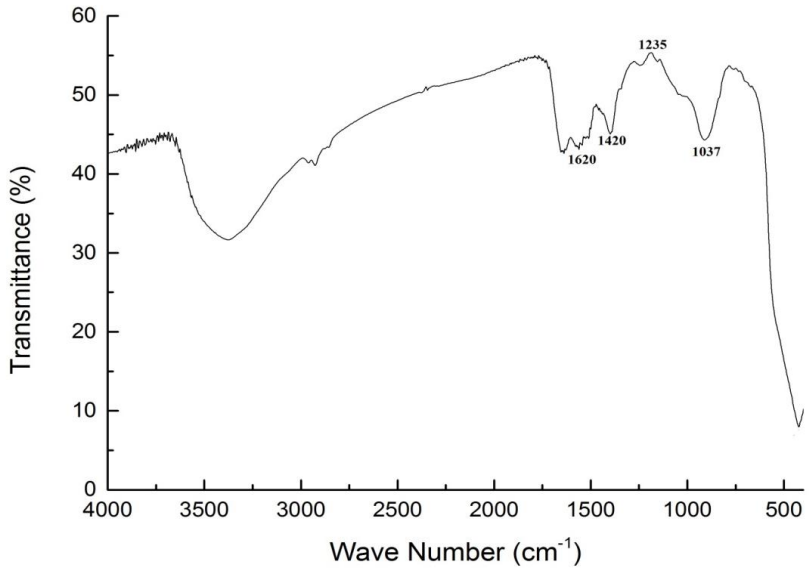


Fig. 12. FTIR spectra of ZnO nanoparticles synthesized from *A. variabilis*

4.3. Synthesis of zinc oxide nanoparticles from *Trichoderma reesei*

T. reesei was inoculated in potato dextrose broth and incubated at 25±2° C on orbital shaker at 120 rpm for 4 days. Biomass was harvested by centrifugation and supernatant was used for synthesis of zinc oxide nanoparticles. Zinc acetate was used as a metal precursor and the formation of ZnO NPs was confirmed through visual judgment as the colour of the mixture reaction changes from pale yellow to cloudy white color after 8-9 hrs indicating the synthesis of zinc oxide nanoparticles (Baskar et al., 2013). In the biosynthesis of metal oxide nanoparticle by a fungus, enzymes are produced which reduce a salt to its metallic solid nanoparticles through the catalytic effect (Jacob et al., 2014,) and later ZnO NPs were synthesised and baked at 90° C for 8 hrs to obtain pale brown powder as shown in the Fig. 13.

Fig. 13. ZnO NPs synthesised from *T. reesei*

4.3.1. Characterisation of zinc oxide nanoparticles

4.3.1.1. XRD

Fig.14 shows the XRD pattern of the biosynthesized ZnO NPs from *Trichoderma reesei*. The appearance of signals was likely due to X-ray emission from carbohydrates/proteins/enzymes

present in the cell wall of the biomass (Chauhan et al., 2014). Positions and intensities of all relative peaks of the ZnO NPs matched the Joint Committee on Powder Diffraction Standard (JCPDS) card number 36-1451. Sharper and stronger diffraction peaks are observed at 31.83°, 33.28°, 33.81°, 36.31°, 48.40°. The shift in the 2Θ peak values of ZnO nanoparticles may be due to the presence of protein molecule from fungal culture filtrate. The distinct diffraction peaks give confirmation of well-crystallized structure of bio-synthesized spherical ZnO NPs. There are no other diffraction peaks detected which illustrates that all the precursors have been completely decomposed and other crystal materials are mixed. The mean particle sizes of the nanoparticles were estimated Scherrer's equation. The average crystallite size of the nZno synthesised by *T. reesei* was 37 nm as calculated by debye sherrer formula (West, 1974).

Debye Sherrer formula $D = K\lambda / \beta_{½} \cos\Theta$ (West, 1974)

Where, K is the Sherrer constant (K=0.89 for spherical particle)

λ is the X-ray wavelength (λ=1.54060 Å)

$\beta_{1/2}$ is the width of the XRD peak at half height

Θ is the Bragg diffraction angle

After substituting these values,

$D = 0.9 (1.54060)/ (0.37402)(\cos 0.3163) = 37.09$ nm

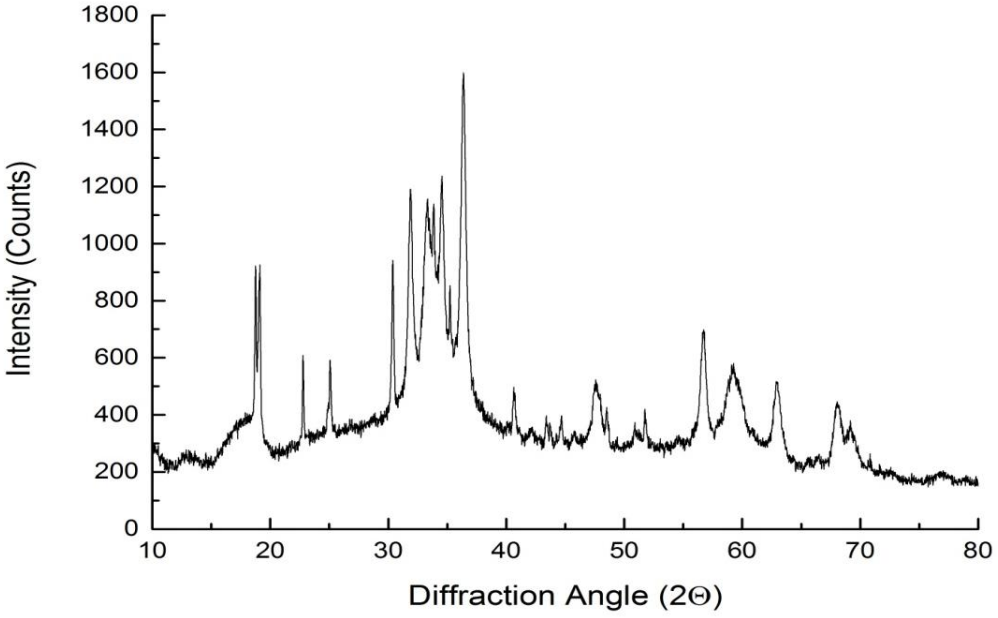


Fig. 14. XRD patterns of ZnO nanoparticles synthesized from *T. reesei*

4.3.1.2. SEM

Scanning Electron Microscopy was used to estimate the particle size and morphology of the synthesized zinc oxide nanoparticles. It was concluded from Fig. 15 that the particles in the samples were too compactly arranged and were almost spherical in shape. The size of the synthesized zinc oxide nanoparticles could not be estimated due to their compaction in arrangement.

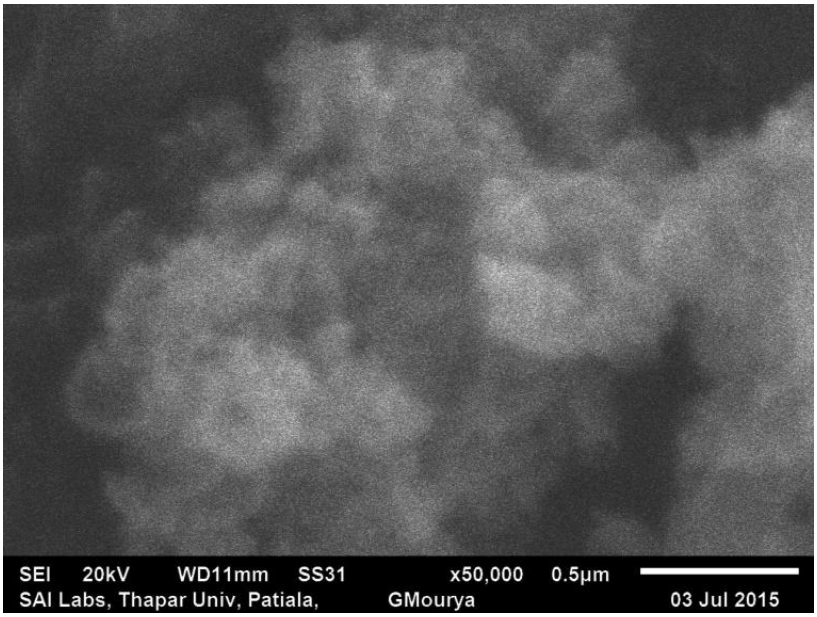


Fig. 15. SEM image of ZnO nanoparticles synthesised from *T. reesei*

4.3.1.3. FTIR

The synthesized zinc nanoparticles (Fig. 16) were subjected to FT-IR analysis to detect the various characteristic functional group associated with the synthesized nanoparticles. The peaks indicate the characteristics functional group present in the synthesized zinc oxide nanoparticles. It is noted from Fig.15 that the samples have absorption peaks in the range of 1615.05 cm^{-1}, 1538.57 cm^{-1}, 1385.57 cm^{-1} and 1052.99 cm^{-1}. The peak at 1052 cm^{-1} is ascribed to the stretching vibration of C-N bond of the primary amine or to the stretching vibration of the C-O bond of the primary alcohol. The peaks at 1538 cm^{-1} are ascribed to the vibrational modes of aromatic nitro compounds and alkyl C=C stretch, amide, open chain imino group (Baskar et al., 2013). The above bonds commonly occur in fungal proteins indicating the presence of proteins as ligands for ZnO nanoparticles, which increase the stability of zinc oxide nanoparticles (Jacob et al., 2014).

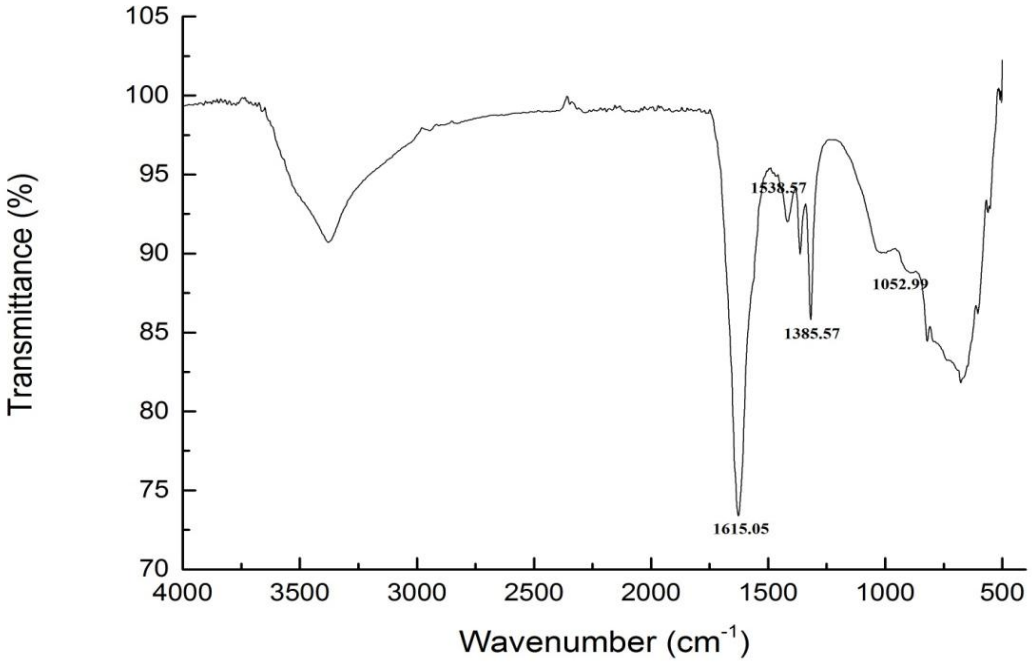


Fig. 16. FTIR spectra of ZnO nanoparticles synthesised from *T. reesei*

4.4. Effect of zinc oxide nanoparticles on the growth of *Anabaena variabilis* and *Nostoc muscorum*

A. variabilis and *N. muscorum* were grown in BG11 medium with different concentrations of nZnO (0-100 ppm) for 30 days. Studies on the effect of ZnO nanoparticle on the growth of *N. muscorum* and *A. variabilis* which reveals that *N. muscorum* is much more sensitive to nanoparticles than *A. variabilis*. The difference of various parameters when observed in both algae indicated that the extent of nanoparticle toxicity is different in different algal species. These metal oxide nanoparticles can have different effects on different organisms and their toxicity depends on their nano structures and high surface to mass ratio as well as the nature of their constitutive element. Based on the studies conducted on various algal species, ZnO nanoparticle had the maximum toxicity followed by aluminum oxide, titanium dioxide and cesium oxide nanoparticles, respectively (Pendashte et al., 2013). In this regard, based on our results *N. muscorum* is sensitive than *A. variabilis* which is visible from Fig. 17 and Fig. 18.

Zinc oxide nanoparticles are toxic to *Chlorella vulgaris* as these nanostructures are adsorbed by cell membranes and a small amount of the NPs can enter the cells that cause damage to chloroplasts and membranes. Hence the toxicity of ZnO NPs was dose-dependent i.e. they also act as growth stimulating at some concentrations (Zhou et al., 2014). ZnO NPs and Zn ions caused mitochondria damage, DNA damage and generation of ROS in algal cells when

present in excess (Lee et al., 2013). Nitrogen fixing cyanobacteria showed inhibition of growth rate in the presence of heavy metals (Gupta et al., 2015). Effect of ZnO NPs and other sources of Zn were studied on the growth of *A. variabilis* and *N. muscorum*. Biomass and chlorophyll content were studied as growth parameters. Whereas their effect on the physiological parameters such as NR activity and IAA production by algae in presence of ZnO NPs was also done and compared with other sources of Zn such as $ZnCl_2$, $ZnSO_4$, $ZnNO_3$, ZnO, $Zn(CH_3COO^-)_2$.

Fig. 17. Effect of zinc oxide nanoparticles on the growth of *Nostoc muscorum*

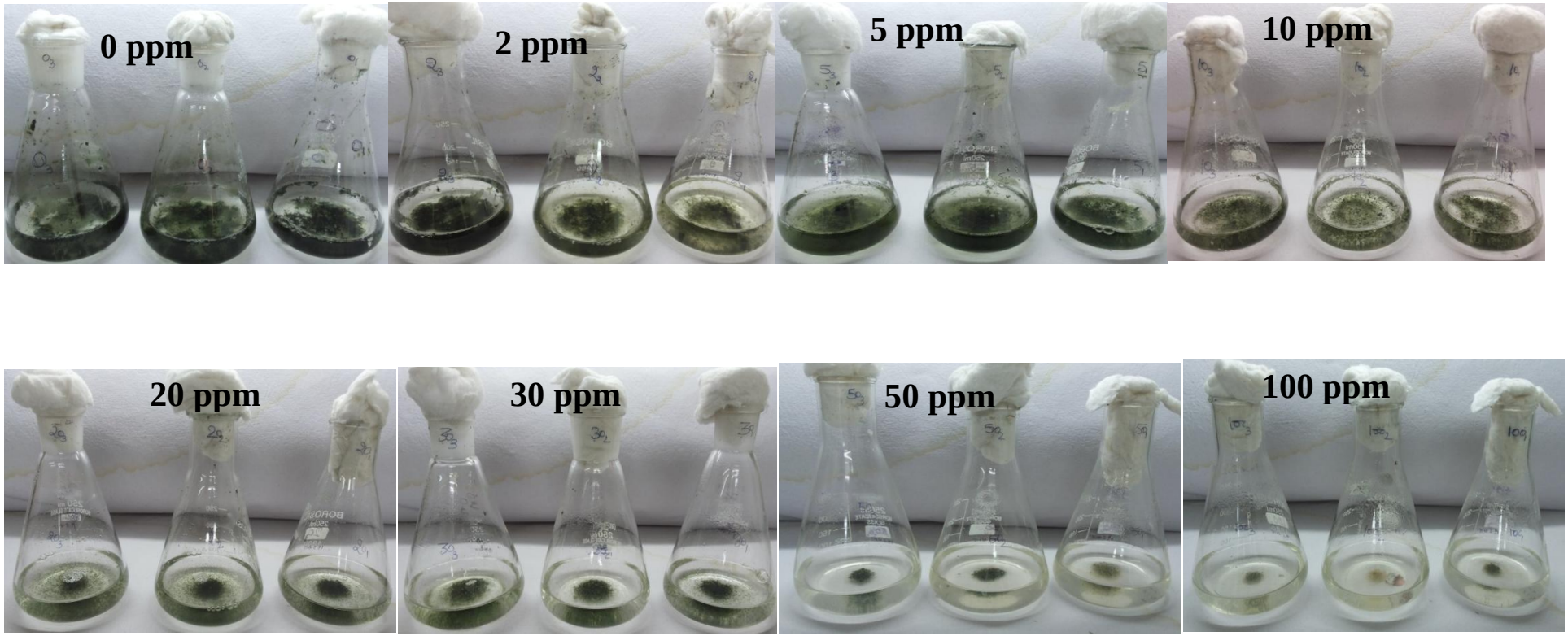
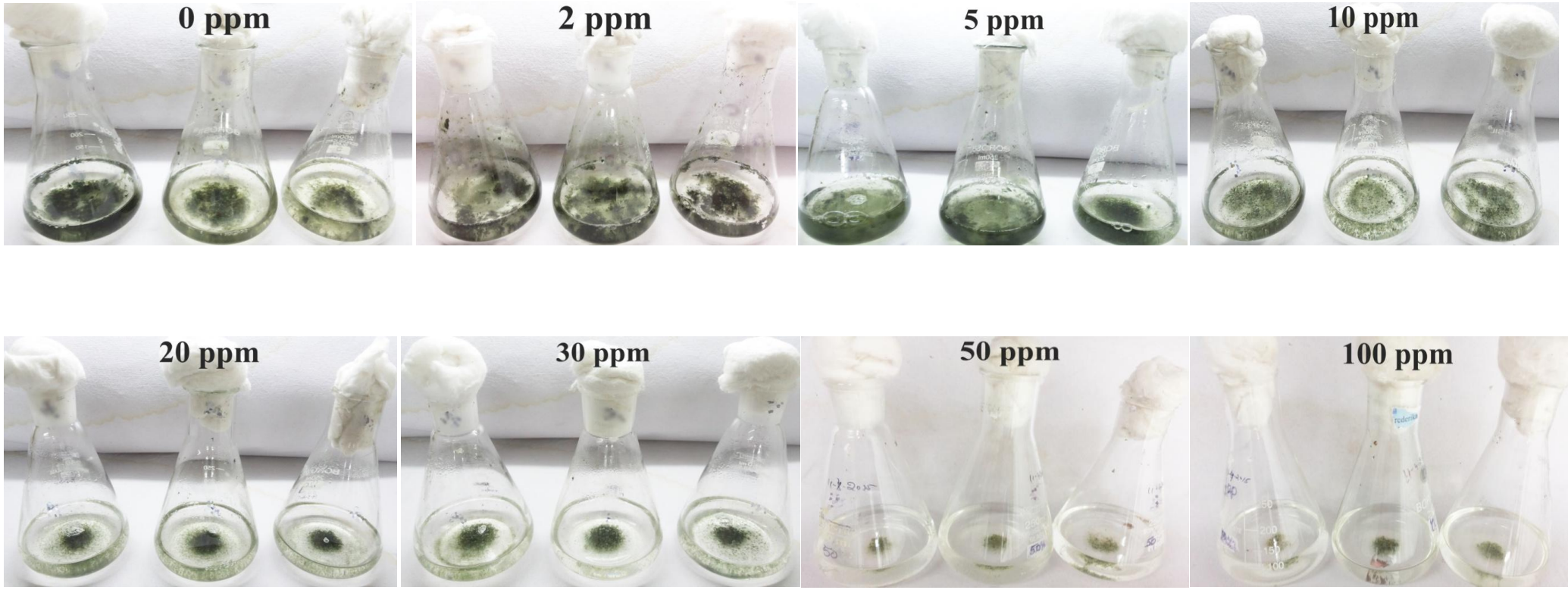


Fig. 18. Effect of zinc oxide nanoparticles on the growth of *Anabaena variabilis*



4.4.1. Wet biomass

The effect of ZnO NPs at different concentration (0-100 ppm) on the growth of *A. variabilis* and *N. muscorum* was studied (Table 7). 2 ppm concentration was found stimulating for the growth in *A. variabilis* and inhibiting at concentration above 5 ppm, Inhibition occurred at 2 ppm in *N. muscorum* as compared to control (Fig. 19).

Table 7. Wet biomass (g/10 ml) of *A. variabilis* and *N. muscorum*

ZnO NPs (ppm)	Wet biomass (g/10 ml) of *A. Variabilis*	Wet biomass (g/10 ml) of *N. muscorum*
0	0.957±0.017	0.15±0.014
2	0.960±0.003	0.13±0.004
5	0.820±0.024	0.09±0.016
10	0.716±0.002	0.07±0.012
20	0.643±0.002	0.05±0.011
30	0.610±0.029	0.02±0.006
50	0.563±0.015	0.01±0.003
100	0.473±0.027	0.001±0.002

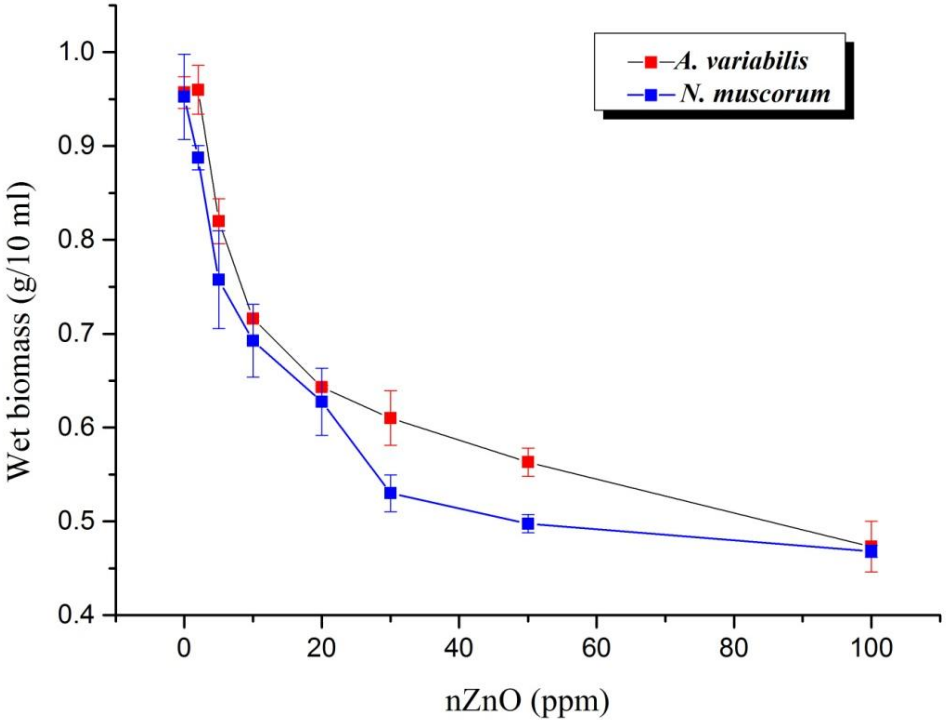


Fig. 19. Wet biomass (g/10 ml) of *A. variabilis* and *N. muscorum*

4.4.2. Dry biomass

The effect of ZnO NPs at different concentration (0-100 ppm) on the growth of *A. variabilis* and *N. muscorum* was studied (Table 8). Growth was found stimulatory for 2 and 5 ppm concentration in *A. variabilis* and inhibition occur above 5 ppm whereas inhibition occur at and above 2 ppm in *N. muscorum* as compared to control where no zinc oxide nanoparticles were provided as shown in the Fig. 20.

Table 8. Dry biomass (g/10 ml) of *Anabaena variabilis* and *Nostoc muscorum*

ZnO NPs (ppm)	Dry biomass (g/10 ml) of *A. variabilis*	Dry biomass (g/10 ml) of *N. muscorum*
0	0.0080±0.001	0.014±0.0019
2	0.0086±0.002	0.010±0.0002
5	0.0083±0.001	0.008±0.0005
10	0.0023±0.000	0.005±0.0005
20	0.0023±0.000	0.006±0.0013
30	0.0016±0.000	0.003±0.0011
50	0.0015±0.000	0.003±0.0012
100	0.0010±0.000	0.001±0.0000

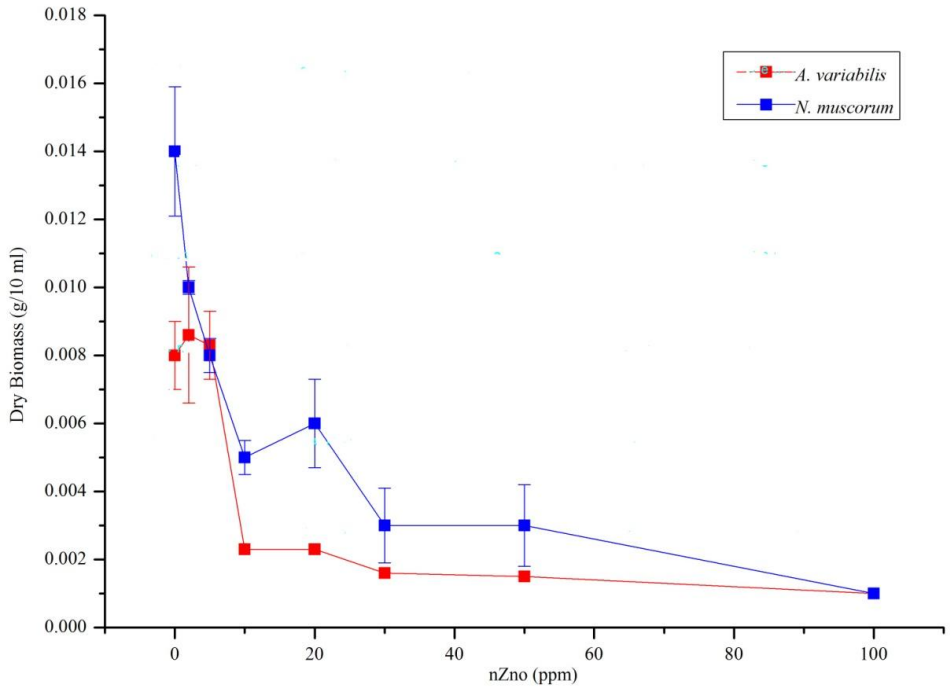


Fig. 20. Dry biomass (g/10 ml) of *A. variabilis* and *N. muscorum*

4.4.3. Chlorophyll

Chlorophyll in the algae relates to the photosynthetic activity. Thus if zinc oxide nanoparticles are sensitive to algae then less chlorophyll content relates to less photosynthetic activity. The effect of ZnO NPs at different concentration (0-100 ppm) on the growth of *A. variabilis* and *N. muscorum* was studied (Table 9). The amount of chlorophyll increased upto 2 ppm in *A. variabilis*, thereafter it decreased with increasing concentration of nZnO and inhibition occured above 2 ppm whereas inhibition occured at and above 2 ppm in *N. muscorum* (Fig. 21).

Table 9. Chlorophyll (mg/ml) of *Anabaena variabilis* and *Nostoc muscorum*

ZnO NPs (ppm)	Chlorophyll (mg/ml) of *A. variabilis*	Chlorophyll (mg/ml) of *N. muscorum*
0	$0.73920\times10^{-4}\pm0.032$	$0.73538\times10^{-4}\pm0.027$
2	$0.86435\times10^{-4}\pm0.043$	$0.67667\times10^{-4}\pm0.013$
5	$0.60992\times10^{-4}\pm0.019$	$0.53173\times10^{-4}\pm0.031$
10	$0.17885\times10^{-4}\pm0.007$	$0.21673\times10^{-4}\pm0.014$
20	$0.14283\times10^{-4}\pm0.037$	$0.19780\times10^{-4}\pm0.006$
30	$0.07195\times10^{-4}\pm0.003$	$0.18005\times10^{-4}\pm0.014$
50	$0.03916\times10^{-4}\pm0.007$	$0.07300\times10^{-4}\pm0.001$
100	$0.02260\times10^{-4}\pm0.003$	$0.05330\times10^{-4}\pm0.003$

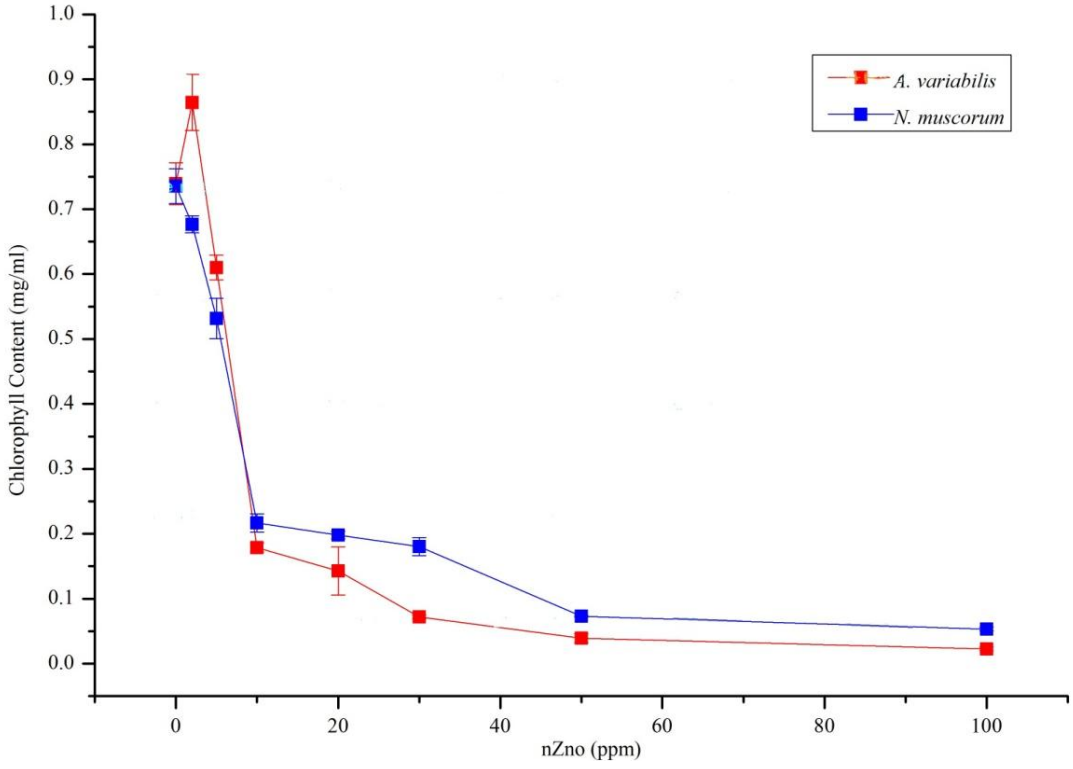


Fig. 21. Chlorophyll (mg/ml) in *A. variabilis* and *N. muscorum*

4.4.4. Indole acetic acid

Indole Acetic Acid (IAA) stimulates the algal growth and multiplication. Heavy metals stimulates the commencement of peroxidase enzyme that degrade the IAA and chlorophyllase activity, disorderness of membrane system and inactivation of electron transport functions in the photosystem which are responsible for inhibition (Gupta et al., 2015). Effect of ZnO NPs at different concentration (0-100 ppm) was observed on IAA production by *A. variabilis* (Table 10). There was decrease in IAA production by cyanobacteria with increase in the concentration of nZnO as compared to control. There was sharp decline upto 10 ppm thereafter it was gradual upto 50 ppm. Inhibition in *A. variabilis* (Fig. 22) relates to the heavy metal stress of zinc oxide (Awasthi, 2015). In *Nostoc muscorum* there was no IAA production found as there was 0 mg/ml of IAA at 2 ppm which suggests that it was more sensitive to zinc.

Table 10. IAA (mg/ml) in *Anabaena variabilis*

ZnO NPs (ppm)	IAA (mg/ml) in *A. variabilis*
0	0.037±0.004
2	0.027±0.001
5	0.019±0.003
10	0.015±0.002
20	0.013±0.002
30	0.012±0.001
50	0.010±0.000
100	0.001±0.000

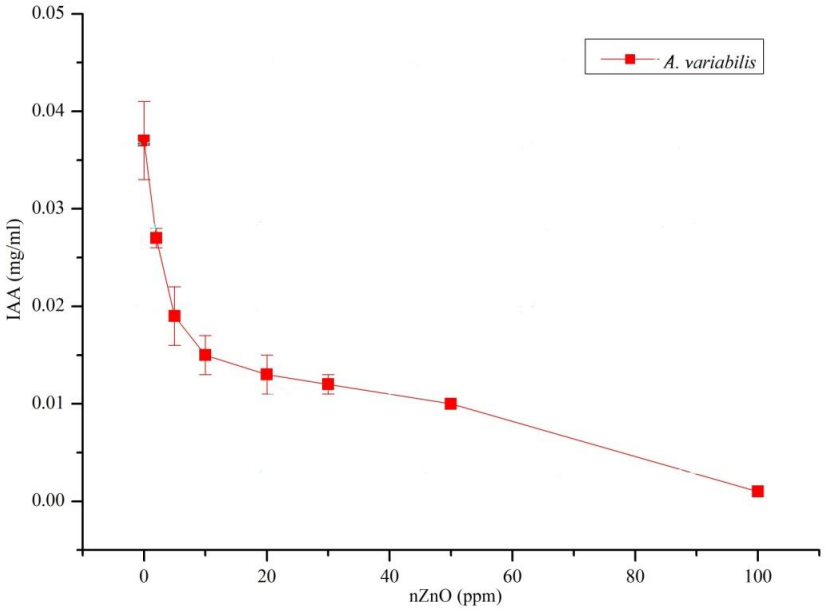


Fig. 22. IAA (mg/ml) in *Anabaena variabilis*

4.4.5. Nitrate reductase activity

The effect of ZnO NPs at different concentration (0-100 ppm) was observed on NR activity of *A. variabilis* and *N. muscorum* (Table 11). Any stimulation in the ATP pool and accessibility to NADPH may rouse ATP dependent processes like, nitrate reductase activity. Thus increase in the photosynthetic activity indicates increase of the enzymatic activities (Awasthi, 2005). *A. variabilis* has higher NR activity as compared to *N. muscorum*. Different organisms have different NR activity. NR activity increases upto 2 ppm of nZnO in *A. variabilis* whereas in *N. muscorum* with increase in nZnO concentration, there was decline in NR activity (Fig. 23).

Table 11: NR (µ mole NO_2/ml) of *Anabaena variabilis* and *Nostoc muscorum*

ZnO NPs (ppm)	NR (µ mole NO_2/ml) in *A. variabilis*	NR (µ mole NO_2/ml) in *N. muscorum*
0	0.401±0.012	0.017±0.0015
2	1.067±0.419	0.006±0.0011
5	0.207±0.034	0.006±0.0021
10	0.077±0.002	0.006±0.0023
20	0.039±0.006	0.001±0.0001
30	0.016±0.001	0.001±0.0007
50	0.014±0.003	0.000±0.0000
100	0.012±0.004	0.000±0.0000

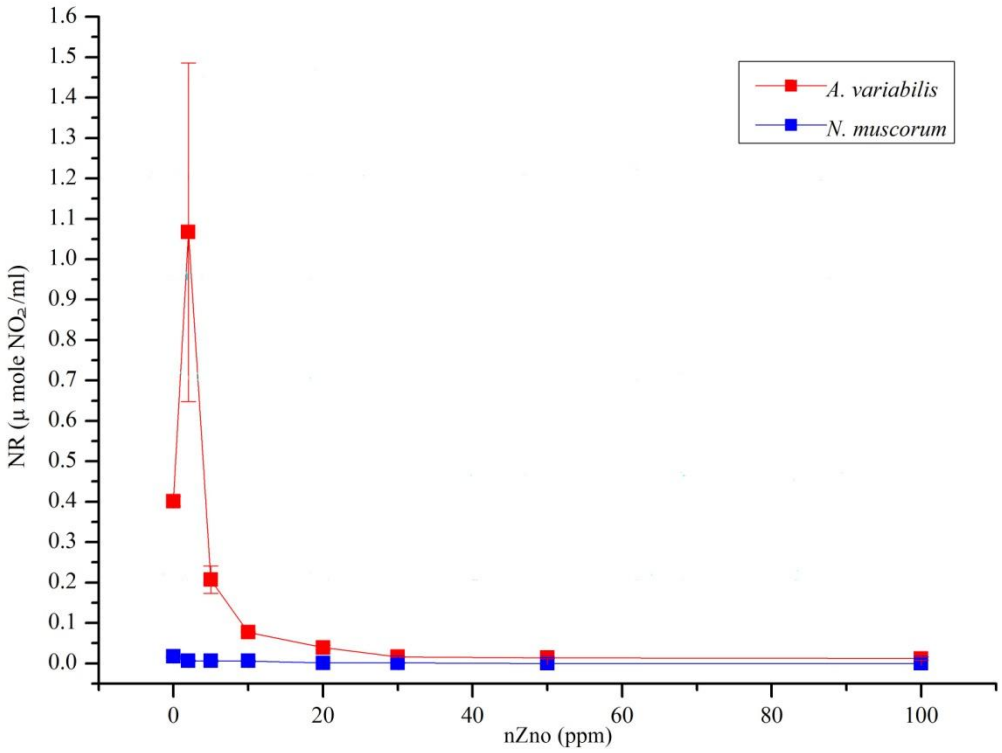


Fig. 23. NR (µ mole NO_2/ml) in *A. variabilis* and *N. muscorum*

4.5. Effect of different Zinc sources on the growth of *A. variabilis* and *Calothrix* sp.

A. variabilis and *Calothrix* sp were grown in BG11 medium replacing zinc with different zinc sources such as $ZnCl_2$, $ZnSO_4$, $ZnNO_3$, ZnO, $Zn(CH_3COO^-)_2$ and nZnO for 21 days. Aquatic organisms exhibit variable sensitivities to zinc, besides zinc being an essential nutrient. This is due to either free hydrated zinc ion (Zn^{2+}) or its labile inorganic complexes or in the form of strong complexes adsorbed to colloidal and particulate matter (Franklin et al., 2007). However a comparable toxicity between different zinc complexes can only occur from the dissolution of these oxides in the bulk media with the release of Zn^{2+} and subsequent diffusion of ions through the algal cell surfaces where it can exert its toxic action depressing cell-division rates, uncoupling of cell division and photosynthesis (Franklin et al., 2007). Arnoja et al., (2009) reported that zinc oxides were equally toxic in bulk and nano formulations in case of *Pseudokirchneriella subcapitata.* However, it was not reported in case of *A. variabilis* where nano and bulk zinc when used as a replacement of the zinc source. Zhou et al., (2014) reported that inhibition of *Chlorella vulgaris* by $ZnSO_4 \cdot 7H_2O$, used as a source of free zinc ions, was greater than that by the same concentration of ZnO NPs which suggests that Zn^{2+} is more toxic than ZnO NPs. We reported similar results in case of *A. variabilis.*

4.5.1. Wet biomass

Zinc is an essential nutrient for algal growth and metabolism so when *A. variabilis* and *Calothrix* sp were exposed to different zinc sources at concentration of 0.222 g/L (Table 12), then it was found that in case of *A. variabilis* maximum growth occurred in the medium supplemented with zinc oxide nanoparticles followed by bulk ZnO as shown in the Fig. 24, whereas $ZnSO_4$ remains the best minor nutrient in BG11 medium in case of *Calothrix* sp. (Fig. 25).

Table 12. Wet biomass (g/10 ml) in *Calothrix* sp. and *A. variabilis*

Type of zinc source (0.222 g/lt)	**Wet biomass (g/10 ml) in *Calothrix* sp.**	**Wet biomass (g/10 ml) in *A. variabilis***
Control	0.6553±0.075	0.3700±0.020
$ZnSO_4$	0.8493±0.014	1.7100±0.030
$ZnNO_3$	0.5900±0.030	-
$ZnCl_2$	0.5581±0.020	1.5300±0.030
$Zn(CH_3COO^-)_2$	0.7172±0.090	-
ZnO	0.3633±0.007	1.8900±0.020
nZnO	-	1.9600±0.030

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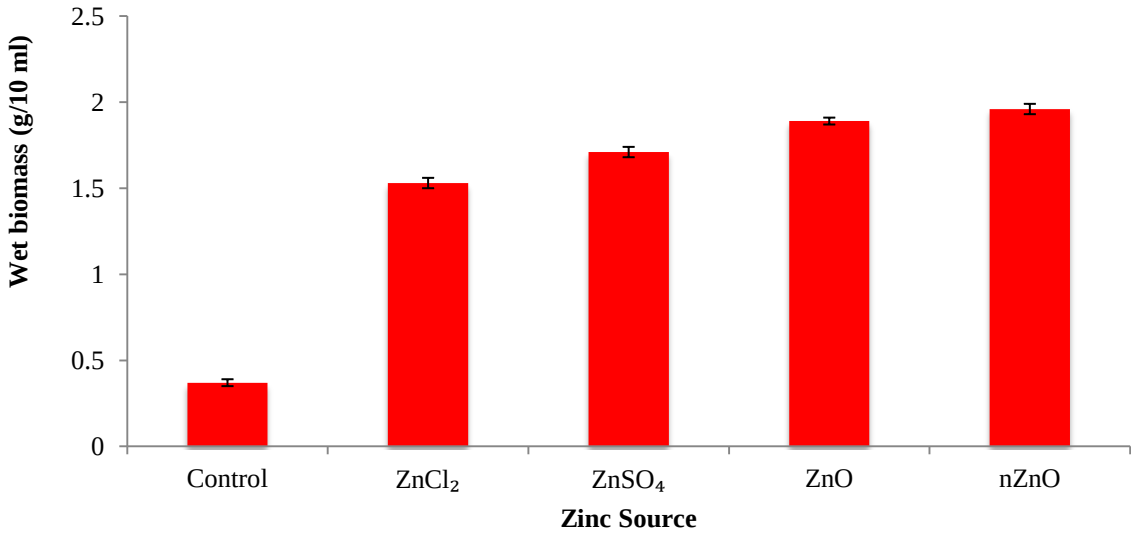

Fig. 24: Wet biomass (g/10ml) in *A. variabilis*

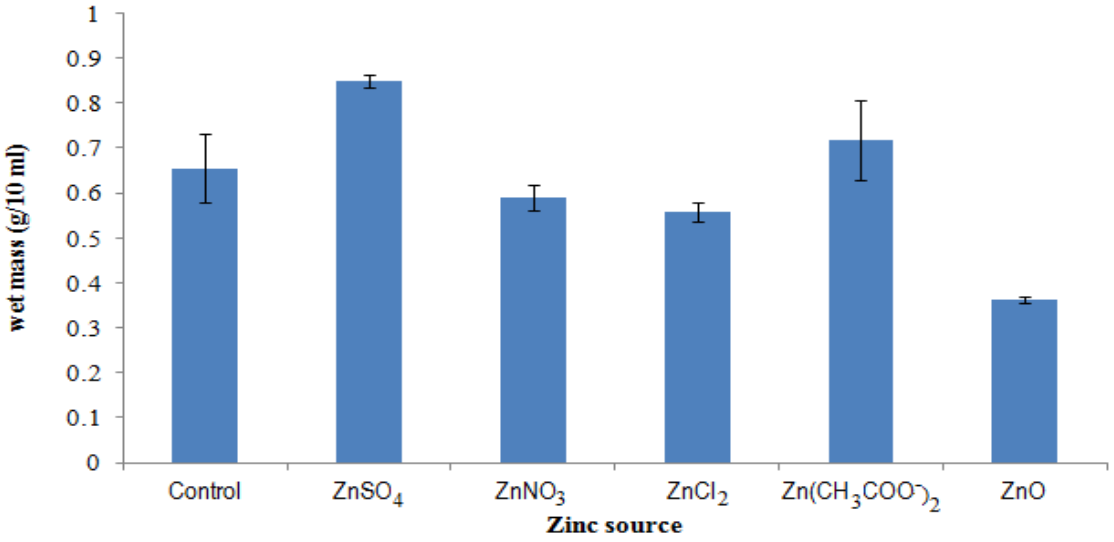

Fig. 25. Wet biomass (g/10 ml) in *Calothrix* sp

4.5.2. Chlorophyll

Chlorophyll relates to the photosynthetic activity of the algae. When *A. variabilis* and *Calothrix* sp were grown in BG11 medium supplemented with different zinc sources at concentration of 0.222 g/L (Table 13), then in case of *A. variabilis* maximum chlorophyll was found in presence of zinc oxide nanoparticles followed by bulk ZnO and $ZnSO_4$ (Fig. 26), whereas bulk ZnO was the best zinc source in BG11 medium in case of *Calothrix* sp. (Fig. 27).

Table 13. Chlorophyll (mg/ml) in *Calothrix* sp. and *Anabaena variabilis*

Type of zinc source	Chlorophyll (mg/ml) in *Calothrix* sp.	Chlorophyll (mg/ml) in *A. variabilis*
Control	$1.058\times10^{-4}\pm0.080$	$0.528\times10^{-4}\pm0.080$
$ZnSO_4$	$1.243\times10^{-4}\pm0.038$	$0.880\times10^{-4}\pm0.030$
$ZnNO_3$	$1.469\times10^{-4}\pm0.075$	-
$ZnCl_2$	$1.311\times10^{-4}\pm0.054$	$0.541\times10^{-4}\pm0.010$
$Zn(CH_3COO^-)_2$	$1.272\times10^{-4}\pm0.064$	-
ZnO	$1.522\times10^{-4}\pm0.058$	$0.931\times10^{-4}\pm0.020$
nZnO	-	$0.964\times10^{-4}\pm0.040$

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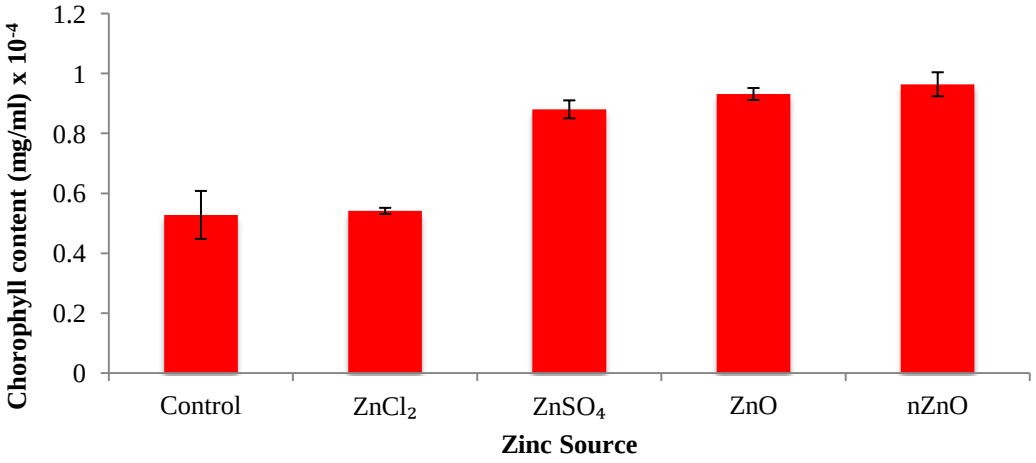

Fig. 26. Chlorophyll (mg/ml) in *A. variabilis*

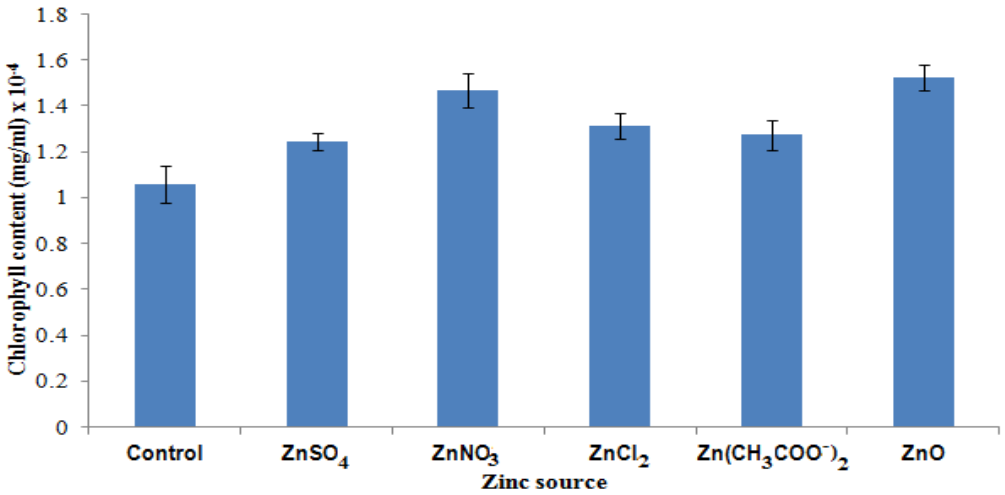

Fig. 27. Chlorophyll (mg/ml) in *Calothrix* sp.

4.5.3. IAA activity

Cyanobacteria secrete various growth promoting substances for their growth like indole acetic acid. *A. variabilis* and *Calothrix* sp were grown in presence of different zinc sources at a concentration of 0.222 g/L (Table 14). Maximum IAA production was found in *A. variabilis* in presence of nZnO (Fig. 28), whereas $ZnSO_4$ was the best minor micronutrient in BG11 medium for *Calothrix* sp. (Fig. 29).

Table 14. IAA (mg/ml) in *Calothrix* sp. and *Anabaena variabilis*

Type of zinc source	**IAA (mg/ml) in *Calothrix* sp.**	**IAA (mg/ml) in *A. variabilis***
Control	0.024±0.006	0.007±0.001
$ZnSO_4$	0.077±0.028	0.012±0.002
$ZnNO_3$	0.017±0.009	-
$ZnCl_2$	0.023±0.007	0.011±0.002
$Zn(CH_3COO^-)_2$	0.017±0.005	-
ZnO	0.014±0.000	0.023±0.001
nZnO	-	0.034±0.003

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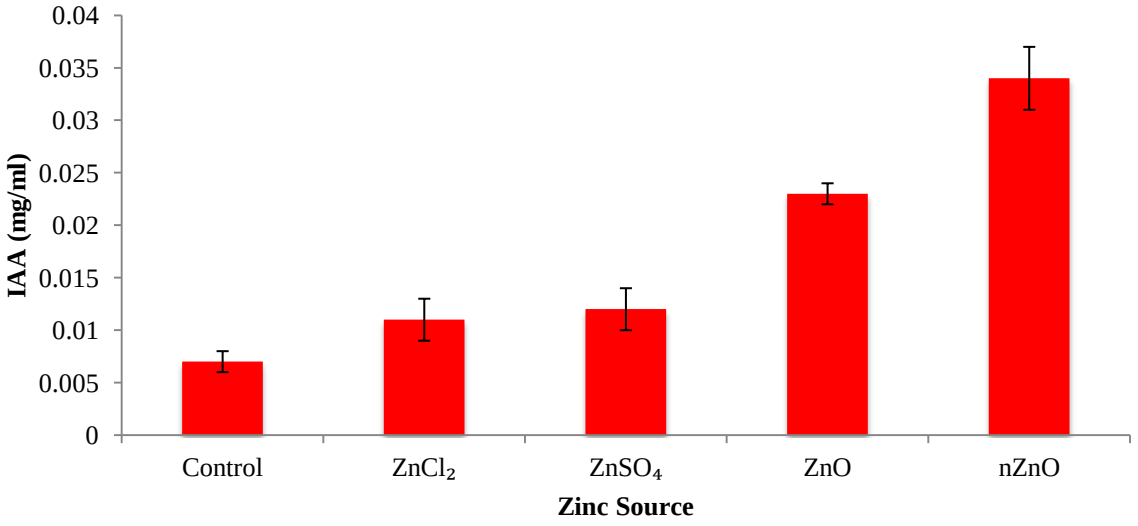

Fig. 28. IAA (mg/ml) in *Anabaena variabilis*

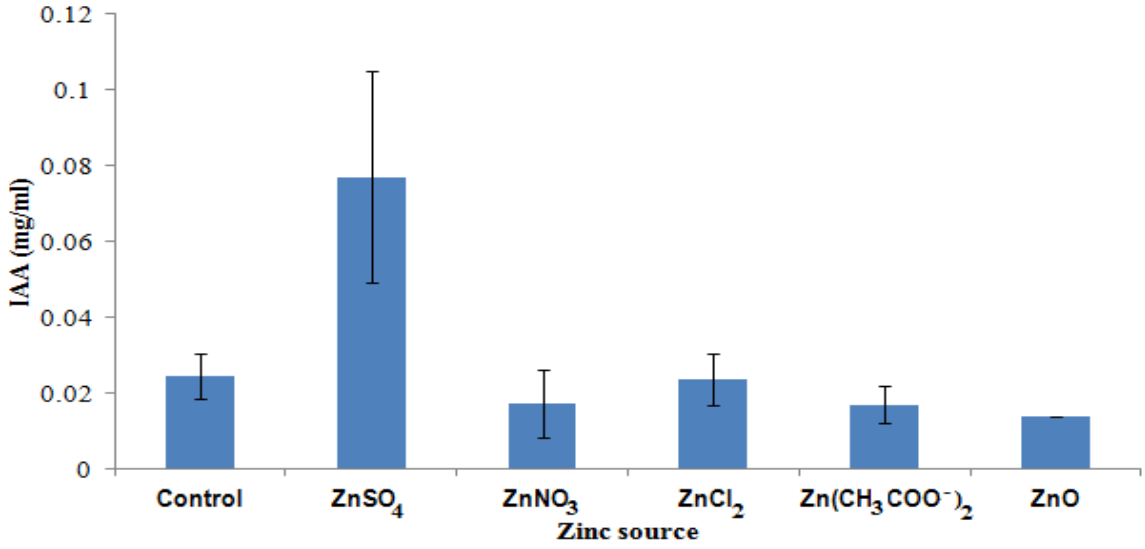

Fig. 29. IAA (mg/ml) in *Calothrix* sp.

4.5.4. Nitrate reductase activity

A. variabilis and *Calothrix* sp were grown in presence of different zinc sources at a concentration of 0.222 g/L (Table 12). In *A. variabilis* maximum NR activity was found in the medium supplemented with bulk zinc oxide followed by nano-ZnO (Fig. 30), whereas different zinc sources has no negative effect on the NR activity in *Calothrix* sp. (Fig. 31).

Table 15. NR (µ mole NO_2/ml) in *Calothrix* sp. and *Anabaena variabilis*

Type of zinc source	**NR (µ mole NO_2/ml) in *Calothrix* sp.**	**NR (µ mole NO_2/ml) in *A. variabilis***
Control	0.311±0.022	0.044±0.007
$ZnSO_4$	0.429±0.014	0.078±0.003
$ZnNO_3$	0.321±0.013	-
$ZnCl_2$	0.408±0.034	0.037±0.001
$Zn(CH_3COO^-)_2$	0.294±0.031	-
ZnO	0.381±0.045	0.117±0.008
nZnO	-	0.081±0.002

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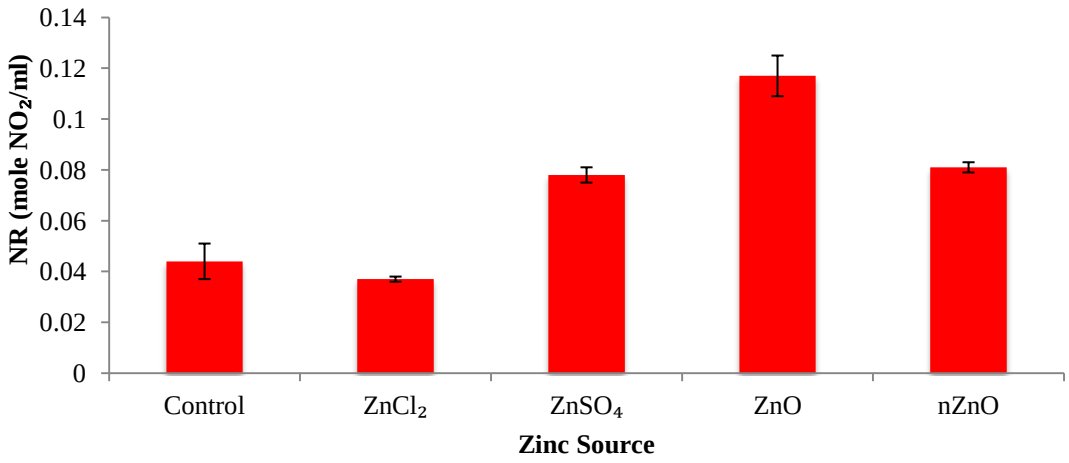

Fig. 30. NR (µ mole NO_2/ml) in *A. variabilis*

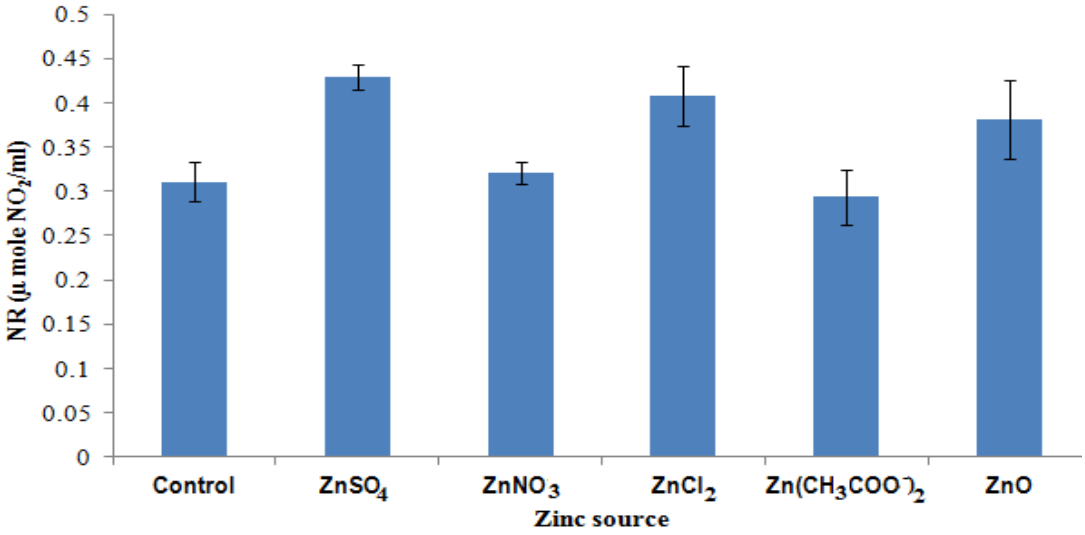

Fig. 31. NR (µ mole NO_2/ml) in *Calothrix* sp.

4.5.5. Total nitrogen estimation

The algal biomass was harvested and total nitrogen was estimated with kjeldahl method (Piper, 1960) at the end of 21 days of incubation. *A. variabilis* was grown in the presence of different zinc sources and was checked for total nitrogen content. Total nitrogen content determines the nitrogen fixing ability. Maximum nitrogen fixing ability was reported in the medium supplemented with zinc oxide nanoparticles followed by bulk ZnO (Fig. 32). This may be due to the reason that surface area to volume ratio of zinc oxide nanoparticles makes it easy for algae to uptake the zinc ions in the medium and further stimulate the growth of cyanobacteria. However it is reported by Shukla et al. (2009) that lower concentration of

heavy metal can stimulate growth of cyanobacteria by acting as growth promoter and may increase nitrogen fixation with increase in heterocyst frequency.

Table 16. Total Nitrogen (in %) in *Anabaena variabilis*

Type of zinc source	**Total Nitrogen (in %) in *A. variabilis***
Control	0.002±0.0005
$ZnCl_2$	0.003±0.0002
$ZnSO_4$	0.004±0.0002
ZnO	0.004±0.0007
nZnO	0.004±0.0003

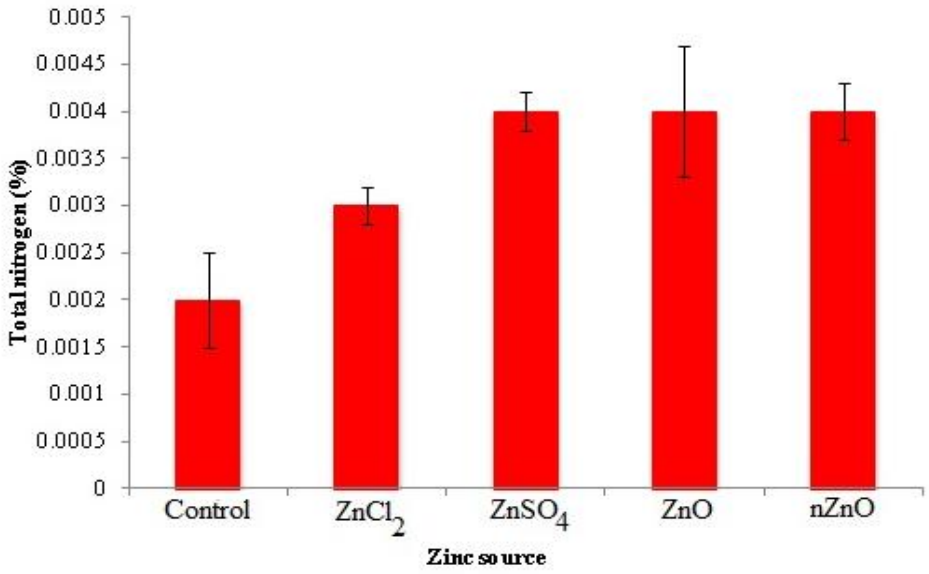

Fig. 32. Total nitrogen (%) in *A. variabilis*

Conclusions

1. Zinc oxide NPs synthesized from *Aspergillius niger* were characterized by FTIR, XRD and SEM. According to the FTIR analysis functional groups present in the ZnO nanoparticles was confirmed through the peaks in the range of 3075 to 859 cm^{-1} and 1615.05 to 1099.99 cm^{-1}. The size of nanoparticles is 64-126 nm as observed from the SEM images. Sharper and stronger diffraction peaks observed in XRD analysis confirmed synthesized zinc oxide nanoparticles.
2. Zinc oxide NPs synthesized from *Anabaena variabilis* were characterized by FTIR, XRD and SEM. According to the FTIR analysis the polysaccharides present in the aqueous extract of *A. variabilis*, are involved in the formation of zinc oxide NPs. The size of nanoparticles is 72-74 nm as observed from the SEM images. Sharper and stronger diffraction peaks observed in XRD analysis confirmed synthesized zinc oxide nanoparticles.
3. Zinc oxide NPs synthesized from *Trichoderma reesei* were characterized by FTIR, XRD and SEM. According to the FTIR analysis functional groups present in the ZnO nanoparticles was confirmed through the peaks in the range of 3075 to 859 cm^{-1} and 1615.05 to 1099.99 cm^{-1}. Their average size could not be estimated as nanoparticles remain so compactly arranged. Sharper and stronger diffraction peaks observed in XRD analysis confirmed synthesized zinc oxide nanoparticles.
4. *A. variabilis* and *N. muscorum* were supplemented with 0 to 100 ppm concentrations of zinc oxide nanoparticles. It was found that 2 ppm of ZnO NPs are stimulating and above 2 ppm were found inhibitory for the growth of *A. variabilis* whereas in *N. muscorum* even 2 ppm of ZnO NPs were inhibitory for its growth. So it can be concluded that *N. muscorum* is far more sensitive than *A. variabilis* for ZnO NPs.
5. When zinc oxide nanoparticles are provided to the *A. variabilis* as a replacement of the zinc source for its growth then the results were found stimulatory when compared to the positive and negative controls. Moreover $ZnSO_4$ remains the best source for the growth of *Calothrix* when grown in the BG11 medium with different zinc sources.

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Appendix I

Table 17. BG11 Medium composition

Constituents	Quantity (gm/l)
$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4$	0.075
$CaCl_2.H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (Disodium magnesium salt)	0.001
Sodium carbonate	0.02
Trace metal	1 ml

Table 18. Trace metal mix composition

Constituents	Quantity (gm/l)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$NaMoO_4.2H_2O$	0.390
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.0494

Table 18. Composition for potato dextrose broth

Constituents	Quantity (gm/l)
Potato	4
Dextrose	20

Appendix II

A. Standard curve for IAA

IAA activity was measured by using indole acetic acid as standard (Glickmann and Dessaux, 1995)

Materials required-

Stock solution: 1 mg/ml IAA
Acetone

Table 19. Composition of stock solution for IAA

IAA	20 mg
Acetone	20 ml

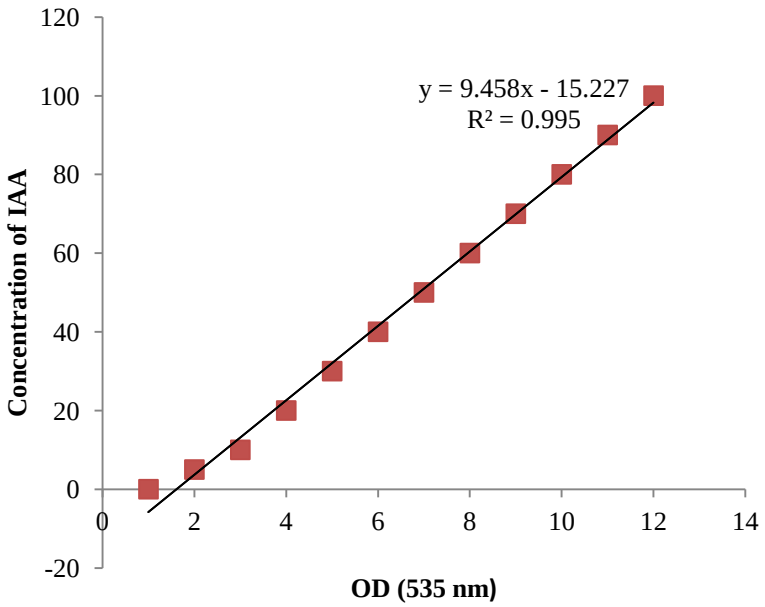

Fig. 33. Standard curve for IAA

B. Standard curve for Nitrate reductase

Standard curve of NR was made by using $NaNO_3$ as standard (Lowe and Evans, 1964**).**

Materials required-

Stock – 1 mg/ml of 10 mM $NaNO_3$

Table 20. Composition of stock solution for NR

Distilled water	10 ml
Sodium nitrate	0.0089 gm

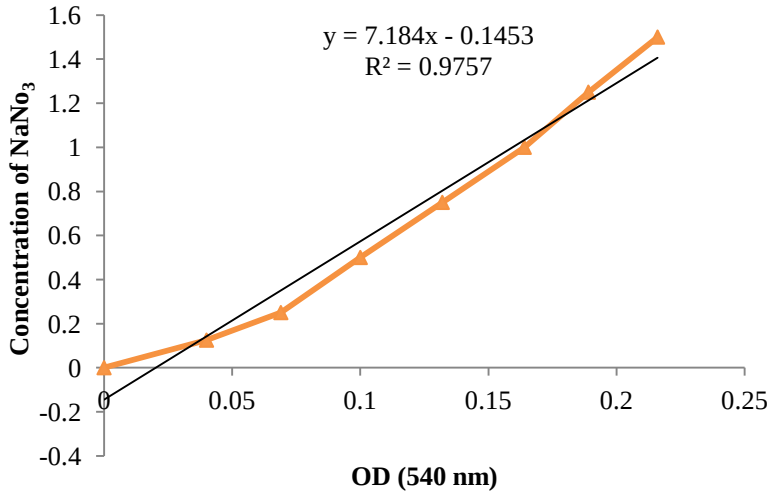

Fig. 34. Standard curve for Sodium nitrate