Studies on Superoxide Dismutase: An antioxidative protein from *Cicer arietinum* seedlings

A Thesis Submitted

By

Sushant Singh

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Centre for The Environment
Indian Institute of Technology Guwahati
Guwahati - 781039, Assam, India
January 2014

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Dedicated

To

My teachers, past and present whose devotion, inspiration and guidance made me capable

My parents, whose sacrifices, motivation and trust have served as a constant source of inspiration

My family whose love, support and encouragement have made everything possible

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

CENTRE FOR THE ENVIRONMENT

CERTIFICATE

I hereby declare that the matter embodied in this thesis entitled "**Studies on Superoxide Dismutase: An antioxidative protein from *Cicer arietinum* seedlings**" is the result of investigations carried out by me at Centre for The Environment, Indian Institute of Technology Guwahati, Assam, India under the supervision of **Dr. Vikash Kumar Dubey** (Supervisor) and **Dr. Anil Verma** (Co-supervisor). In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

January 2014

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Studies on Superoxide Dismutase: An antioxidative protein from *Cicer arietinum* seedlings**" by **Mr. Sushant Singh (**Roll No: 09615202), submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under our supervision at Centre for The Environment, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for any kind of degree.

Dr. Vikash Kumar Dubey
(Supervisor)

Dr. Anil Verma
(Co-supervisor)

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Abbreviations

SOD	:	Superoxide Dismutase
ROS	:	Reactive Oxygen Species
NBT	:	Nitroblue tetrazolium chloride
TCA	:	Trichloro Acetic Acid
TBA	:	Thiobarbituric acid
MDA	:	Malondialdehyde
EDTA	:	Ethylene Diamine tetra Acetic Acid
BSA	:	Bovine Serum Albumin
MALDI	:	Matrix Assisted Laser Desorption Ionization
PCL	:	Polycaprolactone
PVA	:	Polyvinyl alcohol
DCM	:	Dichloro methane
CAT	:	Catalase
DMEM:F12	:	Dulbecco's Modified Eagle Medium: F12 Ham nutrient mixture
FBS	:	Fetal Bovine Serum
PBS	:	Phosphate Buffer Saline
HaCat	:	Human adult low calcium temperature keratinocytes
FESEM	:	Field Emission Scanning Electron Microscopy
FTIR	:	Fourier Transform Infrared Spectroscopy
DLS	:	Dynamic Light Scattering
PDI	:	Polydispersity Index
MTT	:	3-(4,5-dimethyl thiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide
carboxy-H_2DCFDA	:	Carboxy-2′,7′-Dichlorodihydro-fluorescein diacetate
DMSO	:	Dimethyl Sulfoxide
CNT	:	Carbon Nanotube
NHS	:	N-Hydroxysuccinimide
EDAC	:	N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride
MES	:	2-(N-Morpholino) ethanesulfonic acid

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Chapter I

A review of literature on oxidative stress, antioxidative defense and superoxide dismutase.

Abstract

Superoxide dismutase is an important enzyme of the antioxidative defense system. It forms the first line of defense against reactive oxygen species and environment induced oxidative stress. Superoxide dismutase works in coordination with other antioxidative enzymes such as catalase, ascorbate peroxidase and glutathione peroxidase. These enzymes help in maintaining the ionic homeostasis, deplete the level of reactive oxygen species and subsequently limit the oxidative stress condition. Superoxide dismutase enzymes are classified based on their metal cofactor associated with the activity. Because of its high reactive oxygen species scavenging properties, it has numerous therapeutic applications. Recent advancement in the field of proteome research coupled with detailed reaction mechanism, in depth analysis of the crystal structure and *in silico* analysis suggests that superoxide dismutase is indispensible tool for numerous biological applications. However, search of a novel superoxide dismutase with unique properties is always of significant importance.

1.1 INTRODUCTION

World population has been continuously rising since many decades. The most pronounced and significant increase has been observed in the last 50 years. It has been projected that world population which was 7 billion (2011) is going to be more than 9 billion by the year 2050 (*World Population Prospectus: The 2010 Revision, United Nations, 2011*). This accounts for daunting challenges of feeding the growing population which demands the increase in agricultural productivity (Figure 1.1). To overcome these challenges, an additional increase in the agricultural food productivity by nearly 70% globally, while almost 100% increase in developing countries is indispensible (*FAO, 2011*). To achieve this goal of increase in agricultural productivity in order to feed the world, the current prevailing pattern of agricultural practices and its surrounding environment need to be critically reviewed. Over the last 50 years, agricultural lands has grown by 12% and the global irrigated land has doubled, altogether leading to an increase in agriculture production of major crops by 2.5 to 3 times (*FAO, 2011*). However the continuous changing environmental conditions play a major role in the agricultural production. In the present scenario, rapid increase in industrialization as well as numerous anthropogenic activities contributes mostly to the agricultural land pollution (Figure 1.2). Land and water resources are central to agriculture and a major key factor contributing towards increase in agricultural production. These two factors are intrinsically linked and contribute majorly to the world agricultural production and thus needs a special attention with the changing scenario of the global environment. Environmental pollution is a worldwide problem and one of the major issues to be taken seriously as it is turning out to be just more than a health concerned topic. Rapid growth of industrialization, multifarious anthropogenic activities, urban development, inappropriate channeling of industrial effluents, mass consumption of toxic chemicals and fertilizers etc, are the major contributors to this increased environmental pollution surrounding the agricultural habitat *(Schwartz et al., 2001; Bell et al., 2001; Passariello et al., 2002)*. Environmental pollution surrounding agricultural land has turned out to be one of the major topics of debate due to its potential to affect agricultural productivity as well as human health majorly. The term “Pollution” is defined as undesirable change in the physical, chemical and biological characteristic of air, water and soil while “Pollutant” refers to anything which adversely interferes with the environment causing discomfort.

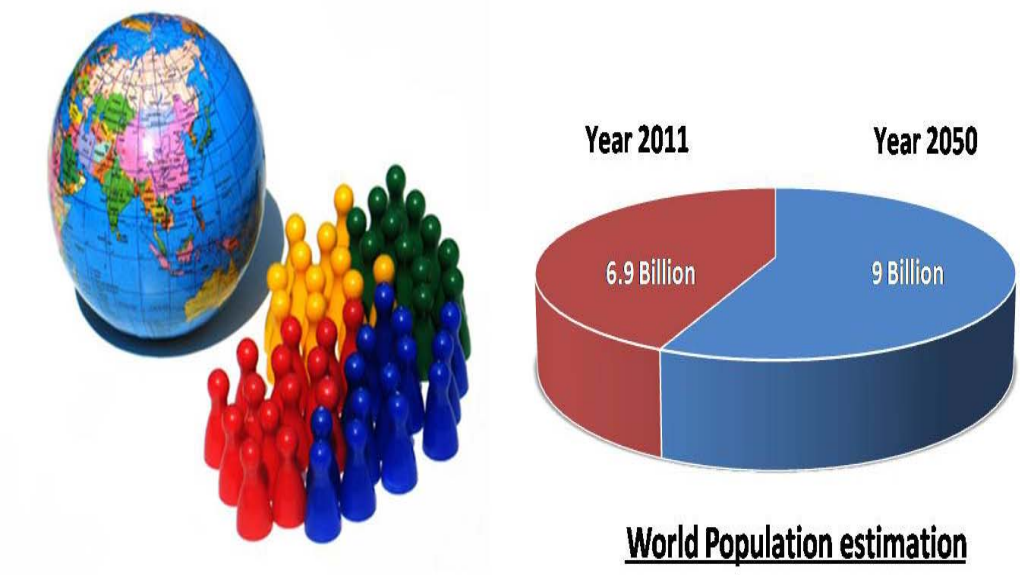

Figure 1.1 Population estimation and projections as reported by World Population Prospectus, (2010 Revision). It has been projected that the world population will increase to more than 9 billion in year 2050 from the current 6.9 billion (estimated in 2011). The tremendous increase in population will have a simultaneous effect on the demand for agricultural productivity (Image is taken from web resource).

Different pollutants get introduced into the environment through wastes, sewage sludge, accidental discharge, inappropriate handling of industrial waste, mismanagement of byproducts of industrial residues etc. Increase in environmental pollution surrounding the agricultural habitat leads to increase in environmental stress on germinating crop plants. There are varieties of environmental stress factors which adversely affect the crop productivity. These can be edaphic or soil factors which include heavy metal toxicity to agricultural land, high salinity, high concentration of herbicide and pesticides, mineral nutrient deficiency or climatic factors involving drought conditions, extreme temperature, UV radiations etc (*Barman et al., 2000; Kisku et al., 2000; Nagajyoti et al., 2008*). These entire environmental stress factors severely affect the crop germination capacity, affecting its growth and development and decreasing the overall agricultural productivity (*Mahajan & Tuteja, 2005*).

1.2 LITERATURE REVIEW

1.2.1 Abiotic environmental stress

A wide range of abiotic stress factors has arisen with the changing environmental conditions surrounding the agricultural lands. These factors have direct as well as indirect negative effects on the germinating crop plants. These environmental stresses are one of the reasons behind significant crop losses and thus are need to be checked. The different environmental stress factors affecting plants as well as agricultural crop productivity include extreme temperature, high soil salinity, soil mineral nutrient deficiency, frequent drought condition, heavy metal toxicity, numerous other environmental oriented pollutants, UV radiations etc (*Noctor & Foyer 1998; Dat et al., 2000*). Plants as well as the germinating crops are unable to avoid these environmental stress conditions and thus have developed certain mechanism to overcome the surrounding external stresses. These abiotic environmental stress factors induce oxidative damage into the metabolic system of the encountering plant (*Braam et al., 1997*).

Oxidative stress condition is a metabolic state of the system where reactive oxygen species (ROS) or free radicals overcome the balanced level and thus hamper the normal metabolic processes. Under normal condition or more appropriately non stressful condition, the level of reactive oxygen species and free radicals are balanced by the antioxidative defense system, thus protecting against oxidative stress conditions. However, under external stress condition, increased production of reactive oxygen species and free radicals takes place which increases the oxidative load onto the system and thus induces oxidative damage (*Mittler, 2002*). Agricultural soil pollution is one of the primary sources of abiotic environmental stress factor affecting the crop plants. Increase in industrialization, numerous anthropogenic activities, inappropriate disposal of sewage sludge as well as industrial effluents has lead to increased pollution surrounding the agricultural land which has direct effect on the agricultural productivity (*Alloway, 1990*).

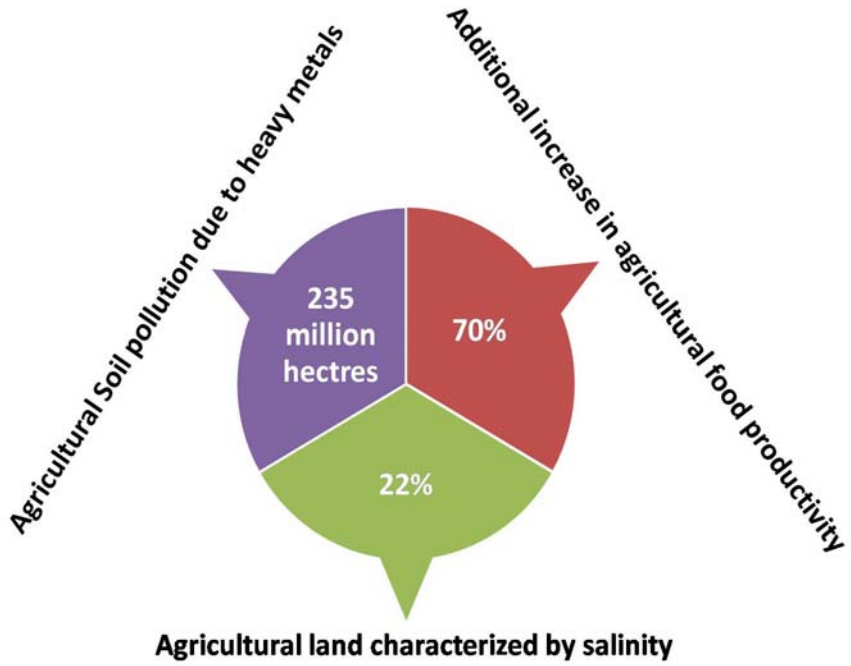

Figure 1.2 Environmental pollution has negative consequence in terms of loss in agricultural productivity. It has been reported that 235 million hectares of agricultural land is being polluted due to variety of heavy metal stress *(Giordani et al., 2005)* while another report suggests increase in soil salinity upto 22% of total agricultural land *(Kolodyazhnaya et al., 2009)*. The increase in world population demands an additional increase in agricultural productivity of upto 70%.

1.2.2 Agricultural soil pollution

Environmental pollution neighbouring agricultural lands has turned out to be a serious issue of concern. The surroundings of agricultural environment including air, water and soil are getting polluted in numerous ways. Agricultural land pollution inflicts the most damage as it is the base of agricultural productivity. Activities surrounding the agricultural lands need to be reviewed and cross checked in order to control the increasing land pollution as well as to maintain the soil fertility and agricultural productivity. Human activities have resulted in the increased level of contamination near the agricultural land which is having a negative impact on the agricultural production. As indicated in Figure 1.3, numerous sources are responsible for the agricultural land pollution (*Hatamzadeh et al., 2012*).

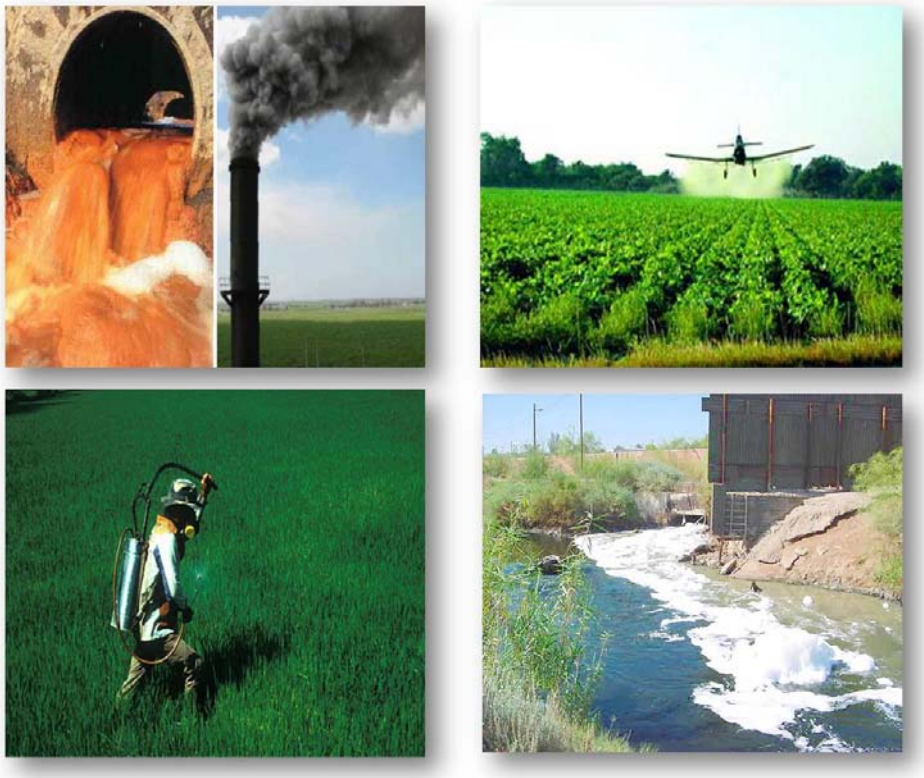

Figure 1.3 Soil pollution towards agricultural land has turned out to be a major issue. The different sources of agricultural soil pollution are generally due to human negligence which includes inappropriate disposal of industrial waste, indiscriminate and excessive use of different chemicals and fertilizers/pesticides as well as mismanagement in the efficient disposal of sewage sludge. All these contaminating factors lead to change in the natural composition of the agricultural soil and thus have a negative impact on the overall agricultural productivity (Images are taken from web resources)

1.2.2.1 Inappropriate disposal of industrial effluents

Industrial waste is one of the major causes of soil pollution surrounding the agricultural land. The industrial waste contains both organic as well as inorganic toxic materials as waste products and their disposal is a matter of serious concern. Industrial waste contains metals which have high potential for toxicity. Chemical industries related to superphosphate, phosphoric acid, aluminium, ceramics and steels release large amount of fluorides into the atmosphere. Thermal plants and other factories release sulfur dioxide which imparts acidity to agricultural soil and thus negatively affects the agricultural

productivity (*Veer & Lata, 1987; Swaminathan et al., 1989*). Cadmium contamination of soil results from the emissions of smelting units (non ferrous), primary iron, steel factories as well as from mining sites (*Nriagu & Pacyna, 1988*). Arsenic toxicity in the soil is common in the regions prone to mining and smelting industries. Effects of arsenic include skin irritation, ulceration and are considered to have carcinogenic effect on human body (*Karim, 2000; Paul et al., 2000*). In case of plants, arsenic leads to reduction in growth of germinating crop plants and thus affects normal metabolic development (*Williams et al., 2007; Rahman et al., 2007*). Agricultural soil pollution of metals like copper, nickel, zinc and lead results from the metal smelting and mining industries. Excessive levels of these metals in the agricultural soil causes phytotoxicity to the germinating crop plants. In addition to these metal toxicity, cyanides and sulphates released from metal processing industries as wastes adversely affects the plant growth and thus induces phytotoxicity (*Nagajyoti et al., 2008*). Organic waste generated as unwanted byproduct from industries contributes to a major part of agricultural land pollution. Phenol and its derivatives constitute the major industrial organic waste generated extensively from petroleum and chemical industries. The inappropriate disposal of mixed wastes generated from chemical and petroleum industries causes higher level of phenol contamination into the land (*Delfino & Dube, 1976*). Phenol does not bind to the soil but easily dissolves into the water available and thus contaminates the agricultural soil. Phenol level is highly toxic to aquatic life even at much lower concentration. In case of plants, phytotoxic effects have been observed under higher phenol concentration (*Hansch et al., 2000*).

1.2.2.2 Inappropriate disposal of sewage sludge

Inappropriate disposal of sewage sludge, surrounding the agricultural land is one of the major causes of agricultural soil pollution. The uncontrolled disposal of sewage and other liquid waste from industries, animal husbandry, urban runoff as well as drainages lead to the increase in the agricultural soil contamination. Soil leaching property, its humus content and porosity are majorly affected by the unwanted soil contamination. Other major changes include increase in acidity, salinity, availability of nutrients like nitrogen, phosphorous and potash etc. Accumulation of different metals like nickel, lead, zinc, cadmium etc occurs due to sewage sludge pollution which leads to phytotoxicity in

plants. Among various metals present in sewage sludge, zinc is a major contaminant followed by copper and nickel. These contaminations in agricultural soil induce phytotoxic effects on the germinating crop plants (*Sterritt & Lester, 1980*). The other parameters which get affected by the increase sewage sludge contamination include pH of soil, organic matter content, and ion exchange capacity.

1.2.2.3 Indiscriminate use of fertilizers/ herbicides/ pesticides/ insecticides

Fertilizers are required to maintain agricultural soil nutrient capacity. Nutrients like carbon, hydrogen and oxygen are being easily available through air and water. However for other nutrient factors such as nitrogen, phosphorous, potassium, magnesium, sulfur and many more, fertilizers are being used to cope up the deficiencies (*Adesmoye & Kloepper, 2009*). Although fertilizers are being used to correct soil nutrient deficiency, their indiscriminate use as well as the raw material involved in its manufacturing contaminates the soil. Excessive use of NPK (nitrogen, phosphate, potassium) fertilizers affect the crop germination capacity and thus decreases the protein and carbohydrate content of the germinating crops (*Tilman et al., 2002*). Excess potassium in soil decreases the vitamin C and carotene content. Arsenic, lead and cadmium rich soils on encountering phosphate get converted into superphosphate fertilizer. As these metals are non degradable, they gets accumulated into the soil due to excessive use of phosphate and thus their concentration beyond a certain range become toxic to germinating crops *(Yadav, 2010)*. Different herbicides, pesticides and insecticides are being used in order to control the agricultural crop from the negative effects of insects, bacteria, fungi, rodents etc. Dichlorodiphenyltrichloroethane (DDT), benzene hexachloride (BHC), organophosphates, aldrin, malathion, dieldrin and chlorinated hydrocarbons etc are some of the major pesticides used commonly in the agricultural practices (*Wasim et al., 2009*). However, their remnants contaminate the soil and thus increase the soil toxicity. It enters the biological system through the root of crops grown in the contaminated soil, adversely affecting the productivity. The effects of pesticides/insecticides/herbicides not only increase the toxicity into the biological system but also negatively affect the agricultural soil capacity. The degradation of pesticides into the biological environment has also been a major issue of health concern (*Brouwer et al., 1999*). It has been observed that some of the pesticides are quite stable and thus their biodegradation takes weeks and even months.

The negative effects associated with the pesticides/ herbicides have limited their use and also forced the scientists to seek alternatives (*Igbedioh, 1991; Forget, 1993*).

1.2.2.4 Heavy metal pollution towards agricultural land

Heavy metals are essential for plants in trace amounts; however its concentration beyond a certain range confers toxicity to the surrounding environment. Agricultural soil pollution due to heavy metal results from numerous sources. These include urban and industrial aerosols, fuel combustions, liquid and solid wastes, industrial, chemical and agricultural wastes (Figure 1.4), and weathering of rocks from the parent material (*Barman et al., 2000; Kisku et al., 2000; Nagajyoti et al., 2008*). Excessive use of fertilizers rich in phosphates, insecticides, pesticides also results in increase in heavy metal concentration in agricultural soils and thus hampers the crops productivity. Heavy metals present in the soil are controlled by both physical and biological processes. It may either pass with the drainage system from the soil or will be taken up by the plant or may also be retained in the soil as sparingly soluble or insoluble form (*Avery, 2001; Schutzendubel & Polle, 2002*).

Agricultural soil is mostly contaminated by heavy metals such as copper, nickel, zinc, cadmium, chromium, arsenic, lead etc. The primary effect of plant exposure to certain heavy metals leads to the development of oxidative stress condition which results from unwanted accumulation of reactive oxygen species (*Dietz et al., 1999*). Transition metals such as Fe^{2+} and Cu^{+} lead to formation of highly reactive hydroxyl radicals (OH^{-}) via Fenton reactions (*Fenton, 1984; Fenton, 1989; Haber & Weiss 1934*). Secondary heavy metals also interact with the antioxidative defense system of the plant, disrupting the electron transport system as well as other essential metabolic pathways. Lipid peroxidation also results due to the heavy metal toxicity causing the degradation of cellular membrane and thus imparting toxicity to the germinating plants (*Scandalios, 1993; Alscher et al., 1997*). In general, heavy metal stress and abiotic environmental stress as a whole induces oxidative stress into the surrounding plants and thus inhibits its normal growth and development. Development of oxidative stress results from the increased production of reactive oxygen species due to the altered metabolism under the stressed condition (*Ahmad, 1995; Dean et al., 1997*).

Figure 1.4 Heavy metal contamination in agricultural soil is one of the major abiotic stress inducing factors to the germinating crop plants. The different sources of heavy metal pollution towards agricultural land includes industrial wastes such as coal, iron and ore industry, smelting unit plants, sewage sludge, chemical and fertilizer industries. Heavy metal pollution also takes place due to inappropriate disposal of sewage sludge. Regular and indiscriminate use of different chemicals/fertilizers/herbicides/pesticides also lead to the increase in heavy metal concentration of agricultural soil, overall leads to soil pollution which ultimately has a negative effect on the growth and normal development of the germinating crop plant (Images are taken from web resources).

1.2.3 Oxidative stress

Plants are exposed to a variety of adverse environmental conditions which lead to the development of oxidative stress. These adverse environmental conditions are also considered as abiotic environmental stress factors which include extreme temperatures, high salinity condition, heavy metal toxicity, UV radiations, frequent droughts, air and

water as well as soil pollution surrounding the agricultural land. All these adverse ambiences lead to the development of oxidative stress condition in plants (*Moller et al., 2007*). Oxidative stress condition is the metabolic state where the formation of reactive oxygen species overcomes its scavenging rate by different antioxidatives present and thus imparts an oxidative load with these highly reactive radicals. The reactive oxygen species (ROS) are also known as active oxygen species (AOS) and free radicals, which includes superoxide ions ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), singlet oxygen species ($^1O_2$) (*Arora et al., 2002*). The formation of these reactive oxygen species is an inevitable part of normal aerobic metabolic reactions. Under normal unstressed conditions, production of reactive oxygen species takes place continuously as byproduct of various metabolic pathways localized in different cellular compartments such as chloroplast, mitochondria and peroxisomes (*Moller, 2001*). However under normal physiological conditions these reactive oxygen species (ROS) produced are scavenged by the antioxidative defense molecules which are confined to the respective cellular locations. Equilibrium exists between the productions and scavenging of the reactive oxygen species, however this equilibrium gets disturbed by the adverse environmental conditions which impart stress onto the plant metabolic system. As a result of this perturbation, increase in the level of reactive oxygen species takes place which increases the oxidative load onto the plant metabolism and thus disturbs the normal homeostasis (*Noctor & Foyer, 1998*).

1.2.3.1 Formation of reactive oxygen species

Reactive oxygen species is continuously produced as a byproduct of different metabolic reactions and these reactions are unavoidable in case of aerobic metabolism (*Foyer & Harbinson, 1994*). Under the normal physiological condition or more appropriately unstressed condition, the level of these reactive oxygen species is counterbalanced through antioxidative defense system involving both enzymatic and non-enzymatic pathways (*Alscher et al., 1997*). External stress condition adversely affecting the normal physiological and metabolic processes induces the byproduct formation and lead to increased level of reactive oxygen species. This increased level of reactive oxygen species lead to the development of oxidative stress condition (*Malan et al., 1990; Prasad et al., 1994*). The different reactive oxygen species involved in the induction of oxidative

stress include superoxide ions (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), singlet oxygen species ($^1O_2$) majorly (*Koltz, 2002*). The production of reactive oxygen species is mainly confined to the metabolic subcellular components which are involved in redox reactions. Cellular components such as chloroplast, mitochondria and peroxisomes are majorly involved in the production of different reactive oxygen species (*Alscher et al., 1997; Bowell et al., 2002*) (Figure 1.5).

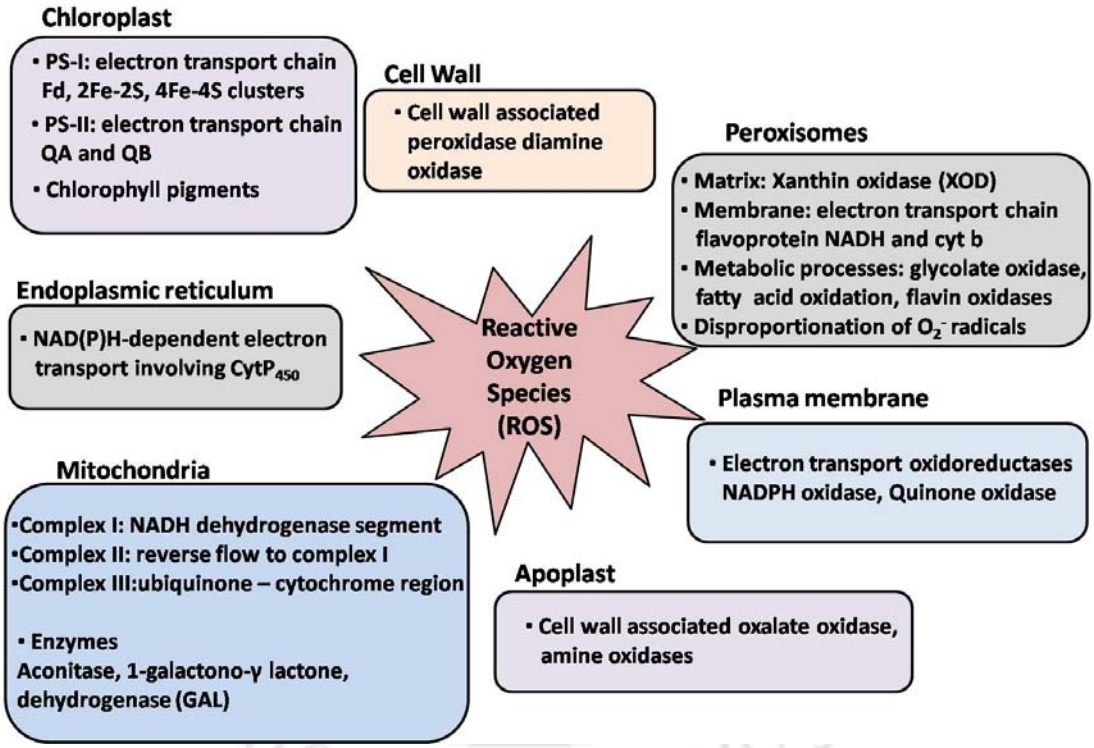

Figure 1.5 Formation of Reactive Oxygen Species (ROS) is an inevitable part of the redox metabolic system. The different sources of ROS formation includes the major cellular components which are involved in the different cellular redox metabolic reactions. The major site of ROS formation includes mitochondria and chloroplast. However other cellular components are also involved such as peroxisomes and endoplasmic reticulum. Formation of ROS also takes place with the help of oxidases associated with cell wall and plasma membrane. (Sharma et al., 2012, Journal of Botany, Article ID 217037)

Photosynthesis in chloroplast is a major source of ROS production. The oxygenase reaction of ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBisCO), directs reduction of molecular oxygen involving photosystem II and the reaction involving NAD(P)H dehydrogenase/ terminal oxidase in chlororespiration are some of the major sites for generation of reactive oxygen species in the chloroplast. In case of peroxisomes, reaction involving glycolate-oxidase and CAT-peroxidase system leads to the formation of ROS molecules (*Nixon, 2000*). Under extreme light intensities, where CO_2 assimilation capacity exceeded, over reduction of electron transport chain leads to photoinhibition of photosystem II and thus inhibition of photosynthesis as whole. In order to protect the photosynthesis apparatus against photoinhibition, non photochemical quenching in form of dissipation of the excess excitation energy takes place in the photosystem II antennae takes place. The photochemical quenching involves the ability of photosystem II to transfer the electrons to various electron acceptors available within chloroplast thus direct reduction of molecular oxygen to form the superoxide anion radical (*Ort & Baker, 2002*). Under different environmental stress such as drought and temperature stress the reduction of oxygen by photosystem I (Mehler reaction) and the photo respiratory pathways involving RiBisCO and glycolate lead to the formation of hydrogen peroxide, which is an another oxidative stress contributing reactive oxygen species secondary to superoxide anion radical. Reactive oxygen species formation during photosynthesis and as byproduct during other redox metabolic reactions are an inevitable process which cannot be neglected or suppressed, thus the plant system needs to evolve an efficient strategy to cope up with increased oxidative stress under extreme conditions (*Mehler, 1951*).

1.2.3.2 Types of reactive oxygen species (ROS)

Aerobic metabolic reactions in the system lead to the formation of numerous reactive oxygen species (ROS) continuously throughout the processes. However, on encountering abiotic environmental stress conditions such as heavy metal contamination, drought, high light intensity etc. increase in the formation of these reactive oxygen species takes place which induces oxidative stress into the system. Formation of these reactive oxygen species (ROS) as a byproduct is an inherent property of aerobic metabolic reactions and thus cannot be neglected. The different types of reactive oxygen

species which get produced as a byproduct of normal metabolic reaction as well as under stress condition are superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH) and singlet oxygen species ($^1O_2$) majorly (Figure 1.6). The formation of these reactive oxygen species takes place within different cellular compartments which are mainly involved in the normal physiological metabolic reactions. The major sub cellular compartments involved are chloroplast, mitochondria and peroxisomes (*Foyer & Harbinson, 1994*).

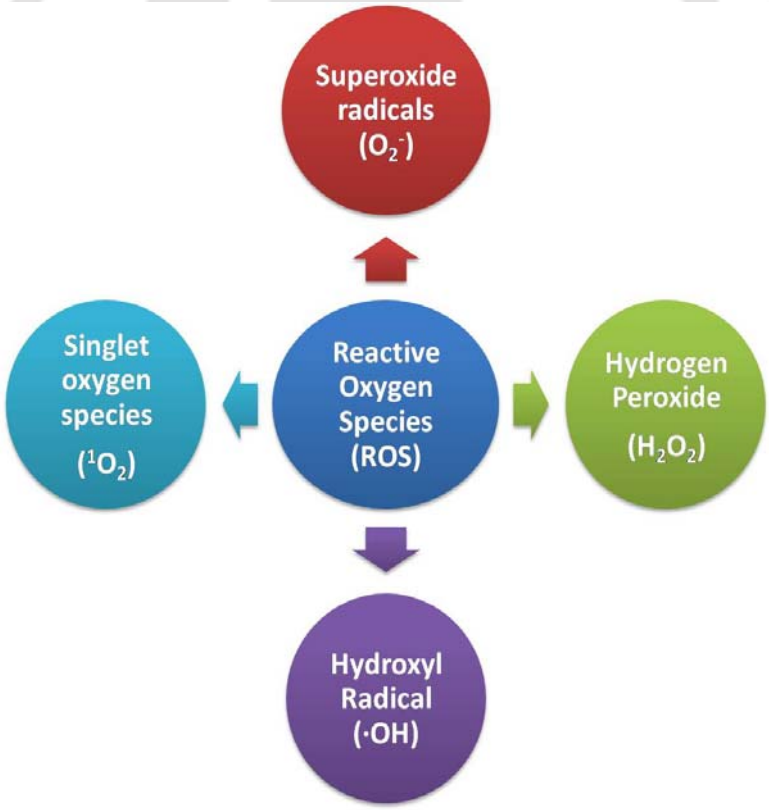

Figure 1.6 Reactive oxygen species (ROS) are the unavoidable byproduct of the redox metabolic reactions which gets produce into the different cellular components. Different forms of ROS exist which are mainly superoxide radicals, hydrogen peroxide radicals, hydroxyl radicals and singlet oxygen species.

1.2.3.2.1 Superoxide anion radicals

Superoxide anion radicals (O_2^-) are one of the key reactive oxygen species formed as byproduct of normal metabolic reactions. In photosynthetic metabolic reactions, molecular oxygen is produced as a result of oxidation of water molecule during photosynthetic electron transport chain reactions (*Asada, 1999*). However, molecular oxygen produced is also being used as an electron acceptor which converts it to superoxide anion radicals. In addition to photosynthetic reaction leading to formation of superoxide anion radical as by product, the photorespiration reactions also produces superoxide anion radicals. Metabolic processes involved in oxygen consumption are the key sources of generation of superoxide anion radical. These metabolic processes include reaction involving cytochrome oxidase of mitochondria, NADH dehydrogenase involved in the respiratory chain reactions as well as oxygen reduction to superoxide anion through ubiquinone-cytochrome respiratory chain reactions (*Bennoun, 1982*). The superoxide anion radicals formed due to sequential reduction of molecular oxygen involved in different metabolic reactions are incompatible and unstable thus need to be removed (*Apel & Hirt, 2004*).

1.2.3.2.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) also contributes to the reactive oxygen species however maximally gets generated into the system due to dismutation of superoxide anion radical by the antioxidative enzyme superoxide dismutase (SOD) (*Asada et al., 1974*). Hydrogen peroxide produced due to dismutation of superoxide anion radical gets scavenged by H_2O_2 utilizing antioxidative enzymes *i.e* catalase and peroxidase present in different subcellular compartments.

1.2.3.2.3 Hydroxyl radical

Major reactive oxygen species such as superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) induces oxidative stress into the metabolic system, however they are much less toxic compared to the effect of hydroxyl radical (·OH). The hydroxyl radical (·OH) formation takes place by Fenton's reaction using transition metals such as

Fe^{2+} and Cu^{+} (*Haber & Weiss 1934;Fenton, 1984; Fenton, 1989*). In the presence of even trace amount of these metal ions such as Fe^{2+}, hydrogen peroxide and superoxide anion radical leads to the formation of highly reactive hydroxyl radical. Hydroxyl radical (·OH) is one of the most highly reactive and unstable molecule in biological system. The increased concentration of hydroxyl radical degrades different cellular components through lipid peroxidation and also denatures protein, DNA and other biomolecules (*Apel & Hirt, 2004*).

1.2.3.2.4 Singlet oxygen species ($^1O_2$)

Singlet oxygen species ($^1O_2$) gets produced into the chlorophyll pigments associated with electron transport system. The photosynthetic reaction involving photosystem II (PSII) is involved in the production of singlet oxygen species. Excess of light stress leads to photo inhibition of PSII which induces the production of singlet oxygen species. Like hydroxyl radical, singlet oxygen species is also one of the destructing reactive oxygen species produced and reacts with most biological molecules (*Knox & Dodge, 1985*). The production of singlet oxygen species also takes place by the lipoxygenase activity.

1.2.3.3 Scavenging reactive oxygen species

Oxidative stress develops due to increased reactive oxygen species within the metabolic system. This increased level of reactive oxygen species includes superoxide anions, hydrogen peroxide, hydroxyl radicals and singlet oxygen species (*Apel & Hirt, 2004*) (Table 1.1). The level of these highly reactive species is to be cross checked in order to maintain the normal homeostatic balance. The plant metabolic system is well equipped with reactive oxygen scavenging system consisting of both enzymatic and non-enzymatic mechanisms (Figure 1.7). The non-enzymatic mechanism of scavenging reactive oxygen species include numerous antioxidant molecules such as glutathione, ascorbate, α-tocopherol, flavonoids, alkaloids, carotenoids etc which acts as reducing equivalents and thus lowers the level of different oxidative radicals in the metabolic system (*Blokhina et al., 2003*). Glutathione (GSH) is oxidized to form reduced glutathione (GSSH) during ROS scavenging; ascorbate is oxidized to mono dehydroascorbate (MDHA) and dehyderoascorbate (DHA).

Table 1.1 The table represents the different production sources of reactive oxygen species and its specific locations as well as the types of ROS molecules produced. It also represents the different ROS scavenging antioxidative enzymes, their locations and the ROS molecule scavenged (Table adopted with permission from *Mittler, 2002, Trends in Plant Science*. 7, 405-410).

Mechanism	Localization	Primary ROI
Production		
Photosynthesis ET and PSI or II	Chl	O_2^-
Respiration ET	Mit	O_2^-
Glycolate oxidase	Per	H_2O_2
Excited chlorophyll	Chl	O_2^1
NADPH Oxidase	PM	O_2^-
Fatty acid β-oxidation	Per	H_2O_2
Oxalate oxidase	Apo	H_2O_2
Xanthine oxidase	Per	O_2^-
Peroxidases, Mn^{2+} and NADH	CW	H_2O_2 , O_2^-
Amine oxidase	Apo	H_2O_2
Scavenging		
Superoxide dismutase	Chl, Cyt, Mit, Per, Apo	O_2^-
Ascorbate peroxidase	Chl, Cyt, Mit, Per, Apo	H_2O_2
Catalase	Per	H_2O_2
Glutathione peroxidise	Cyt	H_2O_2, ROOH
Peroxidases	CW, Cyt,Vac	H_2O_2
Thioredoxin peroxidases	Chl, Cyt, Mit	H_2O_2
Ascorbic acid	Chl, Cyt, Mit, Per, Apo	H_2O_2, O_2^-
Glutathione	Chl, Cyt, Mit, Per, Apo	H_2O_2
α-Tocopherol	Membranes	ROOH, O_2^1
Carotenoids	Chl	O_2^1
Avoidance		
Anatomical adaptations	Leaf structure, epidermis	O_2^-, H_2O_2, O_2^1
C_4 and CAM metabolism	Chl, Cyt, Vac	O_2^-, H_2O_2
Chl movement	Cyt	O_2^-, H_2O_2, O_2^1
Suppression of photosynthesis	Chl	O_2^-, H_2O_2
PS and antenna modulations	Chl	O_2^-, O_2^1
Alternative oxidase	Chl, Mit	O_2^-

[a] ***Abbreviations:*** Apo-apoplast; Chl-chloroplast; CW-cell wall; ET-electron transport; Mit-mitochondria; O_2^1-singlet oxygen; Per-peroxisome; PM-plasma membrane; PS-photosystem; ROI-reactive oxygen intermediate; Vac-vacuole.

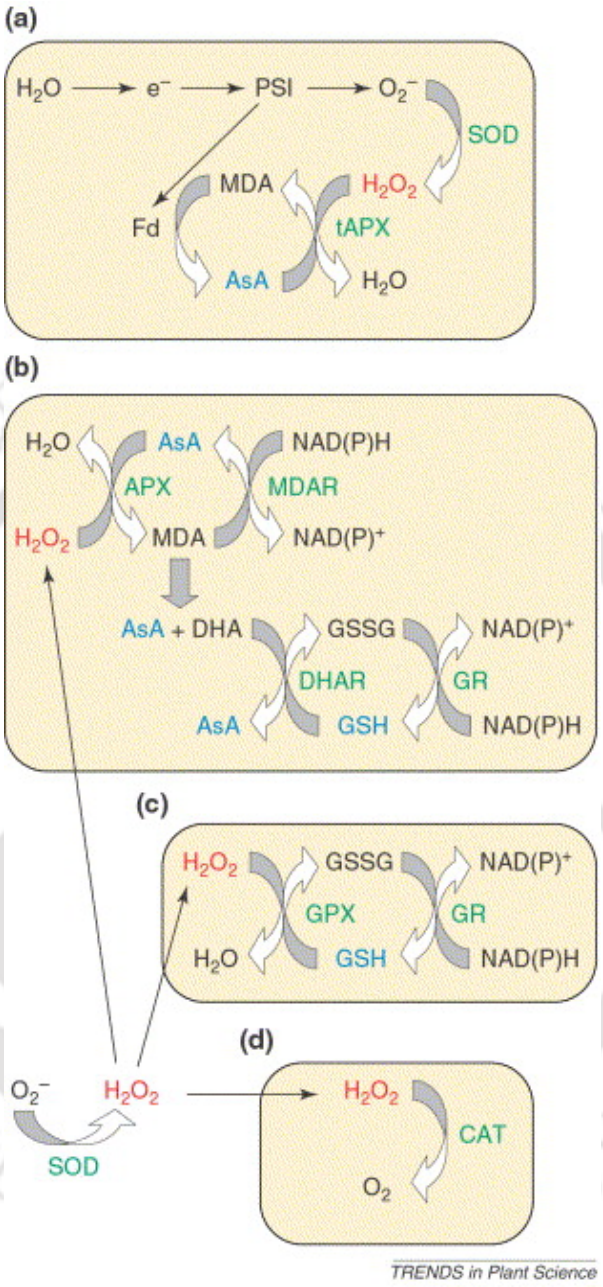

Figure 1.7 In order to protect itelf from the negative effects of reactive oxygen species (ROS), plants possess a well defined antioxidative defense system consisting of different enzymatic and nonenzymtaic molecules. The different scavenging pathways which exist in the plant metabolic system to counter balance the level of reactive oxygen species are **(a)** water-water cycle in chloroplast, **(b)** glutathione-ascorbate cycle found mainly in chloroplast, mitochondria, peroxisomes and cytoplasm, **(c)** glutathione-peroxidase cycle and **(d)** catalase cycle in peroxisomes. Abbreviations: DHA, dehydroascorbate; DHAR, DHA reductase; Fd, ferredoxin; GR, glutathione reductase; GSSG, oxidized glutathione; MDA, monodehydroascorbate; MDAR, MDA reductase; PSI, photosystem I; tAPX, thylakoid-bound APX. (Figure adopted with permission from *Mittler, 2002, Trends in Plant Science*. 7, 405-410).

Ascorbate-Glutathione cycle reduces GSSH, MDHA and DHA leading to formation of GSH and ascorbate. During the external stress such as drought, extreme temperatures, heat shock etc. increase in GSH and ascorbate level as well as enzymes involved in the ascorbate-glutathione cycle has been observed in the plant metabolic system. The enzymatic scavenging system involves different antioxidative enzymes which scavenges reactive oxygen species precisely throughout the metabolic system.

The major ROS scavenging enzymes are superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and catalase (*Mittler, 2002*). These antioxidative enzymes are involved in scavenging ROS molecules and thus maintain the ionic balance. These enzymes are localized in different sub cellular components and scavenge exclusively the ROS molecules generated in their respective compartments. Superoxide dismutase scavenges superoxide anion radical and thus converts it to hydrogen peroxide which is further being used by other antioxidative enzymes such as peroxidases and catalses. Balance of different antioxidative enzymes is required in order to maintain the ROS level and suppress its toxic effects onto the metabolic system of the plant (*Bowler et al., 1992*).

1.2.4 Antioxidative defense system

Oxidative stress conditions develops due to increase in the level of reactive oxygen species such as superoxide radical, hydrogen peroxide and singlet oxygen species (*Apel & Hirt, 2004*). Plant posses an excellent antioxidative defense system to protect it from the negative effects of these ROS radicals (Figure 1.8). The antioxidative defense system of plant consists of low molecular weight non-enzymatic antioxidants such as ascorbate, glutathione, tocopherols and phenolic compounds. The enzymatic antioxidative defense system consists of superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and catalase. External stress condition induces the formation of reactive oxygen species within the metabolic system and simultaneously increases the oxidative stress. However, both the non-enzymatic and enzymatic antioxidative defense system depletes the oxidative stress condition by scavenging the different reactive oxygen species (*Arora et al., 2002*).

1.2.4.1 Non-enzymatic antioxidative defense system

Non-enzymatic antioxidative defense system plays a major role in the scavenging of reactive oxygen species and thus depletes the oxidative stress. The different low molecular weights antioxidants involved are ascorbate, glutathione, tocopherols, and phenolic compounds. These different antioxidants are present throughout the cellular and subcellular components.

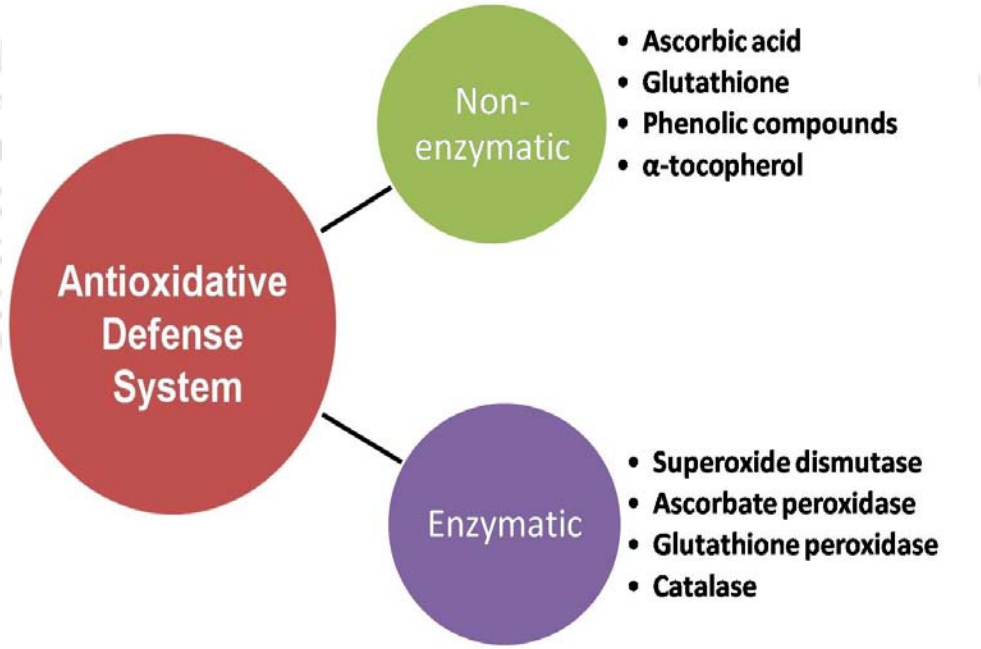

Figure 1.8 Plants possess an excellent antioxidative defense system consisting of both enzymatic and non-enzymatic part to protect itself from the negative effects of reactive oxygen species (ROS). The non-enzymatic part consists of different antioxidative molecules which help in the reduction of ROS. The enzymatic antioxidative defense system consists of different enzymes which are highly specific in their locations and are found throughout the cellular component. They scavenge the ROS in sequential way and thus reduce the oxidative load generated onto the plant metabolic system.

1.2.4.1.1 Ascorbic acid

Ascorbic acid is a very potent antioxidant molecule present throughout the cellular and subcellular components (*Foyer et al., 1991*). It is mainly present in the cytosol, chloroplast, vacuole and apoplastic space of leaf cells. Its major role includes scavenging of hydrogen peroxide and the reaction takes place in two sequential steps i.e. oxidation of ascorbate to monodehydroascorbate (MDHA) and then to dehydroascorbate (DHA). Monodehydroascorbate reductase (MDHA-reductase) and dehydroascorbate reductase enzymes are involved in the regeneration of reduced ascorbate. Regeneration of ascorbate within the chloroplast helps in the electron transport system (*Miyake & Asada, 1992*; *Foyer & Lelandais, 1993*)

1.2.4.1.2 Glutathione

Glutathione or γ-L-Glutamyl-L-cysteinylglycine (GSH) is another major low molecular weight antioxidant molecule present throughout the cellular components (*Jimenez et al., 1998*). The glutathione act as a disulphide reductant, protects thiol groups of enzymes and scavenges singlet oxygen species and hydroxyl radical generated. Glutathione also regenerates ascorbate with the help of enzyme dehydroascorbate reductase. In this reaction glutathione is oxidized to glutathione disulphide (GSSG) and further regenerated to glutathione (GSH) using glutathione reductase (GR) enzyme using NADPH-dependent reaction (*Alscher, 1989*).

1.2.4.1.3 α-Tocopherol

Tocopherols are essential biological components with antioxidative properties. Out of different tocopherols groups (α-,β-,μ-,δ-), α-tocopherol is the most potent membrane associated antioxidant which scavenges singlet oxygen species and lipid peroxides (*Kamal-Eldin & Appelqvist, 1996*). Tocopherols are abundantly found in chloroplast of higher plants and impart protection against photooxidative damage. It is relatively poor electron donor in normal physiological condition and act by transferring single hydrogen atom. It scavenges hydroxyl radical, singlet oxygen species and superoxide radicals (*Arora et al., 2002*).

1.2.4.1.4 Phenolic compounds

Phenolic compounds are secondary metabolites found abundantly in plant tissues. They have been found to possess free radical scavenging activity and thus act as potential antioxidants. The antioxidative property of polyphenols is due to their high ability to delocalized unpaired electrons and chelates transition metal ions, ultimately inhibiting the formation of hydroxyl radical through Fenton reaction. Polyphenols also interfere with the lipid peroxidation reaction and inhibit the hydrogen peroxide formation cascades in plants (*Rice-Evans et al., 1997*).

1.2.4.2 Enzymatic antioxidative defense system

To balance the increasing oxidative condition developed due to a variety of external stress factors, plant possess a very excellent antioxidative defense system consisting of enzymatic and non-enzymatic part. The enzymatic part of antioxidative defense system consists of different enzymes which scavenges the free radical throughout the metabolic system and compensates the induced oxidative stress. The different enzymes involved are superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT). Superoxide dismutase scavenges superoxide radical (O_2^-) and convert it to hydrogen peroxide (H_2O_2). Ascorbate peroxidase, glutathione peroxidase and catalase further scavenge hydrogen peroxidase into water and oxygen molecule and thus balance the induced oxidative stress (*Mittler, 2002*).

1.2.4.2.1 Superoxide dismutase (SOD)

Superoxide dismutase belongs to group of metalloenzymes which scavenges superoxide radicals (O_2^-) and converts it to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) which itself is a reactive oxygen species (*Takahashi & Asada, 1983*). The initial scavenging of superoxide radical into hydrogen peroxide decreases the chances of formation of highly reactive hydroxyl radical (OH^-). Based on the metal cofactor involved, three different forms of SOD enzymes exist in plant metabolic system. First is copper-zinc superoxide dismutase (Cu-Zn SOD) which is found in the choloroplast, mitochondria as well as cytoplasm. Another is iron superoxide dismutase (Fe-SOD)

which is majorly found in chloroplast, however mitochondrial and cytoplasmic Fe-SOD has also been observed. And the third one is manganese superoxide dismutase (Mn-SOD) which is mitochondrial and chloroplastic specific in existence. The different isomers of SOD have been found from almost all the essential and metabolically active cellular components, indicating its significance in the antioxidative defense system (*Kanematsu & Asada, 1990*).

1.2.4.2.2 Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) is another antioxidative enzyme found majorly in cytoplasm and chloroplast (*Chen & Asada, 1989*). Ascorbate peroxidase scavenges hydrogen peroxides which are generated through dismutation of superoxide radicals using SOD enzymes. APX and SOD works in coordination within chloroplast as SOD dismutate superoxide radicals into hydrogen peroxide and further APX reduces the formed hydrogen peroxide into water and oxygen molecule. APX is also involved with low molecular weight antioxidant molecule ascorbate and other different antioxidative enzymes in scavenging of hydrogen peroxide through Asada-Haliwell pathway, which also involves the regeneration of ascorbic acid (*Noctor & Foyer, 1998*).

1.2.4.2.3 Glutathione peroxidase (GSH-POD)

Glutathione peroxidase (GSH-POD) scavenges hydrogen peroxide into water molecule using glutathione as reducing equivalents. The GSH-POD cycle which mainly exists in cytoplasm converts glutathione (GSH) into glutathione disulphide (GSSH) which is again converted back to GSH using glutathione reductase (GR), an another antioxidative enzyme and reducing agent NAD(P)H (*Alscher, 1989*). The different antioxidative enzymes work in co-ordinated way to scavenge the reactive oxygen species and free radicals generated through different metabolic reactions steps.

1.2.4.2.4 Catalase (CAT)

Oxidative stress is determined by the level of O_2^{-}, H_2O_2, and ·OH radical however ionic balance of this redox metabolic system is maintained by the different antioxidative enzyme such as superoxide dismutases and other peroxidases. Catalase is

another important antioxidative enzyme regulating the intracellular level of H_2O_2. It scavenges H_2O_2 into H_2O and O_2 and is also found in peroxisomes (*Willekens et al., 1995*).

1.2.5 Superoxide dismutase: An overview

Reactive oxygen species (ROS) or Free radicals formation takes place under both, stressed and unstressed conditions. Different molecules generated during the redox metabolic reactions and contributing to the increase in oxidative load includes superoxide anions, hydrogen peroxide, hydroxyl radical and singlet oxygen species specifically. However, the plant metabolic system has very well developed antioxidative system to balance the increased oxidative load. The antioxidative enzymes scavenge the free radicals produced and thus maintain the ionic balance, overall maintaining oxidative stress condition (*Mittler, 2002*). Out of the different antioxidative enzymes, superoxide dismutase (SOD) forms the very first line of defense against the induced oxidative stress. Superoxide anion (O_2^-) is produced by the activation of oxygen molecule and takes place in almost all the cellular components such as mitochondria, chloroplast, cytosol, peroxisomes, glyoxysomes, apoplast and microsomes. However, the major cellular component involved in the formation of superoxide anions is mitochondria, chloroplast and peroxisomes (*Scandalios, 1983*). Electron transport chain reaction is mainly involved in the production of superoxide anion radical and thus found exclusively in these components (*Apel & Hirt, 2004*). Due to the formation of superoxide anion radical in numerous subcellular components, existence of superoxide dismutase enzyme throughout the subcellular components is desirable. SOD is found in all the cellular components and exclusively in mitochondria, chloroplast and peroxisomes as they are the major site for superoxide anion formation (*Kanematsu & Asada, 1990*). Superoxide anion radicals are membrane impermeable and thus cannot diffuse across the lipid bilayer membrane. SOD antioxidative enzyme which scavenges the superoxide anion into hydrogen peroxide and depletes the concentration of superoxide anion radical. Thus lowering the level of highly reactive superoxide anion radical further avoids formation of hydroxyl radical, most toxic and highly unstable reactive oxygen species (*Takahashi and Asada, 1983*). Hydrogen peroxide is membrane permeable, thus formed at one location *i.e* inside mitochondria or chloroplast and gets easily diffused into the surrounding medium to be further scavenged

by other H_2O_2 utilizing peroxidase and catalase enzymes. Superoxide dismutase (SOD) belongs to metallo group of enzymes and based on its different metal cofactor associated with it the enzyme gets divided into three different groups namely copper-zinc superoxide dismutase (Cu/Zn SOD)**,** iron superoxide dismutase (Fe SOD)**,** manganese superoxide dismutase (Mn-SOD) (*Bannister et al., 1987*) (Figure 1.9).

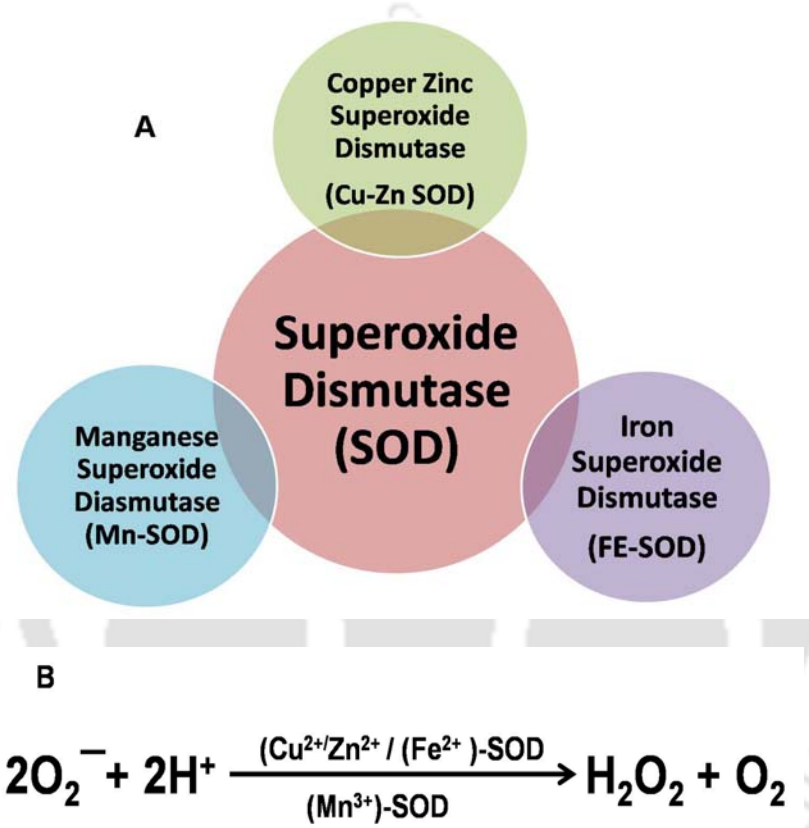

Figure 1.9 Superoxide dismutases are differentiated based on their metal co-factor associated with its activity. **(A)** Represents different types of plant superoxide dismutase. The antioxidative superoxide dismutase (SOD) protein scavenges superoxide radicals and converts it to hydrogen peroxide which is much less toxic and less reactive as compared to a typical superoxide anion. **(B)** Represents the scavenging activity of plant superoxide dismutases involving the metal cofactor.

1.2.5.1 Mechanism of action of superoxide dismutase

Superoxide dismutase (SOD) enzyme, irrespective of the source obtained and type is very excellent scavenger of superoxide radicals. The enzyme is ubiquitous in nature and found especially in O_2 consuming organisms. Superoxide dismutase was first isolated

from bovine blood and thought to be a copper storage protein (*Mann & Kelin 1938*). The catalytic function of dismutating superoxide radicals into hydrogen peroxide and O_2 was discovered by *McCord & Fridovich (1986)*. SODs reaction mechanism employs alternate reduction/ oxidation of the respective metal associated with the enzyme and catalyzes disproportionate reaction very close to diffusion (*McCord & Fridovich 1969*).

1.2.5.2 Types of superoxide dismutase

Based on their metal cofactor involved in the active site which is responsible for oxidation/ reduction reaction, superoxide dismutase is of three different types. These are, SODs containing copper-zinc (Cu/Zn-SOD), manganese (Mn-SOD) and iron (Fe-SOD) (Figure 1.9). These entire different metal ions containing SODs are found in different sub cellular compartments. Fe-SOD is found exclusively in chloroplast, Cu/Zn-SOD in cytosol while Mn-SOD in mitochondria (Figure 1.10).

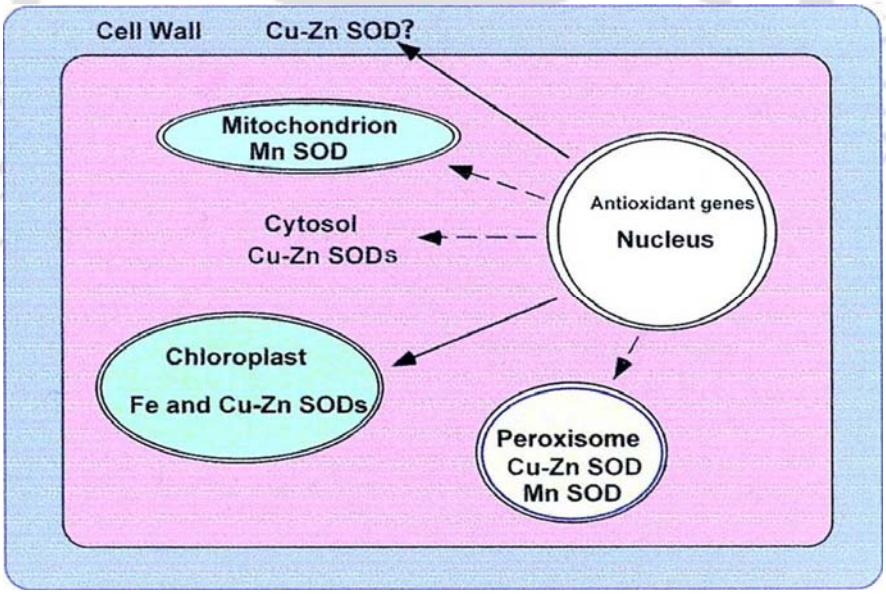

Figure 1.10 The antioxidative superoxide dismutase (SOD) forms the initial first line of defense against the increased oxidative stress and thus has a major role to play. The figure represents the different cellular location of superoxide dismutase protein. Being an important part of the antioxidative defense machinery, SOD proteins are found throughout the different cellular locations within a cellular system. (Figure adopted with permission from *Alscher et al., 2002. Journal of Experimental Botany*. 53, 1331-1341)

Complete amino acid sequence comparison reveals that Mn-SOD and Fe-SOD are interrelated and are of most ancient type while Cu/Zn-SOD is the most newly evolved superoxide dismutase. The difference in their evolution could be attributed to the availability of different soluble transition metal ions in the atmosphere during different geological eras (*Bannister et al., 1991*).

1.2.5.2.1 Iron superoxide dismutase (Fe-SOD)

Iron superoxide dismutase (Fe-SOD) is generally considered to be the most ancient type of SOD evolved. Fe-SOD is found in both prokaryotes as well as eukaryotes where it has been isolated from *Euglena gracilis* (*Kanematsu & Asada, 1979*). In plants, Fe-SOD has been strictly confined to chloroplast and had been verified using raised polyclonal antibody reaction in water lily (*Neuphar luteum*). Fe-SOD had been found in variety of plants including *Arabidopsis thaliana, Glycin max, Nelumbo plumbaginifolia* etc. It exists in two different groups *i.e* one group belongs to homodimer of identical 20 kDa subunit as reported from *E. coli* (*Yost & Fridovich, 1973*), *Photobacterium sepoa* and *P. leiognathi* (*Puget and Michelson, 1974*) and from plant species like *Ginkgo biloba*, *B. campestris* and *Nupher luetum* (*Salin & Bridges, 1980*). While the second group of Fe-SOD belongs to tetrameric with four equal subunits having molecular weight of 80-90 kDa and generally found in higher plants. The unique property of Fe-SOD is that it gets inactivated by H_2O_2 and is resistant to KCN.

1.2.5.2.2 Manganese superoxide dismutase (Mn-SOD)

Manganese Superoxide dismutases (Mn-SOD) are found exclusively in mitochondria and peroxisomes and only one metal atom per subunit has been found to be responsible for the activity. The presence of mitochondrial and peroxisomal Mn-SOD has been confirmed using immunolocalization assay in watermelon (*del Rio et al, 1992*). Mn-SOD bears much similarity to Fe-SOD in terms of its primary, secondary and tertiary structure however the Mn atom cannot be replaced with Fe atom for its activity (*Fridovich, 1986*). Mn-SOD exist in either homodimer or homotetrameric form with one Mn(III) atom per subunit. The enzyme in both prokaryotes and eukaryotes is neither inhibited by KCN neither inactivated by H_2O_2. Plant Mn-SODs have almost 65%

sequence similarity and also high similarity in case of bacterial species (*Bowler et al, 1994*). In addition to Mn-SOD identification in maize, it has also been obtained from mitochondria of other plant species such *Nicotiana tabacum* (*Bowler et al, 1994*), *Vigna mungo* (*Reddy & Venkaiah, 1982*), *Pisum sativum* (*Foster & Edward, 1980*) *Spinacia oleraceae* (*Jackson et al., 1982*) and many more.

1.2.5.2.3 Copper-Zinc superoxide dismutase (Cu-Zn SOD)

Copper-Zinc containing superoxide dismutase forms another important category of antioxidative SOD enzymes which are found throughout the plant cells such as cytosol, chloroplast, glyoxisomes, peroxisomes and extracellular spaces, irrespective of presence of Fe-SOD and Mn-SOD enzyme. Each Cu-Zn SOD subunit enzyme molecule contains an atom of copper and an atom of zinc in its structure. Cu-Zn SOD enzyme exists in both homodimeric and homotetrameric forms. Cytoplasmic and periplasmic forms of Cu-Zn SOD are found to be homodimeric while chloroplastic and extracellular Cu-Zn SODs are majorly homotetrameric in nature. It has also been observed that each subunit function independently and the interaction between the subunits are not essential for full catalytic activity. Tetrameric extracellular SOD has been exclusively found in mammals in the intestinal tissue matrix and glycocalyx of cell surface. However, small fractions of extracellular SODs are also found in the extracellular fluids such as lymph, plasma, synovial fluid and cerebrospinal fluid (*Carlson et al 1995*). In case of plants, Cu-Zn SODs exists in two major forms *i.e* chloroplastic and cytoplasmic. The amino acid sequencing suggests around 68% sequence similarity among these two forms of Cu-Zn SODs. Chloroplastic Cu-Zn SODs shows 90% sequence similarity among itself while the cytoplasmic form shows 80-90% sequence similarity. Cu-Zn SODs are the most prevalent forms of SOD and it has been studied from different sources such as spinach, maize, cabbage, *Radix lethospermi, Olea europea, Nicotiana plumbaginifolia*, Garlic (*Allium sativum* L.) etc (*Hadji et al 2007*).

1.2.6 Physiological significance of superoxide dismutase in plants

Superoxide dismutase catalyzes the dismutation of superoxide radicals into hydrogen peroxide and thus forms the first line of defense against oxidative stress condition. The formation of reactive oxygen species in plant system takes place in

mitochondria, chloroplast, peroxisomes, glyoxisomes etc, subcellular components which are majorly involved in redox metabolic reactions leading to the development of oxidative stress condition. These redox reactions are unavoidable as they are required for the different metabolic process leading to the generation of increased level of reactive oxygen species. Increase in the level of reactive oxygen species further gets enhanced under external stress condition. Different environmental stress conditions affecting the normal plant growth and development includes drought, high light intensity, heavy metal contamination, excessive use of fertilizers/ herbicides/ insecticides etc. Tolerance to wide variety of environmental stress has been associated with increased level of antioxidative enzymes of which superoxide dismutase, a metalloenzyme dismutating superoxide radical into hydrogen peroxide and molecular oxygen forms an integral part. Numerous reports are available which confirm the higher induction of antioxidative enzymes and insertion of highly inducible SOD gene help crop plants to tolerate adverse environmental condition. Winter survival of alfa-alfa transgenic plant over-expressing SOD has been reported (*McKersie et al., 1999*). Transgenic *Brassica napus* with Mn-SOD from *Triticum aestivum* has been reported having higher tolerance to Aluminium metal concentration (*Basu et al., 2001*). Transgenic tobacco plant over-expressing Fe-SOD in chloroplast (*Van camp et al., 1996*) and another tobacco transgenic over-expressing Cu/Zn SOD in chloroplast was found to be tolerant to high oxidative stress condition (*Gupta et al., 1993*). All these reports indicate the physiological significance and importance of superoxide dismutase enzyme towards maintenance of redox metabolic balance as well as the oxidative load which gets developed under different unwanted environmental stress conditions.

1.2.7 Physiological significance of superoxide dismutase in humans

Superoxide dismutase, the metal containing antioxidative enzyme has a very important role to play against induced oxidative stress in human health. SOD scavenges superoxide anions into molecular oxygen and hydrogen peroxide. Depending on the metal ions associated, two different types of superoxide dismutase are present namely Mn-SOD and Cu-Zn SOD constituting the total superoxide anion scavenging activities. Fe containing SODs have not yet been reported from higher eukaryotes and are generally present in prokaryotes and some green plant. SODs are present mainly in the nucleus,

mitochondria, cytoplasm and extracellular spaces. In higher eukaryotes these SODs are differentiated based on their localization *i.e* SOD1 represents cytoplasmic SOD, SOD2 represent mitochondrial SOD while SOD3 represent extracellular SOD. In case of humans it has been observed that cytoplasmic SODs are homodimeric in nature while mitochondrial and extracellular SODs are homotetrameric. Oxidative stress develops in humans, due to enhanced ROS production as well as due to deficiency in both enzymatic and non enzymatic antioxidative defense system. Increase in the oxidative stress condition in human is related to numerous pathological conditions of acute and chronic nature. Ischemia-reperfusion injury is an example of acute pathological condition while diabetes mellitus refers to chronic conditions leading to uncontrolled increase in ROS and simultaneously oxidative stress. Normal human ageing process also leads to increase in ROS level in the body. The superoxide anions produced into the metabolic system reacts with nitric oxide to produce peroxynitrite ($OONO^-$), a very powerful oxidant molecule. This peroxynitrite has very high reactivity and its conjugated form (OONOH) crosses the cellular membrane and reaches to other cellular components. Superoxide dismutase enzyme works in coordination with other antioxidative enzyme such as catalase and peroxidase (Figure 1.11). Superoxide anions dismutated into hydrogen peroxide is further being scavenged into oxygen and water with the help of these H_2O_2 scavenging enzymes.

Imbalance between SOD, catalase and peroxidase enzymes will lead to the accumulation of H_2O_2 into the cellular environment which can induce the production of highly reactive hydroxyl radical and reactive oxygen species. Superoxide dismutase also dilutes the oxidative load by dismutating superoxide anions into hydrogen peroxide which permeates through the cell membrane and thus balances the oxidative stress throughout the cellular system. Dysfunction of superoxide dismutase or its decreased activity within the human cellular system is caused by a number of factors. Human pathological condition such as amyotrophic lateral sclerosis (ALS) is one of the genetic disorders found to have decreased SOD activity through mutation in SOD genes. Metal ions deficiency also contribute to the decreased activity of different superoxide dismutase enzyme as well as age related disorders and certain pathological condition such as obesity shows dysfunction in SOD activity. In order to increase ROS scavenging activity within the metabolic system different approaches has been followed. Increasing the cells native

SOD activity, increasing the gene expression, using SOD mimetic as well as supplementary enzyme therapy are being followed for the enhanced scavenging of reactive oxygen species.

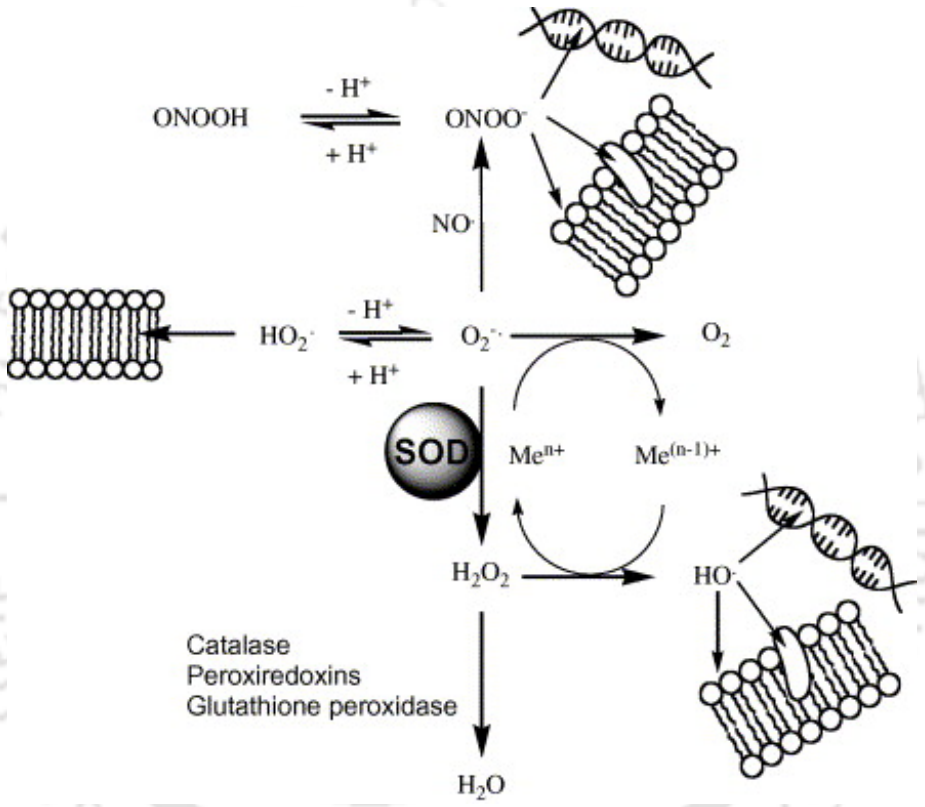

Figure 1.11 Superoxide dismutase is an important part of antioxidative defense machinery. It scavenges the initial superoxide anion radical and converts it to less reactive and comparatively much less reactive hydrogen peroxide. The figure represents the different roles of superoxide dismutase protein against the negative consequences due to increase level of reactive oxygen species (ROS) *i.e* superoxide anion radicals (Figure adopted with permission from *Johnson and Giulivi, 2005, Molecular Aspects of Medicine*. 26, 340 – 352)

1.2.8 Superoxide dismutase and its therapeutic potentials

Oxidative stress results from the high production of reactive oxygen species under adverse metabolic conditions as well as due to ineffective antioxidative defense system. Superoxide dismutase, the metal containing enzyme has a very critical role to play towards limiting the oxidative stress condition and is considered to be one of the enzymes of therapeutic importance (*Johnson and Giulivi, 2005*). The highly specific nature of superoxide dismutase towards reducing superoxide anion and its presence throughout the cellular compartments indicate its critical role towards maintenance of oxidative stress conditions (*Giulivi et al., 1999*). Low levels of superoxide dismutase enzyme has also been observed in complexity with numerous human pathologic conditions which includes age related as well as organ specific conditions. Dysfunction of superoxide dismutase activity has been associated with genetic disorder such amylotrophic lateral sclerosis (ALS) where the destabilization of SOD enzyme takes place due to its impaired affinity with zinc metal ions (*Crow et al., 1997*). Thus, the induced oxidative stress is considered to be the underlying condition under numerous diseased states which further elevates the pathogenic conditions.

Superoxide dismutase enzymes have been explored for its therapeutic potentials towards reducing the induced oxidative stress and maintaining the ionic balance. Therapeutic treatment includes inducing the cells ability towards high SOD activities using natural extracts (*Ng et al., 2005*), as well as use of SOD mimetics (*Izumi et al., 2002; Pong et al., 2002*). However, the external supplementation of SOD enzyme had been observed with significant advantages. Various delivery approaches has been used towards overcoming the cellular barrier and higher reduction of oxidative stress condition (*Reddy et al., 2008*). The Nanoparticulate system has been observed with significant interest towards overcoming the drug deliveries. Nanosystem increases the stability in the biological environment as well as enhances the cellular and tissue uptake (*Panyam and Labhasetwar, 2003*). Therapeutic potentials of SOD enzyme can be achieved with the significant use of an efficient nanoparticulate system towards reducing the induced oxidative stress.

1.3 SIGNIFICANCE OF THE WORK

The significance of superoxide dismutase is intimately involved in their role against the increase in reactive oxygen species and subsequent induction of oxidative stress condition. Formation of reactive oxygen species within the plant cellular metabolism is unavoidable due to various redox metabolic reactions involved in normal energy synthesizing cellular processes. Increase in these reactive oxygen species also gets induced under external stress condition leading to the development of oxidative stress. Environmental pollution surrounding the agricultural lands plays a major role in the induction of oxidative stress within the germinating plants and thus leads to improper growth and development. To avoid the negative effects of oxidative stress, plant possess a variety of antioxidative enzymes of which superoxide dismutase forms the first line of defense. High activity of superoxide dismutase depletes the initial superoxide anions formed and decreases oxidative stress simultaneously. Superoxide dismutase also induces other antioxidative enzymes by formation of hydrogen peroxide as by product which easily permeates through cellular membranes and dilutes its overall effects. Superoxide anions are membrane impermeable and thus get accumulated at the sites of production specifically in mitochondria, chloroplast and peroxisomes. As they are highly unstable and reactive, they need to be dismutated and thus superoxide dismutase plays its essential role in dismutation and converting it to hydrogen peroxide and molecular oxygen. Antioxidative enzymes such as catalase, ascorbate peroxidase, glutathione peroxidase scavenge hydrogen peroxide into oxygen and water molecule thus depletes the oxidative stress completely. The current work report antioxidative enzymatic analysis of *Cicer arietinum* seedlings induced under aluminium metal stress, purification and detailed biochemical characterization of a novel superoxide dismutase enzyme induced under aluminium metal stress. Further the work was also explored using nanotechnological advances such as conjugating superoxide dismutase onto carbon nanotubes towards reducing the induced oxidative stress as well as polymeric nanoencapsulating superoxide dismutase in combination with another antioxidative enzyme catalase and exploring its potentiality towards reducing the induced oxidative stress in human cell line.

The work presented in the thesis is divided into four parts as follows:

I. Aluminium metal stress induced antioxidative enzymatic analysis in germinated Cicer arietinum seedlings.

Agricultural land pollution in terms of high metal concentration has been found to affect the normal growth and development of germinating crop plants. Physiological and biochemical response were observed under increasing aluminium stress at different time intervals in chickpea seedlings (*Cicer arietinum*). Germination percentage and root length were found to be highly reduced under increasing metal stress. Antioxidative enzymatic fluctuations were also observed under aluminium metal treatment. Roots were found with higher antioxidative activity compared to shoots. Low concentration of aluminium after a brief treatment induced higher superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activity whereas longer duration of treatment led to decrease in these activities. Higher oxidative damage in roots compared to shoots was observed based on malondialdehyde concentration. Overall, the results indicated that high concentration and long exposition of aluminium increases oxidative stress and impairs antioxidative defense system, leading to poor growth and low survival of seedlings.

II. Isolation, purification and characterization of aluminium metal stress induced novel superoxide dismutase protein from Cicer arietinum seedlings.

A copper zinc superoxide dismutase (Cu-Zn SOD) was purified and characterized from *Cicer arietinum* seedlings germinated under aluminium (Al^{3+}) stress. The enzyme was purified upto 28 fold with specific activity of 158 units mg^{-1}. The SOD was found to be homodimeric with approx subunit molecular weight of 33.27 kDa. Cu-Zn category of superoxide dismutase was identified by H_2O_2 induced inhibition of in-gel activity and presence of quantifiable copper and zinc ions. The optimum pH range for purified Cu-Zn SOD activity was observed within 6.5-8.5 (highest at pH 8.0) and the pH stability was in the range of 6.0-8.5. The enzyme was more stable at low temperature (below 30°C). The Km of purified Cu-Zn SOD for riboflavin as substrate was 10.16 ± 2.5 µM. The N-

terminal amino acid sequence showed homology with conserved residues of other plant Cu-Zn SODs.

III. Superoxide dismutase-multiwalled carbon nanotubes biocatalytic conjugate towards alleviating oxidative stress in human skin cell line.

MwCNT(SOD) biocatalytic conjugate was synthesized by two step process involving diimide activated amidation reaction chemistry. The conjugates obtained were highly aqueous dispersible and characterized further using field emission scanning electron microscopy and Fourier transformed infrared spectroscopy. MwCNT(SOD) conjugates were found to retain significant superoxide anion scavenging activity even after 96 hours time period. pH optima and stability was found in the range of 6 to 9 with highest activity at pH 8. Low temperature optima and stability was obtained (upto 30°C). Unrefined (raw) and oxidized MwCNTs were found to induce significant toxicity on human skin HaCat cell line, however MwCNT(SOD) biocatalytic conjugate was found to be comparatively nontoxic when applied on human skin HaCat cells. Oxidative stress condition on human skin HaCat cells was optimized using H_2O_2 (1mM) as an external stress factor for further experimental purpose. MwCNT(SOD) biocatalytic conjugate was found to alleviate the increased oxidative stress conditions on human skin HaCat cell lines. Increasing concentration of MwCNT(SOD) conjugate was observed to be significantly increasing the cell viability in human skin cell lines reducing the increased oxidative stress level.

IV. Polycaprolactone nanopshere encapsulating superoxide dismutase and catalase enzyme towards elevating antioxidative defense against induced oxidative stress.

Polycaprolactone (PCL) nanosphere encapsulating superoxide dismutase (SOD) and catalase (CAT) were successfully synthesized using double emulsion (w/o/w) solvent evaporation technique. Characterization of the synthesized nanosphere was done using dynamic light scattering, field emission scanning electron microscope, and Fourier transform infrared spectroscopy. A spherical-shaped nanosphere in a size range of 812±64 nm with moderate protein encapsulation efficiency and high in vitro protein release was synthesized. Human skin HaCat cells were used for analyzing antioxidative properties of SOD and CAT

encapsulated PCL nanospheres. Hydrogen peroxide as external stress factor was used for oxidative stress condition in HaCat cells and verified through reactive oxygen species (ROS) analysis using H_2DCFDA dye. PCL nanosphere encapsulating SOD and CAT together indicated better antioxidative defense against H_2O_2 induced oxidative stress in human skin HaCat cells in comparison to PCL encapsulating either SOD or CAT alone as well as against direct supplement of SOD and CAT proteins together.

Chapter II

Materials and Methods

2.1 MATERIALS

Chickpea (*Cicer arietinum*) var. Avrodhi seeds were obtained from certified sources. $Al_2(SO_4)_3.16H_2O$, $Ca(NO_3)_2.4H_2O$, KNO_3, KH_2PO_4, $MgSO_4.7H_2O$, H_3BO_3, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $NaMoO_4$, $FeSO_4.7H_2O$, KOH, H_2O_2, Disodium-ethylenediaminetetraacetic acid (EDTA.2Na) and Trichloro acetic acid (TCA) were obtained from Merck chemicals. L-methionine, riboflavin and ascorbate were purchased from Loba chemicals. Guaiacol, Thiobarbituric acid (TBA) was obtained from Spectrochem. DEAE-Sepharose, Sephacryl S300, Bovine Catalase (MW, 250 kDa), Bovine Serum Albumin (BSA) was obtained from Sigma-aldrich. Multiwalled Carbon Nanotubes (MwCNTs), N-Hydroxysuccinimide (NHS), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 2-(N-Morpholino) ethanesulfonic acid (MES), Polycaprolactone (PCL; MW, 14000), Polyvinyl alcohol (PVA; MW, 13,000–23,000), dichloromethane (DCM) were also purchased from Sigma-aldrich. Dimethyl sulfoxide (DMSO), sodium bicarbonate ($NaHCO_3$), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin–streptomycin antibiotic mixture were also procured from Sigma-aldrich. Nitroblue tetrazolium chloride (NBT), Dulbecco's modified Eagle's medium (DMEM)/Nutrient mixture F-12 Ham (DMEM/F12, 1:1 mixture) with L-glutamine, 15 mM HEPES buffer, and phosphate-buffered saline (PBS; pH 7.4, 1×) were obtained from HiMedia

Laboratories Pvt. Ltd. All chemicals and reagents were of highest purity commercially available.

2.2 METHODS

2.2.1 Aluminium metal treatment and *Cicer arietinum* seeds germination

Chickpea (*Cicer arietinum*) seeds var. Avrodhi obtained from certified sources were used for experiment. Seeds after imbibition for overnight were germinated in glass container (100 mm diameter) containing Hoagland's nutrient solution (pH 5.0) with varying concentration of Al^{3+} (*i.e.* 25, 100, 200, 400, 1000, 2000 and 3000 μM). A separate control plate was also maintained with nutrient solution only. Desired aluminium concentration was prepared from $Al_2(SO_4)_3.16H_2O$ using Hoagland's nutrient solution. Germination was done in dark for initial 2 days after that regular exposure of light: dark condition was provided and analyzed after 8^{th}, 15^{th} and 21^{st} day of germination for root length, shoot length, antioxidative enzymes and lipid peroxidation. The control and experimental seeds were germinated in Hoagland's nutrient by keeping other parameters constant (pH, temperature, humidity etc.) while only variable was aluminium concentration.

2.2.2 Crude enzyme extraction from germinated seedlings

Enzyme extraction from the germinated chickpea (*Cicer arietinum*) seedlings was carried out at 4°C. Samples (0.25 g) taken from root and shoot of Al^{3+} treated plant was homogenized separately in 3 mL phosphate buffer (100 mM, pH 7.8) containing 1 mM disodium-EDTA in a prechilled mortar and pestle. The homogenate was centrifuged at 10,000 *g* for 20 minutes at 4 °C and supernatant was collected for SOD, APX, GPX and lipid peroxidation assay.

2.2.3 Determination of superoxide dismutase activity

Riboflavin mediated nitro blue tetrazolium (NBT) reduction method was followed for SOD activity measurement (*Giannopolitis and Ries, 1977; Gunes et al., 2009*). In the

presence of fluorescent light, methionine and riboflavin generates superoxide radicals which reduce NBT into blue colored formazan having absorption maxima at 560 nm. SOD dismutates superoxide radicals and thus inhibits NBT reduction into formazan, leading to a decrease in absorbance. Higher SOD activity leads to high inhibition of NBT reduction and successively low absorbance at 560 nm under these assay conditions. The reaction mixture consists of 10 mM Tris-HCl buffer (pH 8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 4 µM riboflavin and quantified enzyme extract. Riboflavin was last to be added in reaction mixture and the test tubes were moved into foil-lined box illuminated with 13 W fluorescent light for 10 min. Blank and control samples were prepared in identical manner but without illumination and enzyme, respectively. The inhibition in NBT reduction was measured at 560 nm and the assay protocol was standardized till reproducibility was consistent. One unit of SOD activity was defined as the amount of enzyme that produced 50% inhibition of NBT reduction under these assay conditions.

2.2.4 Activity analysis for ascorbate peroxidase (APX) enzyme

Ascorbate peroxidase activity was determined using ascorbate oxidation method (*Gracia-Limones et al., 2002*) with minor modification. In brief, reaction mixture of 1 mL consisted of 100 µL ascorbate solution (5 mM), 100 µL H_2O_2 (100 mM), 100 µL EDTA solution (1 mM) and 100 µL of crude plant extract in 600 µL of phosphate buffer (100 mM, pH 7.0). The oxidation of ascorbate was measured with decrease in absorbance at 290 nm for 3 minutes and activity was calculated with help of extinction coefficient (2.8 mM^{-1} cm^{-1}).

2.2.5 Activity analysis for guaiacol peroxidase (GPX) enzyme

Guaiacol peroxidase assay was performed using guaiacol oxidation method (*Roy et al., 1996*). Reaction mixture of 1 mL consisted of 100 µL guaiacol (34 mM), 100 µL H_2O_2 (100 mM), 100 µL plant extract in phosphate buffer (100 mM, pH 7.0). Oxidation of guaiacol was measured by increase in absorbance at 470 nm for 3 minutes and activity was calculated with the help of extinction coefficient (26.6 mM^{-1} cm^{-1}).

2.2.6 Estimation analysis of total lipid peroxidation

Lipid peroxidation was determined by the thiobarbituric acid (TBA)-trichloro acetic acid (TCA) method (*Dazy et al., 2009*). Malondialdehyde (MDA) on reacting with TBA forms MDA-TBA complex known as TBA reactive substances (TBARS) which has specific absorbance at 532 nm and non-specific absorbance at 600 nm. MDA concentration was determined with the help of extinction coefficient (155 mM^{-1} cm^{-1}). Protein content determination in all the above enzymatic preparation was done by the method of Bradford (*Bradford, 1976*) taking bovine serum albumin (BSA) as standard.

2.2.7 *Cicer arietinum* seedlings germination and aluminium metal treatment for purification of superoxide dismutase enzyme

Chickpea (*Cicer arietinum*) seeds, obtained from certified sources were washed with distilled water. They were transferred into petri-dish and allowed to germinate in Hoagland's nutrient solution supplemented with 100 µM $Al_2(SO_4)_3.16H_2O$ as an external metal stress inducing factor. The seeds in the petri-dish were kept in dark for initial period of 48 h and then were exposed with light and dark conditions at regular intervals. All other parameters including temperature and humidity were kept constant. The seeds germinated till 10-12 days were used for crude enzyme extraction.

2.2.8 Determination of protein concentration

The protein content was determined throughout the experiment at every purification step by the method of Bradford (*Bradford, 1976*). In detail, Standard protein solutions were prepared of different concentration ranging from 1µg/ml to 10 µg/ml using Bovine Serum Albumin (BSA). A straight line graph was obtained for its absorbance at 595 nm after addition of Bradford's reagent and the plot was used for determining protein concentration in unknown solutions.

2.2.9 Optimization of superoxide dismutase purification procedure

The crude enzyme extract was prepared in 10 mM Tris-HCl buffer (pH 8.0) by grinding 125 gm germinated Chickpea seedlings after removal of cotyledons. The homogenate was centrifuged at 12000 rpm at 4 °C for 30 min and the clear supernatant

was used for further purification steps. In order to avoid any enzymatic degradation, all purification steps were performed at 4 °C and a commercially available cocktail of protease inhibitors (mixture of inhibitors of different classes of proteases) was added in the extraction buffer. The crude extract was subjected to ammonium sulfate fractionation in two steps. In the first step ammonium sulfate was added slowly into the clear supernatant till 50% saturation and the mixture was centrifuged at 12000 rpm, 4 °C for 20 min to remove precipitated proteins. The clear supernatant was subjected to further fractionation up to 90% saturation. After centrifugation the precipitated proteins were re-dissolved in 10 mM Tris-HCl buffer (pH 8.0) and dialyzed extensively against the same buffer with regular change for a time period of 24 h. The protein concentration and SOD activity of dialyzed sample was determined and the sample was used for the ensuing purification steps. Further purification was achieved through anion exchange chromatography using DEAE-Sepharose as matrix. The column (1.0 cm x 25.0 cm) was pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) and the dialyzed sample, obtained in the previous step was loaded onto the column. After sample loading the column was washed with loading buffer and eluted with a linear gradient of NaCl (0.0 to 0.5 M) in 10 mM Tris-HCl buffer (pH 8.0) with a flow rate of 3.0 mL/min. Fractions of 6.0 mL were collected and analyzed for protein content (absorbance at 280 nm) and SOD activity. The fractions having SOD activity were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 8.0) overnight at 4 °C. After dialysis the sample was lyophilized and loaded on Sephacryl S-300 column (1.6 cm x 40 cm) for gel filtration chromatography (*Singh et al., 2010*). The sample was eluted by 0.125 M NaCl in 10 mM Tris-HCl buffer (pH 8.0) with a flow rate of 1 mL/min and fractions of 3 mL were collected. The collected fractions were analyzed for protein content (absorbance at 280 nm) and SOD activity. The SOD active fractions were loaded onto SDS-PAGE to determine their homogeneity. The pure fractions were used for further characterization.

2.2.10 SDS gel electrophoresis

The homogeneity of the purified protein and its molecular weight were determined by SDS-PAGE technique (*Laemmli, 1970*). Purified protein was loaded onto 12% SDS-PAGE gel along with molecular weight markers and a graph for log molecular weight (log Mw) *vs.* relative mobility (R_f) was plotted. The different molecular weight markers

used were phosphorylase b (97.4 kDa), bovine serum albumin (BSA; 66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean tyrosine inhibitor (20.1 kDa), and lysozyme (14.3 kDa).

2.2.11 MALDI-TOF analysis

Matrix-assisted laser desorption/ ionization (MALDI) mass spectrometry (MS) was performed for determination of the exact molecular mass of purified SOD with an AB SCIEX 4800 Plus MALDI TOF/ TOF analyzer. For the analysis, α-cyano-4-hydroxycinnamic acid (CHCA) was used as matrix and aldolase was used as external standard for mass scale calibration. In mass spectrometry (MS), the ionization process is observed to dissociate oligomers in multimeric proteins. Thus, the oligomeric mass was also determined by native PAGE.

2.2.12 Native (non-denaturing) gel electrophoresis and NBT-zymography staining

Native polyacrylamide gel electrophoresis (PAGE) and NBT activity staining were performed to determine the number of subunits of purified enzyme. The electrophoresis was performed in 10% polyacrylamide gel at 4°C, 100V and the SOD zymography was performed (*Beauchamp and Fridovich, 1971*). The gel after electrophoresis was divided into three different parts. The first part containing BSA and purified SOD was subjected to 0.1% Coomassie brilliant blue R-250 staining, while the second and third parts containing purified SOD were subjected to NBT activity staining with and without H_2O_2 inhibition, respectively.

2.2.13 Determination of UV-visible absorbance spectra and metal content analysis

The UV absorbance spectrum of purified superoxide dismutase was performed on Varian carry-100 double beam spectrophotometer. A conc. of 0.05mg/ml of purified SOD was subjected to UV-Visible absorbance scan. Metal content of purified superoxide dismutase was determined by flame atomic absorption spectrophotometer (Varian AA240-AAS). The purified protein in concentration of 0.05 mg/ml was dialyzed extensively against 10 mM Tris-HCl (pH 8.0) buffer containing 0.1mM EDTA and further buffer lacking EDTA. Copper and Zinc standards were prepared in deionized

water in the range of 2-10 ppm. From the AAS obtained data, number of moles and atoms of respective Cu and Zn metals were determined against applied SOD protein concentration and presented finally in terms of number of atoms/ SOD protein molecule.

2.2.14 Determination of temperature optima and stability

The effect of varying temperature on the activity of purified superoxide dismutase was measured by NBT reduction. The purified SOD (5 µg) was used for measurement and the reaction mixture was incubated for 15 min at defined temperatures ranging from 10 °C – 90 °C. The results were expressed as percentage residual activity to that of maximum activity within the temperature range studied. For determining the temperature stability, the purified enzyme in 10 mM Tris-HCl (pH 8.0) was incubated at different temperatures ranging from 10 °C – 90 °C for 15 minutes and aliquots of enzyme were used for the activity assay. The SOD activity was characterized by NBT reduction and the percentage residual activity was calculated. Measurements were carried out in triplicates for temperature optima and temperature stability experiments. Results were plotted in form of residual activity considering maximum activity as 100%.

2.2.15 Determination of pH optima and stability

The pH optimum for the purified superoxide dismutase enzyme was determined by the NBT reduction method in terms of its activity under varying pH conditions in universal buffers. The buffers used were: 0.01 M Glycine–HCl (pH 2.0–3.5); 0.01 M sodium acetate (pH 4.0– 5.5); 0.01 M sodium phosphate (pH 6.0–7.0); 0.01 M Tris-HCl (pH 7.5–10.5) and 0.01 M sodium carbonate (pH 11–12.0). The purified superoxide dismutase enzyme (5 µg) was used for activity measurement and presented in the form of percentage residual activity to the maximum activity within the examined pH range. In order to determine the pH stability, the purified enzyme (5 µg) was incubated in different pH buffers within range of 2.0-12.0 for overnight at 4 °C and then percentage residual activity was measured as described earlier. Measurements were carried out in triplicates for estimating the pH optima and pH stability.

2.2.16 Effect of substrate concentration on reaction velocity

The kinetic parameters of the purified enzyme were calculated with increasing substrate (riboflavin) concentrations (2-24 µM). The activity assay was performed as described earlier with 5 µg of purified enzyme, in 10 mM Tris-HCl (pH 8.0) at 37 °C. A Lineweaver- Burk plot was drawn from the results and the Michaelis-Menten constant (*Km*) was determined.

2.2.17 N-terminal amino acid sequencing

The purified enzyme was subjected to N-terminal amino acid sequencing using a PROCISE-492 model protein sequencing system available (Intas Biopharmaceuticals Ltd., India). The enzyme was blotted onto PVDF membrane (*Choli et al., 1989*). The obtained amino acid sequence was further analyzed through sequence homology search tools. Amino acids designated as 'X' could not be unambiguously identified.

2.2.18 Effect of metal ions and compounds on superoxide dismutase activity

Effect of metal ions and other compounds on the activity of purified enzyme was studied in the range of 0.1-1.0 mM concentration for $Al_2(SO_4)_3.16H_2O$, $CaCl_2$, $MgCl_2$, $NaNO_3$, $HgCl_2$, EDTA.Na_2, H_2O_2, SDS and 1-10 mM for urea and guanidine hydrochloride (Gu-HCl). The purified SOD (5 µg) was incubated with the metal ions and other compounds for 30 min. The SOD activity was measured as described earlier and the percentage residual activities were calculated in comparison to activity in absence of metal ions and compounds. The measurements were carried out in triplicates and the data used is the average of three independent experiments.

2.2.19 Maintenance of human skin keratinocyte (HaCat) cell line

Human adult low calcium temperature keratinocyte (skin HaCat) cell lines were obtained from the National Centre for Cell Science, Pune, India. The cells were grown in DMEM/F12 (1:1 mixture) containing 1.2 g/L $NaHCO_3$, 10% FBS, penicillin (1,000 UI/mL), streptomycin (1,000 UI/mL) at 37 °C in 5% CO_2. The medium was changed on every 48 h and splitting was done when cell growth reached 90% confluency.

2.2.20 Synthesizing oxidized multiwalled carbon nanotubes (MwCNTs)

Oxidized Multiwalled carbon nanotubes (MwCNTs) were prepared according to the method reported by *Asuri et al.,* (*2006*) with minor modifications. In brief, 100 mg of MwCNTs were subjected to ultrasonication bath in 400 ml of concentrated H_2SO_4:HNO_3 (3:1 ratio, v/v) for 6 hours. Post ultrasonication, the MwCNTs suspension was diluted using milli-Q water upto 1 liter volume. The obtained suspension was filtered using 0.2 µm filter membrane and the nanotube film deposited (a mat or thin film of cabon nanotubes formed, tangled with each other over the filter membrane) was collected. It was resuspended in milli-Q water using ultrasonication and was filtered again to remove the acid. This nanotube film collection, ultrasonication and filtration step was repeated to remove the acid completely (a total of five cycles were performed). The oxidized nanotubes were then subjected to lyophilization and stored further at room temperature as a dry powder. The dry oxidized MwCNTs were found to be easily re-dispersed in water with minimal ultrasonication. Specified quantity of dry oxidized MwCNTs was sonicated in milli-Q water for 2 hours. The solution was subjected to centrifugation for 5 min at 10000 rpm to remove any aggregates formed. The supernatant was carefully collected, absorbance was measured at 500 nm and quantified using extinction coefficient of 3.93×10^4 cm^2/g (*Asuri et al., 2006*). In case of MwCNTs-enzyme conjugate, sonication step was limited to ≤ 1min to avoid enzyme denaturation and inhibition of biocatalytic property.

2.2.21 Covalent immobilization of SOD enzyme onto oxidized MwCNTs using EDAC/ NHS reaction chemistry.

Superoxide dismutase enzyme was covalently linked onto oxidized MwCNT using EDAC/ NHS diimide activated amidation reaction chemistry (*Jiang et al., 2004*). In brief, 5 ml of 2mg/ml suspension of oxidized MwCNTs was prepared in MES buffer (50 mM, pH 6.2). The suspension was mixed with equal volume of NHS solution (400 mM) in same MES buffer. The mixed suspension was sonicated for 30 min before the addition of 20 mM EDAC. The suspension was stirred at 400 rpm for 30 min to initiate NHS coupling to carboxylated functional groups onto oxidized MwCNTs. The activated MwCNTs were filtered using 0.2 µm filter membrane, and the nanotubes mat/thin film collected was further washed thoroughly (3 times) with MES buffer to remove excess

NHS and EDAC. The nanotube suspension in Tris HCl buffer (10 mM, pH 8) was incubated with 5 ml of enzyme solution (2.5 mg/ml). The suspension was sonicated for ≤ 1 min and further incubated in orbital shaker (150 rpm, 37°C) for protein conjugation onto nanotubes. After 1 hour, the nanotube-protein suspension was filtered using 0.2 µm filter membrane and washed thoroughly to remove nonspecific protein binding using the same buffer. Any flocculates formed were removed and the suspension was used for further experiments. Control experiments were also performed without using EDAC and NHS respectively.

2.2.22 FE-SEM imaging and IR analysis of MwCNT(SOD) biocatalytic conjugate.

Size and morphology of MwCNT(SOD) conjugate was studied using FE-SEM imaging. Unrefined (raw) MwCNTs in comparison with superoxide dismutase conjugated MwCNTs were subjected to FE-SEM imaging using Sigma Carl-Ziess microscope operating at 4kV ETH. A thin film of the respective sample was prepared on aluminum foil with vacuum dried overnight and further subjected to gold plating after mounting onto a metal stab sealed with carbon tape. To confirm the covalent immobilization of SOD enzyme onto MwCNTs infrared spectroscopic (IR) analysis was done after each respective reaction step. The samples were prepared following KBr disc methodology where respective sample was blended with KBr powder and compressed to form disc under pressure of 5 tons before being placed in sample holder. Spectral scanning was done within 400 cm^{-1} to 4000 cm^{-1} and analyzed further.

2.2.23 SOD enzyme stability, temperature and pH characterization of MwCNT(SOD) biocatalytic conjugate.

Activity of superoxide dismutase (SOD) enzyme upon covalent immobilization with MwCNTs was confirmed using riboflavin mediated NBT reduction method (*Giannopolitis and Ries, 1977)*. In brief, the reaction mixture consists of 10 mM Tris-HCl buffer (pH 8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 4 µM riboflavin and 100 µl of quantified MwCNT(SOD) conjugate solution. Riboflavin was last to be added in reaction mixture and the test tubes were kept into foil-lined box illuminated with 13 W fluorescent light for 10 minutes duration. Blank and control were prepared in similar manner but without illumination and MwCNT(SOD) conjugate respectively. The

inhibition in NBT reduction was measured at 560 nm and one unit of SOD activity was defined as the amount of enzyme producing 50% inhibition of NBT reduction under assay condition. SOD enzyme stability for MwCNT(SOD) conjugate was analyzed under different time duration of treatment where the synthesized conjugate was shaken at 100 rpm at room temperature. Aliquots from the sample were taken periodically and analyzed for SOD activity as detailed above. Temperature and pH optima/ stability were also determined for the synthesized MwCNT(SOD) biocatalytic conjugate. Temperature analysis was done in the range of 10 - 90 °C while pH characterization was analyzed in the range of pH 2-12 using riboflavin mediated NBT reduction method.

2.2.24 Cytocompatibility of MwCNTs, oxidized MwCNTs and MwCNT(SOD) biocatalytic conjugate on human skin HaCat cells.

Cytocompatibility analysis of unrefined (raw) MwCNTs and oxidized MwCNTs onto human skin HaCat cells were studied. Human skin HaCat cells ($2.5x10^4$ per well) were seeded for overnight in 96 well micro plate. The cells were incubated with unrefined MwCNTs and oxidized MwCNTs separately in concentration ranging from minimum 1 µg/ml to maximum 500 µg/ml prepared in DMEM/F12 (1:1 mixture) culture media without serum from a stock dispersion of 1 mg/ml. Cytocompatibility of synthesized MwCNT(SOD) biocatalytic conjugate was also analyzed onto normal human skin HaCat cells in a concentration range of 1 µg/ml to maximum 200 µg/ml separately. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed after the respective incubation period to determine the percentage (%) cell viability (*Mosmann, 1983*). In brief, the medium was removed after desired time period of incubation and HaCat cells were washed twice with PBS. After addition of 100µl of MTT (0.5 mg/ml), the 96 well micro plates were incubated for another 4 hour at 37 °C. MTT was removed thereafter without disturbing formazan crystals formed. DMSO (100 µl) was added to dissolve the purple colored formazan crystal and absorbance was taken at 570 nm in micro plate reader. Absorbance value obtained at 570 nm is a measure of viability of cells and the data was presented in terms of percentage (%) cell viability in comparison to untreated cells which was considered to be 100% viable.

2.2.25 Optimizing oxidative stress condition using H_2O_2 in human skin HaCat cells

H_2O_2 was used as external source of oxidative stress inducing factor in HaCat cells, and condition was optimized to induce sufficient oxidative stress that would not cause excessive cell death (<75 %). Human skin HaCat cells (2.5×10^4 per well) were seeded overnight in 96 well plates in DMEM/F12 (1:1 mixture) with 10 % FBS. The effect of the different concentration of H_2O_2 on human skin HaCat cells was observed and percentage (%) cell survival was measured after a respective 3, 6, 12, and 24 h incubation period. This study was undertaken to determine H_2O_2 concentration and incubation period for optimal oxidative stress induction in comparison to untreated cells, leading toward further studies on the antioxidative efficacy of MwCNT(SOD) biocatalytic conjugate and enzyme encapsulated PCL nanosphere. Oxidative stress condition in human HaCat cells were also verified using carboxy-2′, 7′-dichlorodihydro-fluorescein diacetate (carboxy-H_2DCFDA) dye. After the exposure of H_2O_2 (1 mM), cells were incubated with carboxy-H_2DCFDA (10 μM) for 30 min at 37 °C and collected for FACS analysis (*Saha et al., 2009*).

2.2.26 Antioxidative effect of MwCNT(SOD) biocatalytic conjugates against H_2O_2 induced oxidative stress

Superoxide dismutase (SOD) was covalently immobilized onto oxidized MwCNTs using diimide activated amidation reaction chemistry. A stock dispersion (1 mg/ml) of synthesized MwCNT(SOD) biocatalytic conjugate was made and appropriately diluted further in the range of 0.1 μg/ml to 100 μg/ml accordingly. Human skin keratinocytes, HaCat cells seeded overnight ($2.5x10^4$ per well) in 96 well micro plate were exposed to H_2O_2 (1mM) for optimal oxidative stress induction. These HaCat cells were further analyzed for antioxidative response of synthesized MwCNT(SOD) conjugates. Oxidative stress induced HaCat cells were incubated with respective concentration of synthesized MwCNT(SOD) conjugate i.e 0.1 μg/ml to 100 μg/ml. Treatment at higher concentration of 1 μg/ml for varying time period of 12, 24 and 48 hours was also performed separately. MTT assay was performed thereafter as described earlier and absorbance value at 570 nm as a measure of percentage (%) cell survival was recorded in micro plate reader. Control analysis was done for cells without any treatment and also with only 1mM H_2O_2 treatment respectively.

2.2.27 Reactive oxygen species (ROS) analysis post MwCNT(SOD) conjugate treatment in HaCat cells

Reactive oxygen species level was also analyzed in human skin HaCat cells post MwCNT(SOD) biocatalytic conjugate treatment. The analysis was done using carboxy-2′, 7′-dichlorodihydro-fluorescein diacetate (carboxy-H_2DCFDA), a ROS specific dye. In brief, H_2O_2 induced oxidative stress human in skin HaCat cells were treated with MwCNT(SOD) conjugate at specific concentration of 10 µg/ml for 24 hour time duration. Post treatment, cells were incubated with carboxy-H_2DCFDA dye (10µM) for 30 minutes at 37 °C and thereafter collected for fluorescence analysis. Both, untreated HaCat cells and oxidative stress induced HaCat cells were also anylzed for reactive oxygen species using ROS specific carboxy-H_2DCFDA dye separately.

2.2.28 Superoxide dismutase (SOD) activity analysis post MwCNT(SOD) conjugate treatment in HaCat cells

Antioxidative enzyme superoxide dismutase (SOD) activity was analyzed in HaCat cells post treatment with MwCNT(SOD) biocatalytic conjugate. H_2O_2 induced oxidative stress optimized human skin HaCat cells ($2.5x10^5$ per well in 6 well plate) were incubated with MwCNT(SOD) biocatalytic conjugate at concentration of 10 µg/ml for 24 hour time period. Cells were washed twice with PBS, detached using trypsin (1X) and centrifuged at 5,000 rpm, 4 °C into 1.5 ml tubes. HaCat cells pellet obtained were dissolved in adequate volume of cell lysis solution prepared in PBS buffer containing 1% Triton-X100, 10 mM sodium tetrathionate as protease inhibitor. The cell lysate prepared was centrifuged for 5 minutes at 10,000 rpm, 4 °C. The clear supernatant obtained was further subjected to determination of protein concentration and superoxide dismutase activity. Superoxide dismutase activity was determined by riboflavin mediated NBT reduction method as described in materials and methods section. Superoxide dismutase activity in untreated HaCat cells and H_2O_2 induced oxidative stress HaCat cells were also analyzed separately.

2.2.29 Formulation of antioxidative enzyme-loaded PCL nanospheres

PCL nanospheres encapsulating SOD and CAT were prepared by double emulsion (w/o/w) solvent evaporation technique with a minor modification (*Coccoli et al., 2008*). In brief, SOD and CAT in defined concentration (1:1 mM ratio) were mixed with 1 mL of

PBS (pH 7.4) containing 2% PVA in internal aqueous phase and emulsified for 1 min with 5 mL of DCM containing 3% PCL using ultra-sonicator. The first emulsion (w/o) was administered dropwise into 100 mL of PBS containing 1% PVA in external aqueous phase under magnetic stirring at 1,200 rpm for producing a double w/o/w emulsion and instantly subjected to sonication for 30 s. To hasten the evaporation of organic solvent, another 200 mL of external aqueous phase solution was added dropwise continuously and stirred at 1,200 rpm until complete evaporation of organic solvent is assured. The double emulsion (w/o/w) prepared nanospheres were centrifuged at 5,000 rpm, resuspended in sterile PBS buffer, and stored at 4 °C. PCL nanospheres were also synthesized in a similar manner without SOD and CAT to serve as blank.

2.2.30 Size and morphology analysis of synthesized polycaprolactone nanosphere

Size and morphology of the polycaprolactone (PCL) nanosphere was determined by dynamic light scattering (DLS) as well as field emission scanning electron microscope (FE-SEM) imaging. The synthesized nanosphere were adequately resuspended in PBS buffer and subjected to DLS for size and polydispersity index (PDI). For FE-SEM imaging, a drop of synthesized nanosphere suspension was vacuum dried on aluminum foil overnight and subjected to gold plating after mounting onto a metal stab sealed with carbon tape. FE-SEM imaging of nanosphere were done using Sigma Carl-Ziess microscope operating at 4 kV ETH with magnification at ×33 K and ×132 K, respectively.

2.2.31 FTIR characterization of synthesized polycaprolactone nanosphere

To confirm the antioxidative enzyme encapsulation through polycaprolactone (PCL) nanosphere, Fourier transform infrared spectroscopy (FTIR) using UNICAM Mattson 1000 FTIR spectrophotometer was done. FTIR spectroscopy was performed on free SOD, CAT and blank polycaprolactone nanosphere and compared with PCL(SOD), PCL(CAT), and PCL(SOD+CAT) synthesized nanosphere. The samples were prepared following KBr disc methodology where respective samples were blended with KBr powder and compressed to form disc under pressure of 5 tons before being placed in sample holder. Spectral scanning was done within 400 to 4,000 cm^{-1} and analyzed further.

2.2.32 Encapsulation efficiency, *in vitro* enzyme release and activity studies

Determination of encapsulation efficiency was done by indirect method, *i.e.* determining the total amount of protein added into the formulation and subtracting the amount which was not encapsulated (remains leftover in supernatant solution). For this, supernatant and washed solution during the course of nanosphere formation was analyzed for protein concentration (*Davda and Labhasetwar, 2002*). Determination of *in vitro* protein release from encapsulated PCL nanosphere was also performed where synthesized PCL nanosphere was diluted up to 20 mL with PBS (pH 7.4) and kept in orbital incubator shaker at 37 °C at 60 rpm. At respective time interval, 2 mL of aliquot was replaced with PBS, centrifuged at 5,000 rpm to pellet out nanosphere, and stored at 4 °C for analysis of protein content and SOD and CAT activities.

2.2.33 Cytocompatibility of synthesized PCL nanosphere with human skin HaCat cells

Human skin HaCat cells (2.5×10^4 per well) seeded overnight in 96-well plates were incubated with blank PCL nanosphere in concentration ranging from minimum 10 μg/mL to maximum 1,000 μg/mL prepared in DMEM/F12 (1:1 mixture) culture media without serum from a stock dispersion of 5 mg/mL. In a different set of experiment, HaCat cells were incubated with blank PCL nanosphere at higher concentration of 500 μg/mL for different periods (6, 12, and 24 h) and MTT assay was performed thereafter (*Mosmann, 1983*). In brief, the medium was removed after a desired period of incubation and washed twice with PBS. After addition of 100 μL of MTT (0.5 mg/mL), the plate was incubated for another 4 h at 37 °C and was removed thereafter without disturbing formazan crystals formed; 100 μL of DMSO was added to dissolve the purple colored formazan crystal and absorbance was taken at 570 nm in 96-well microplate reader. The absorbance value obtained at 570 nm is a measure of viability of cells, and the data were calculated in terms of percentage cell survival in comparison to untreated cells (the value obtained was considered as 100 % viability).

2.2.34 Antioxidative effect of PCL(SOD+CAT) nanospheres against oxidative stress

Biodegradable PCL nanospheres were synthesized encapsulating SOD, CAT, and (SOD+CAT) independently by double emulsion (w/o/w) solvent evaporation technique,

as described earlier. A stock dispersion (5 mg/mL) of PCL(CAT), PCL(SOD), and PCL(SOD+CAT) nanospheres were made and appropriately diluted further in the range of 25 to 1,000 μg/mL accordingly. Human skin HaCat cells (2.5×10^4 per well) seeded overnight in 96-well plates were exposed with H_2O_2 (1 mM) for inducing oxidative stress condition, as optimized earlier. These HaCat cells were further used for analyzing antioxidative response of PCL(CAT), PCL(SOD), and PCL(SOD+CAT) nanospheres. Treatments to cells were given at respective concentration of specified PCL nanosphere, i.e. 25 to 1,000 μg/mL and at a higher concentration of 500 μg/mL for varying periods of 6, 12, and 24 h. MTT assay was done thereafter as described earlier and absorbance value at 570 nm as a measure of cell survival was recorded in 96 well microplate reader. MTT assay was also performed subsequently for cells without any treatment (to be taken as control) and with only 1 mM H_2O_2 treatment, respectively. A similar set of experiment was performed separately where H_2O_2-exposed oxidative stress human skin HaCat cells were incubated with CAT, SOD, or (SOD+CAT) protein solution instead of PCL encapsulated form at concentration ranging from 0.01 nM with 10-fold subsequent increase of up to 1 μM. HaCat cells were also incubated under different periods of 6, 12, and 24 h at protein concentration of 0.1 μM and MTT assay as a measure of percentage (%) cell survival was performed after each incubation period respectively.

2.2.35 SOD and CAT activities after PCL(SOD+CAT) nanosphere treatment

SOD and CAT activity was determined for H_2O_2 exposed (1 mM) human skin HaCat cells (HaCat cells under oxidative stress) treated with PCL(SOD+CAT) nanosphere. A six well plate was overnight seeded with human skin HaCat cells (2.5×10^5 per well) and subsequently incubated with H_2O_2 (1 mM) for optimal oxidative stress induction. Furthermore, the cells were treated with PCL(SOD+CAT) nanosphere at concentration of 500 μg/mL. After different time duration of incubation (6, 12, and 24 h), cells were washed twice with PBS, detached with trypsin (1 X), and were centrifuged at 5,000 rpm, 4 °C into 1.5 mL tubes. The pellet was dissolved in adequate volume of cell lysis solution prepared in PBS buffer containing 1 % Triton-X100, 10 mM sodium tetrathionate as protease inhibitor. The lysate was centrifuged for 5 min (10,000 rpm and 4 °C), and the clear supernatant was subjected to protein content determination, SOD and CAT activities. SOD activity was measured by riboflavin mediated NBT reduction

method (*Giannopolitis & Ries, 1977*). The inhibition in NBT reduction was measured at 560 nm and one unit of SOD activity was defined as the amount of enzyme producing 50% inhibition of NBT reduction under assay condition. CAT activity was determined by the decomposition of H_2O_2 and spectroscopic measurement at 240 nm. Reaction mixture of 3 mL was prepared in PBS (pH 7.4) having 100 mM H_2O_2 concentration and 50 µL of cell lysate was added to start the reaction. The decrease of H_2O_2 monitored at 240 nm was used to quantify CAT activity (*Beers & Sizer, 1952*). Protein concentration throughout the experiment was determined by the method of Bradford (*Bradford, 1976*).

Chapter III

Results and Discussion*

Abstract

We have studied the antioxidative enzymatic response of *Cicer arietinum,* a major legume crop plant, under aluminium metal as external stress factor. It has been observed that aluminium metal induced oxidative stress condition in germinating chickpea plant, leads towards change in the enzymatic antioxidative response. Further, we have purified a novel superoxide dismutase enzyme (SOD) from *Cicer arietinum* seedlings induced under aluminium metal stress. The purified SOD was extensively characterized for its different physiochemical parameters. The purified enzyme was estimated to be of high molecular weight, dimeric Cu-Zn SOD with broad pH, temperature optima and high substrate specificity. The unique characteristics of purified SOD motivated us to explore its therapeutic potentials towards scavenging superoxide anion radicals and to overcome induced oxidative stress conditions. Multiwalled Carbon Nanotube (MwCNTs) immobilized novel SOD from *Cicer arietinum* was studied. Furthermore, biodegradable polycaprolactone (PCL) nanospheres encapsulating antioxidative SOD isolated from *Cicer arietinum* together with commercially available catalase (CAT) enzymes was also evaluated. The study indicates that the aluminium metal stress induced SOD enzyme from *Cicer arietinum* may have therapeutic potentials in overcoming pathological conditions resulting from reactive oxygen species due to various environmental pollutants.

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2. Protein and Peptide Letters. 20, 741-748, 2013.
3. Brazilian Journal of Plant Physiology. 24, 47-54, 2012.
4. Submitted for Publication

3.1 ALUMINIUM METAL STRESS INDUCED ANTIOXIDATIVE ENZYMATIC ANALYSIS IN GERMINATED *CICER ARIETINUM* SEEDLINGS

Increase in human population demands more agricultural food productivity. The current prevailing pattern of agricultural practice and its surrounding environment needs to be critically reviewed for maintaining sustained agricultural practices. The continuous changing environmental conditions play a major role in the agricultural production. Environmental pollution is on continuous rise with increase in industrialization, inappropriate disposal of wastes as well as increase usage of insecticides and pesticides on the agricultural lands. Rapid growth of industrialization, multifarious anthropogenic activities, urban development, inappropriate channeling of industrial effluents, mass consumption of toxic chemicals and fertilizers etc, are the major contributor to this increased environmental pollution surrounding the agricultural habitat *(Schwartz et al., 2001; Bell et al., 2001; Passariello et al., 2002)*. Towards agricultural food crops and germinating plants, the abiotic stress in terms of metal toxicity remains unavoidable with changing environmental conditions where toxicity in agricultural land is increasing. Among the different toxic metals, aluminium, in toxic Al^{3+} ionic form is one of the most common pollutants found in acidic soils. Effects of aluminium on few plants have been previously studied by Delhaize and Ryan (1995) indicating its toxicity in germinating plants. However, not much study has been reported on *Cicer arietinum.* We have analyzed variations in the antioxidative response of *Cicer arietinum* seedlings due to aluminium (Al^{3+}) toxicity.

3.1.1 Physiological response of *Cicer arietinum* seeds under aluminium metal treatment

In the present work, toxic effects of aluminium on chickpea (*Cicer arietinum*) were studied. Aluminium (Al^{3+}) treatment was found to inhibit seedlings germination and decreased root/shoot length (Figure 3.1.1). Interestingly, shooting was affected at much higher concentration. In rice and wheat varieties, aluminium (Al^{3+}) toxicity was also reported to casue growth retardation in root and shoot (*Shri et al., 2009; Liu et al.,2005*). It has been hypothesized that metal stress toxicity induces the inhibition of cell division on shoots (*Pan et al., 2001*). Rooting gets inhibited due to direct contact with metals in nutrient media which interfere with normal cellular division and growth pattern of germinating crop plants.

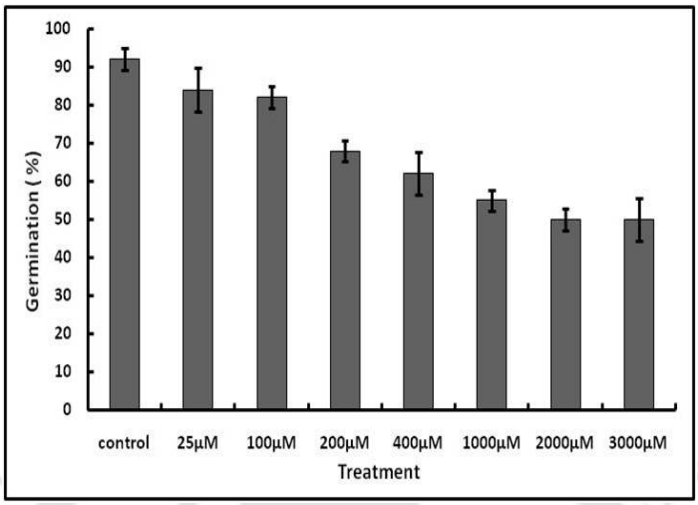

Figure 3.1.1.1 Germination (%) percentage of chickpea seeds under Al^{3+} stress. Germination was considered when plumule and radical grown over 10 mm long and counted on 8th day after treatment. Results are mean values of multiple experiments and plotted in form of seed response ± standard deviation.

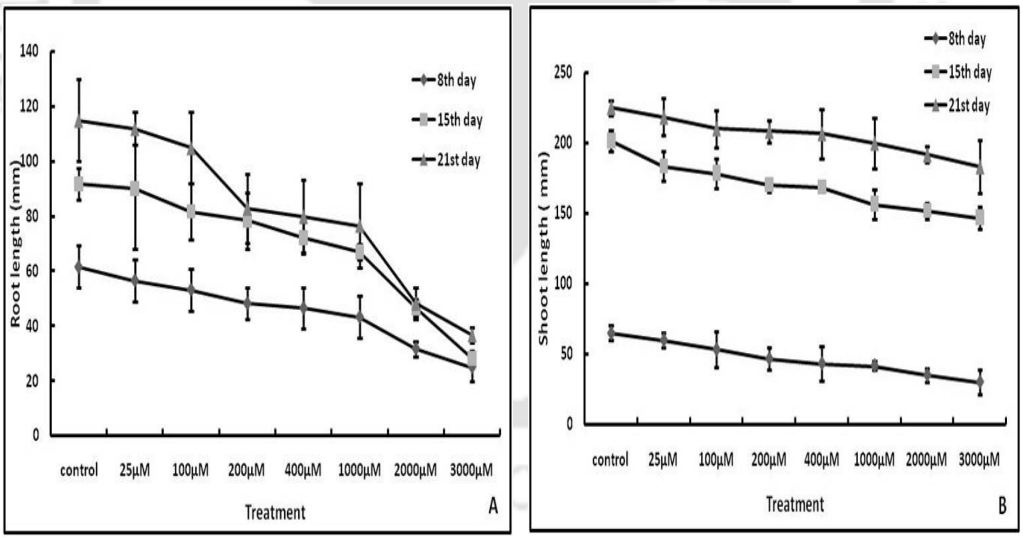

Figure 3.1.1.2 Graph showing **(A)** root and **(B)** shoot length of Al^{3+} treated chickpea seeds. Data obtained on 8^{th}, 15^{th} and 21^{st} day are the mean values of three randomly selected samples and plotted in form of root length ± standard deviation.

3.1.2 Superoxide dismutase activity analysis

Higher concentration of toxic metal concentration intensifies oxidative stress condition which gets counteracted by an efficient antioxidative defense system consisting of ROS scavenging enzymes (*Liu et al., 2007*). SOD is the primary enzyme acting as a first line of defense involved with the dismutation of superoxide radicals (O_2^-) into H_2O_2 and O_2. SOD response was collected on 8th, 15th and 21st day of germination for root and shoot. Increased SOD activity was observed at lower aluminium concentration of 25, 100 and 200 µM (Figure 3.1.2A & B). The initial increase in SOD activity was consistent with reports on maize and barley, in which heavy metal stress leads to increase in SOD activity with respect to control (*Boscolo et al., 2003; Guo et al., 2004*). However beyond 100 µM, a decrease in SOD activity in chickpea seedlings was observed in roots and shoots over a longer exposure time period. As SOD activity was decreased with various aluminium concentrations over a long time, this might be possible due to high concentration of H_2O_2 produced via an unknown pathway affecting SOD induction. Arsenic toxicity in crop plants has been observed to decrease SOD activity while maintaining POD activities (*Gunes et al., 2009*), which might indicate that high levels of H_2O_2 were produced by another pathway. Similar conclusions on SOD activities of getting impaired at higher aluminium concentration, as much as 3.5 mM had also been reported previously (*Sahu et al., 2010*). In our subsequent studies, we have also found lower activity of purified SOD at higher concentration of aluminium. Thus, the lower activity of SOD at higher concentration of aluminium is likey to be due to inhibition of SOD due to aluminium.

3.1.3 Ascorbate peroxidase activity analysis

The initial high activity of SOD leads to accumulation of H_2O_2 and as a result, higher H_2O_2 concentration induces different peroxidases such as APX and GPX to balance its level (*Olmos et al., 2003*). Ascorbate peroxidase activity was found to be increased under aluminium stress in both root and shoot (Figures 3.1.3A & B) initially, with high activity in root. In case of shoots, APX activity gets saturated later which is in accordance with decrease in SOD activity. The APX activity in roots showed increase from 8th day to 21st day while gradually decreasing at higher aluminium concentration i.e. above 200 µM. High APX activity in root was in consistence with other reports (*Schutzendubel et al., 2001*) and its pattern was synchronous to SOD activity in roots.

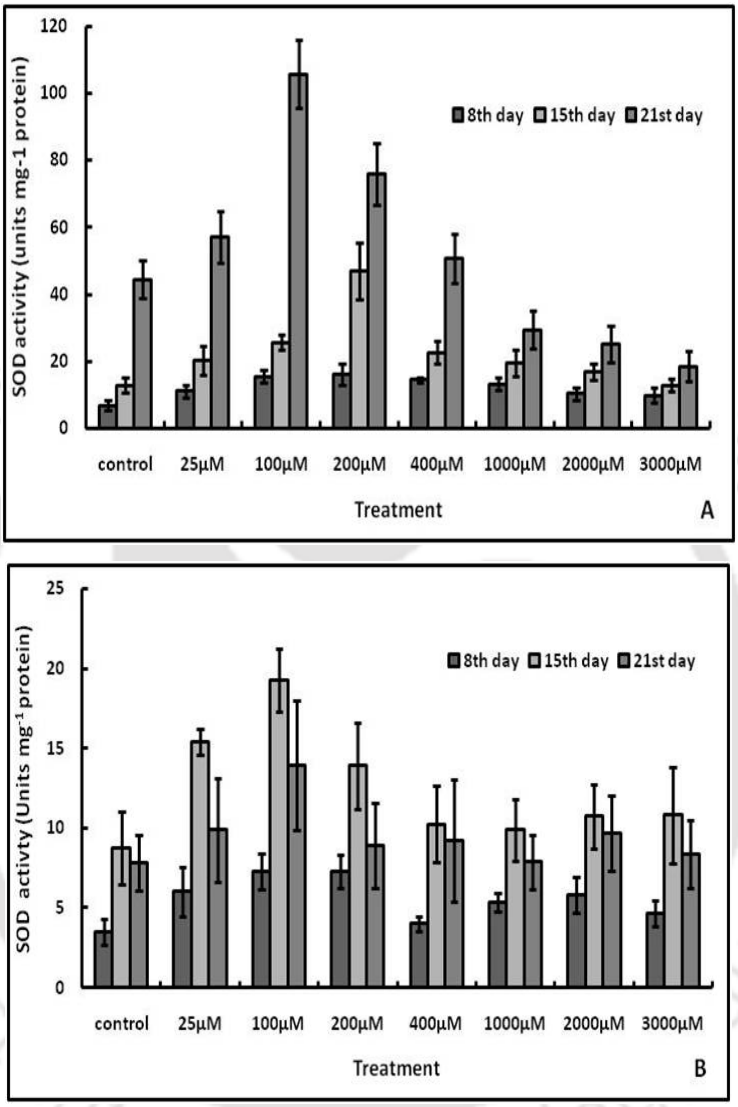

Figure 3.1.2 Superoxide dismutase is one of the most important antioxidative enzyme which forms the first line of defese against increased oxidative stress. Superoxide dismutase activities of **(A)** root and **(B)** shoot samples of chickpea seeds germinated under Al^{3+} stress. Assay performed by the NBT reduction method. Results obtained are mean values of multiple experiments ± standard deviation.

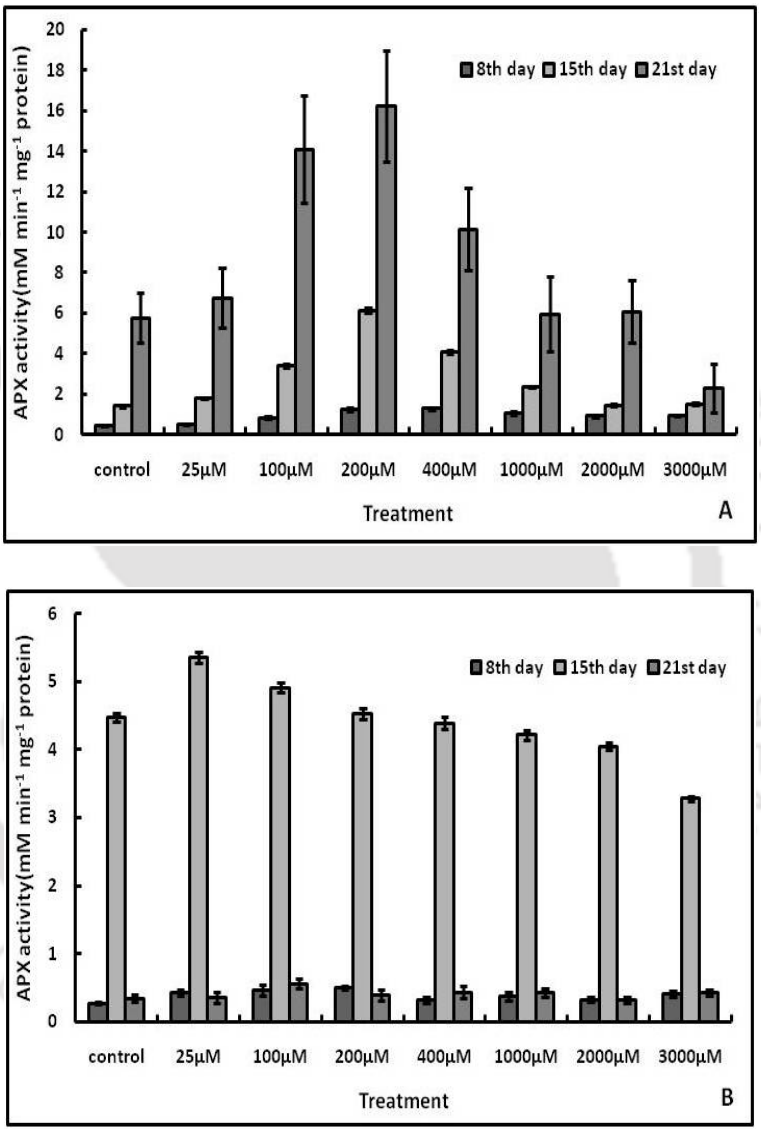

Figure 3.1.3 Ascorbate peroxidase is an important enzyme of antioxidative defense system which scavenges hydrogen peroxide. Ascorbate peroxidase activities of **(A)** root and **(B)** shoot samples of Al^{3+} treated chickpea seeds. The assay includes oxidation of ascorbate, a substrate for APX enzyme activity. Results are presented as mean values of multiple experiments ± standard deviation.

3.1.4 Guaiacol peroxidase activity analysis

Guaiacol peroxidase is another antioxidative enzyme which hydrolyzes H_2O_2 and depletes oxidative load generated. In case of root (Figure 3.1.4A), an increase in guaiacol peroxidase activity has been observed with increasing aluminium concentrations and in a similar manner to other antioxidative enzymes, however at higher metal concentration it gets saturated. The 21st day response reveals saturated guaiacol peroxidase activity in all treatments under metal stress which is due to high oxidative load and thus leading to its inhibition. The duration of treatment with increasing metal concentration induced high oxidative load onto germinating plant, leading to saturation of antioxidative enzymes.

3.1.5 Lipid peroxidation analysis

Lipid peroxidation estimates membrane damage under oxidative stress and malondialdehyde (MDA), a product of lipid peroxidation is used as indicator for oxidative damage. Result indicates increase in malondialdehyde concentration with increasing aluminium treatment both in roots as well as in shoots (Figures 3.1.5A & B). Malondialdehyde concentration was found to be higher in root which indicates higher peroxidation effect due to direct contact with heavy metals and thus the overall increase in malondialdehyde concentration indicates oxidative damage. Increase in antioxidative activity of superoxide dismutase, ascorbate peroxidase and guaiacol peroxidase saturates when damage to lipid peroxidation is high, as observed in case of 15th day data. Similar response under heavy metal stress has been reported in mangrove plant seedlings (*Zhang et al., 2007*).

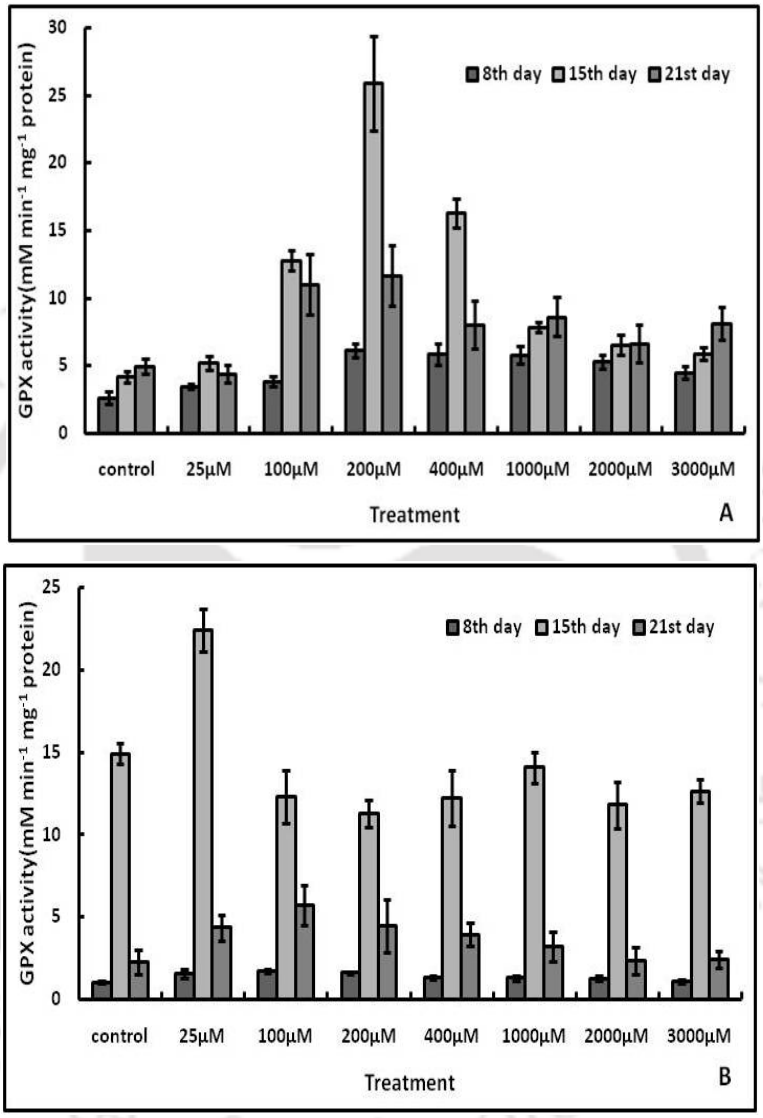

Figure 3.1.4 Peroxidase enzymes are present throughout the cellular compartments effectively scavenges hydrogen peroxide induced under oxidative stress conditions. Guaiacol peroxidase activities of **(A)** root and **(B)** shoot samples under Al^{3+} stress in chickpea seeds. Results are presented as mean values of multiple experiments ± standard deviation.

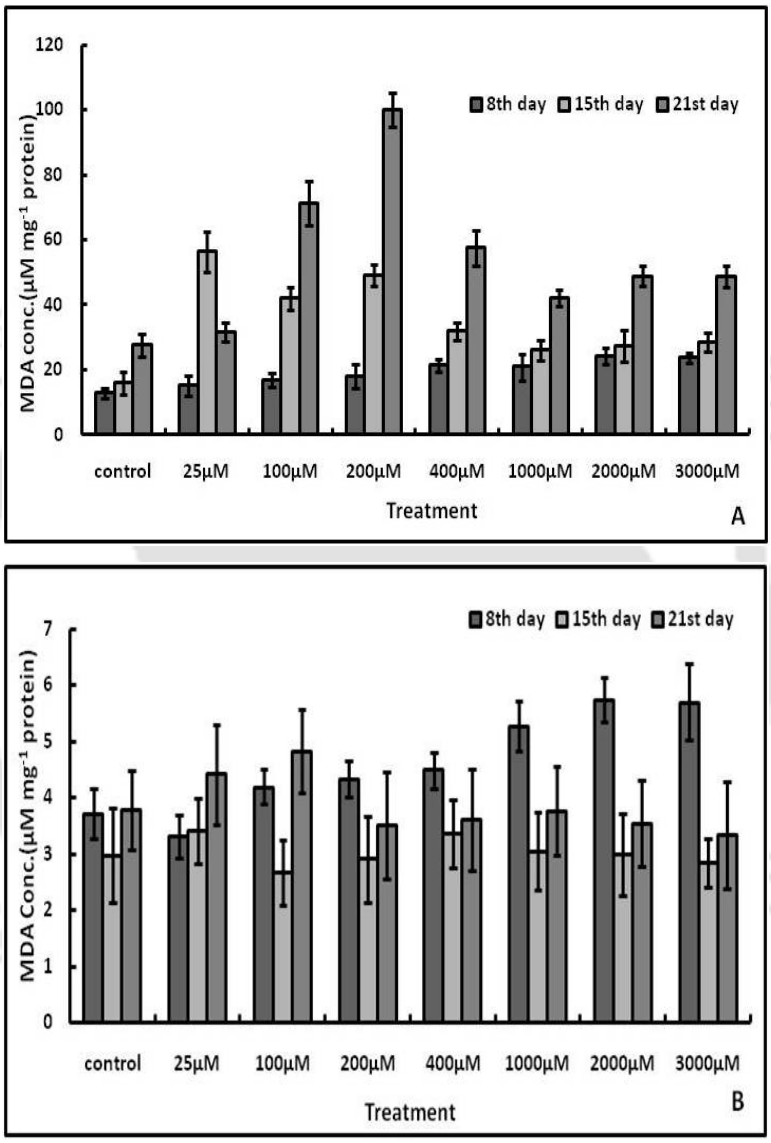

Figure 3.1.5 Lipid peroxidation estimates extent of oxidative damage induced under stress conditions. Graph showing malondialdehyde concentration **(A)** root sample and **(B)** shoot sample of Al^{3+} metal treated chickpea seeds. Lipid peroxidation was determined by the thiobarbituric acid (TBA)-trichloro acetic acid (TCA) method (*Dazy et al., 2009*). Results obtained are mean values of multiple experiments ± standard deviation.

3.2 ISOLATION, PURIFICATION AND CHARACTERIZATION OF ALUMINIUM METAL STRESS INDUCED, A NOVEL SUPEROXIDE DISMUTASE PROTEIN FROM *CICER ARIETINUM* SEEDLINGS

Superoxide dismutase (SOD, EC. 1.15.1.1), a group of metalloenzymes, has an essential role in anti-oxidative defense system against generated oxidative stress (*Mittler, 2002*). SOD enzyme acting as the very first line of defense and play important role by converting superoxide radicals to lesser toxic H_2O_2, which further gets converted to H_2O by other antioxidative enzymes such as catalases and peroxidases (*Giannopolitis and Ries, 1977*). Based on its metal cofactor, SOD enzymes had been classified into three distinct groups which are Cu-Zn SOD, Fe-SOD and Mn-SOD. These SODs are widely distributed among the prokaryotes and eukaryotes. Out of all these, Cu-Zn SOD is the most prevalent form present mainly in prokaryotes as well as in chloroplast and eukaryotic cytoplasm (*McKersie et al., 1993*). Superoxide dismutase obtained from different sources has wide range of physicochemical properties and always had been subject of widespread interest for numerous applications (*Bafana et al., 2011*). Major applications of SOD is related to its higher expression in transgenic plants leading towards its high survival rate under oxidative stress conditions as reported in case of transgenic Alfalfa (resistant to low temperature stress) and transgenic tobacco inducing higher germination rates as well as seeds longevity period under stress conditions (*McKersie et al., 1999; Lee et al., 2010*). There are several studies for superoxide dismutase purification and characterization from plant sources (*Sheng et al., 2004; Hadji et al., 2007; Lai et al., 2008*). The presented report is about isolation, purification and characterization of a novel superoxide dismutase from *Cicer arietinum* seedlings induced under aluminium metal as external abiotic stress factor.

3.2.1 Purification of superoxide dismutase from *Cicer arietinum* seedlings

Superoxide dismutase which acts as the very first line of defense against generated oxidative stress was purified to homogeneity from 10-12 days old chickpea seedlings germinated under Al^{3+} metal stress in Hoagland's nutrient solution. The entire purification includes three sequential steps (ammonium sulphate fractionation, ion exchange chromatography and gel filtration chromatography). The crude supernatant was extracted by grinding the seedlings in ice chilled 10 mM Tris-HCl buffer (pH 8.0) and the clear supernatant was subjected to ammonium sulfate fractionation. The precipitate

obtained within 50-90% saturation showed high SOD activity with 2.3 fold increase in specific activity (Table. 3.2.1). The precipitate was dissolved in same buffer and dialyzed extensively. The dialyzed sample was loaded on DEAE Sepharose column pre-equilibrated with 10 mM Tris-HCl (pH 8.0) buffer and the bound proteins were eluted with a linear gradient of 0-0.5 M NaCl. The sample was eluted in form of two successive peaks (Figure. 3.2.1A). Most of the SOD activity was confined to the first peak, within 10^{th} to 25^{th} fraction. All the fractions of this peak were pooled and lyophilized. The lyophilized sample was subjected to gel filtration chromatography with Sephacryl S300 as matrix. The sample was eluted in form of a single asymmetrical peak of which the fractions of descending arm (in between the arrows) were found to be homogeneous (Figure. 3.2.1B). These homogenous fractions were pooled and stored at 4 °C for further experiment. The purification results showing total protein content, specific activity, fold purification and percentage recovery were summarized in Table 1. The specific activity of purified SOD was increased to 28 fold (158 units/mg) in comparison to crude extract (Table. 3.2.1)

Table 3.2.1 Purification table of superoxide dismutase (SOD) from Chickpea (*Cicer arietinum*). The enzyme was purified with ~28 fold purification

Procedure	**Total Protein (mg)**	**Total Activity (units)**	**Specific Activity (units/mg)**	**Fold Purification**
Crude sample	267.80	1499.68	5.6	1
Ammonium sulfate fractionation (50%-90%)	58.85	759.165	12.9	2.3
DEAE Sepharose Ion exchange chromatography	7.9	697.57	88.3	15.76
Gelfiltration chromatography	0.692	108.99	157.5	28.125

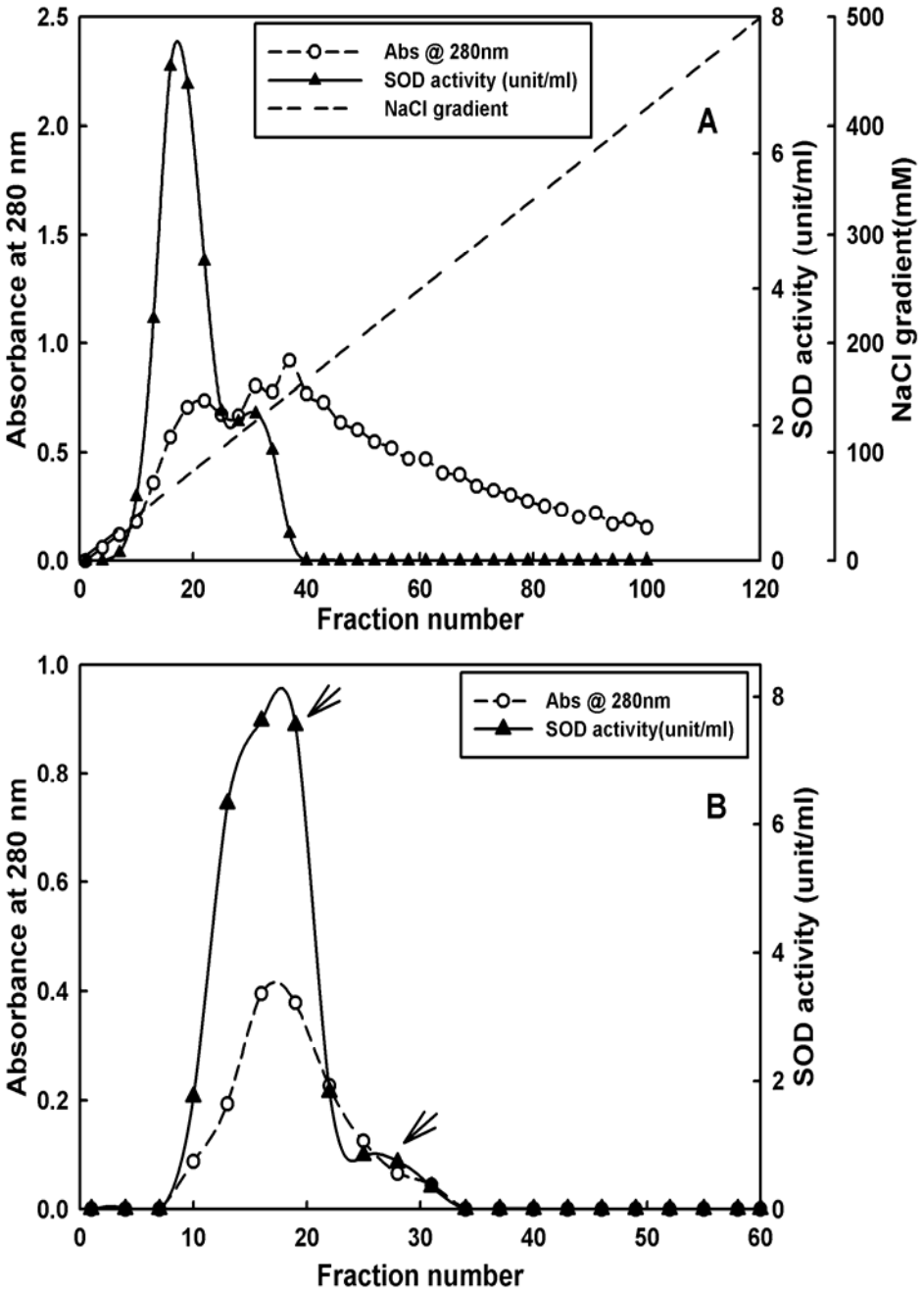

Figure 3.2.1 **(A)** Elution profile of 50% ammonium sulfate supernatant on DEAE Sepharose column. Bound proteins were washed with equilibration buffer and then eluted with linear salt gradient in range of 0.0-0.5M NaCl. Fractions of 6 mL were collected. **(B)** Elution profile of first peak of DEAE Sepharose column on Sephacryl S300 gel filtration chromatography column. The flow rate was 1 mL/min and fractions of 3 mL were collected. In both cases the fractions were assayed for protein content and SOD activity.

3.2.2 Molecular weight determination

The purified SOD enzyme showed single band when subjected to SDS-PAGE analysis in coordination with standard protein molecular weight markers (Figure. 3.2.2A) confirming the homogeneity of enzyme. The approx molecular weight of purified SOD enzyme was found to be 33.5 kDa based on relative mobility (Rf) versus log molecular weight (Log Mw) graph (Figure. 3.2.2B). This was further confirmed by the MALDI-TOF mass spectrum as the m/z ion lies at 33.272 kDa (Figure 3.2.2C) which corresponds to the monomeric subunit molecular mass of purified SOD approximated to be 33.5 kDa in SDS PAGE. The molecular weight of purified native SOD enzyme was approximated to be 67 kDa based on native gel electrophoresis in comparison with bovine serum albumin (BSA) taken as standard marker (Figure 3.2.3A). All the above results confirm the homodimeric nature of this purified SOD enzyme.

3.2.3 Native PAGE and superoxide dismutase NBT-zymography staining

Native polyacrylamide gel electrophoresis (PAGE) and NBT activity staining were performed. The molecular weight of purified native SOD enzyme was approximated to be 67 kDa based on native gel electrophoresis in comparison with bovine serum albumin (BSA) taken as standard marker (Figure 3.2.3A). The detection of prosthetic group of purified SOD enzyme was done by native-gel nitro-blue tetrazolium (NBT) activity staining in presence and absence of H_2O_2 which acts as inhibitor for Cu-Zn SOD. A concentration of 5 mM H_2O_2 completely inhibits SOD activity, validating Cu-Zn as prosthetic group (Figure. 3.2.3 B & C). Different SODs have been purified from various plant sources, such as Cu-Zn SOD isoenzymes and Mn-SOD from pea and corn, SOD from maize (31-33 kDa), Cu-Zn SODs from mungbean (31 kDa) and tobacco (33.2 kDa), respectively (*Ragusa et al., 2001*). Few other SODs with comparatively lower molecular weight having subunit molecular weight in the range of 15-24 kDa have also been purified from garlic (15.5 kDa), tobacco (23 kDa), and soyabean(18 kDa) (*Clarkson et al., 1989*). However the current report indicates presence of nearly a 67 kDa homodimeric Cu-Zn SOD from chickpea and is the highest molecular mass Cu-Zn SOD reported so far.

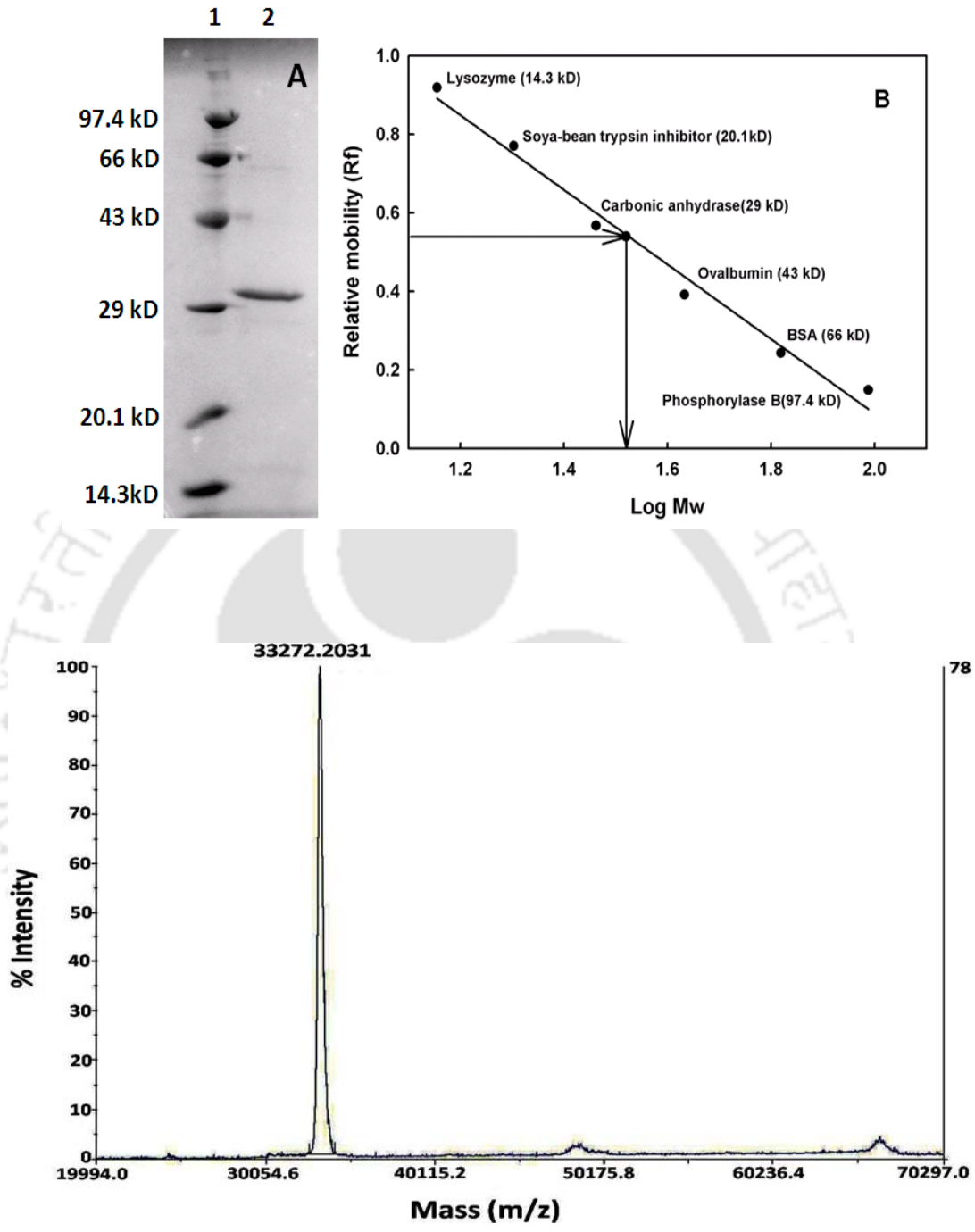

Figure 3.2.2 Purified superoxide dismutase enzyme was subjected to molecular weight determination using different techniques. **(A)** SDS PAGE of protein sample. Lane 1: Protein molecular weight marker. Lane 2: Purified SOD protein. **(B)** Molecular weight determination of purified SOD enzyme by relative mobility (Rf) versus log molecular weight (log MW) plot. **(C)** Represents the MALDI-TOF mass spectrum.

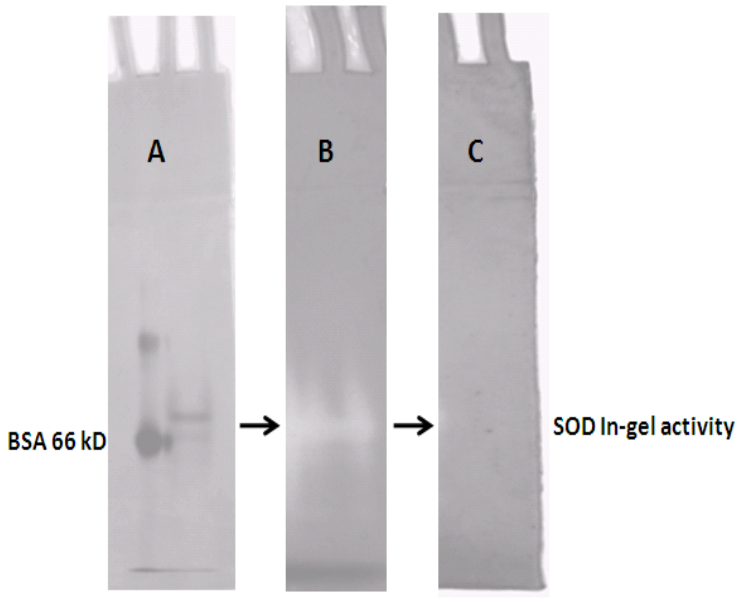

Figure 3.2.3 Determination of native mass (by native PAGE) and prosthetic group (by H_2O_2 inhibition) for purified SOD enzyme. The purified SOD was electrophoresed on 10% native PAGE gel in triplicate and further divided into three sections. **(A)** Coomassie brilliant blue (R-250) staining. Lane 1: BSA, as marker, Lane 2: Purified SOD enzyme. **(B)** SOD zymography by NBT staining showing activity in form of clear zone. **(C)** SOD zymography by NBT staining after H_2O_2 inhibition. Disappearance of clear zone indicates complete inhibition of SOD activity, confirming it as Cu/Zn SOD.

3.2.4 UV-visible absorption spectra and metal content of purified superoxide dismutase

The ultraviolet absorption scan of purified SOD was similar to most of the reported Cu-Zn category of superoxide dismutase (*He et al., 2008*). The UV absorption peak of purified SOD was found to be at 268 nm and accompanied by a broad shoulder at longer wavelength, typical to most of Cu-Zn SOD (Figure 3.2.4). However no absorption zone was observed in the visible range. UV spectral characteristic along with H_2O_2 induced inhibition, sequencing data and Copper-Zinc metal analysis, further validates the purified protein to be Cu-Zn category of SOD.

The copper and zinc metal ions in purified SOD enzymes were detected by Atomic Absorption spectrophotometer. The result of metal content analysis reveals two

atoms of Cu and two atoms of Zn per molecule of enzyme (experimental data showed 1.60 and 2.17 per molecule of enzyme for Cu and Zn, respectively). The presence of Cu and Zn metals validates the Cu-Zn SOD nature of purified protein.

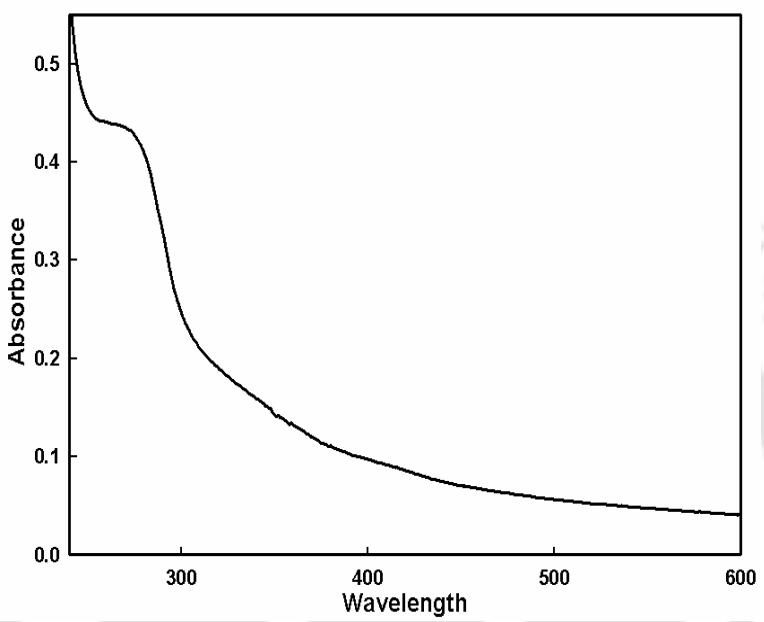

Figure 3.2.4 Ultraviolet absorption spectrum of Cu-Zn superoxide dismutase protein purified from chickpea seedlings. The enzyme was at 0.05 mg/ml concentration in 10mM Tris-HCl (pH 8.0) buffer. The UV absorbance scan of purified SOD had absorbance peak at 268 nm and a broad shoulder at higher wavelength, typical to most of Cu-Zn SODs.

3.2.5 Effects of pH and temperature on purified SOD

The optimum pH for the activity of purified SOD was analyzed in the range of pH 2-12. The enzyme was found to be highly sensitive at low pH as no activity was observed below pH 5.0. The purified SOD had a functional pH range of 6.5-8.5 with more than 70% activity (Figure 3.2.5A). The highest activity was observed at pH 8.0. The pH stability of purified SOD was observed in the range of pH 5-12 (Figure 3.2.5B). As mentioned earlier at lower pH the enzyme was not active and most of the activity was confined mainly in basic range. It retains more than 60% activity upto pH 11. The optimum temperature for the purified enzyme was found to be in the range of 10-30°C

having more than 85% activity (Figure 3.2.5C). The enzyme showed higher temperature sensitivity as after 40°C it retains less than 50% residual activity (Figure 3.2.5D).

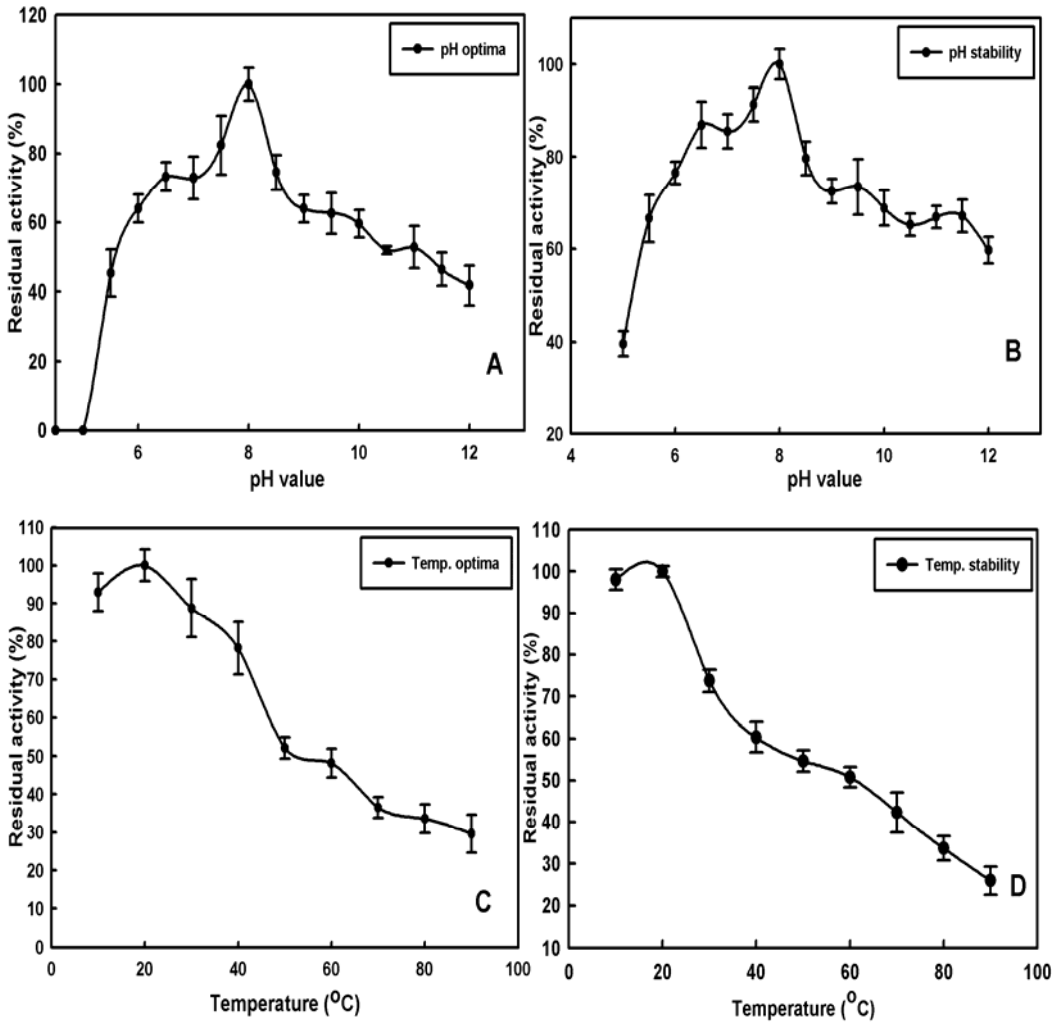

Figure 3.2.5 pH optima **(A)** and stability **(B)** of purified superoxide dismutase. Optimum temperature **(C)** and temperature stability **(D)** of purified superoxide dismutase. For temperature optima, the enzyme was incubated at different temperatures for 15 min and substrates pre-equilibrated at respective temperatures were added to carry out reaction at corresponding temperatures. Stability was determined by overnight incubating the enzyme at room temperature at different pH conditions and next day activity was taken as mentioned in method section For temperature stability measurements, enzyme was incubated at required temperature for 15 min and activity was measured as mentioned in method section.The data presented are mean values of three independent experiments±S.E.

3.2.6 Effect of substrate concentration on superoxide dismutase activity

The effect of increasing substrate concentration reveals that the purified SOD follows Michaelis–Menten kinetics (Figure 3.2.6). The K_m of enzyme was calculated from Lineweaver-Burk plot. The K_m calculated was 10.16 ± 2.5 µM which indicates its high substrate specificity and efficiency in controlling superoxide ion level and overall balancing oxidative stress.

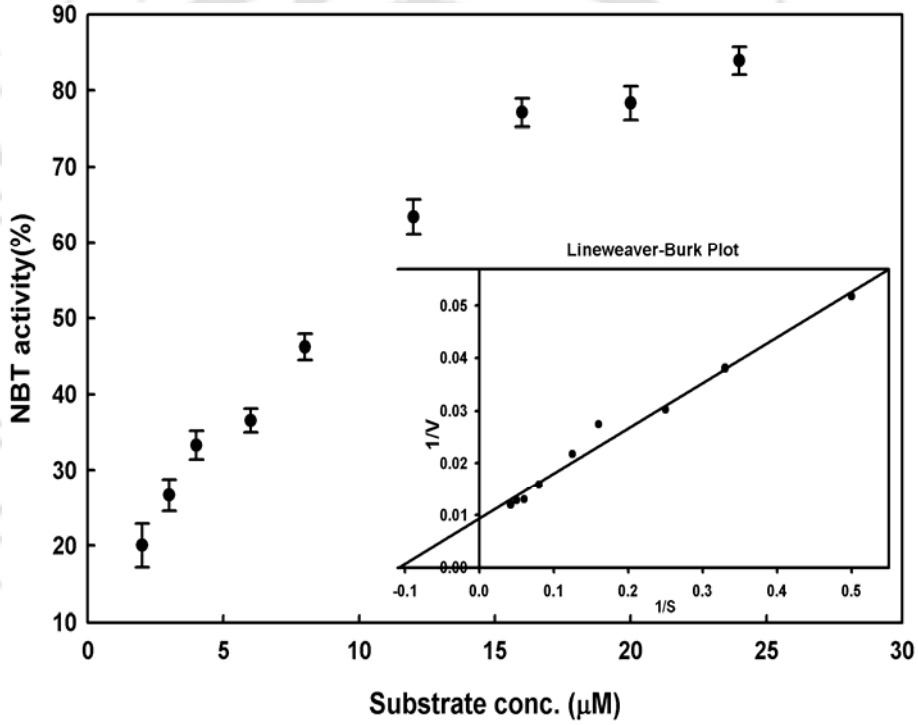

Figure 3.2.6 Effect of substrate concentration on reaction velocity of purified SOD enzyme using riboflavin as substrate. The Km value was calculated with the help of Lineweaver-Burk plot prepared from the NBT activities. The presented results are mean values of three independent experiments with ± S.E.

3.2.7 Effect of metal ions and compounds on superoxide dismutase activity

The SOD belongs to metalloenzymes where the metals (Cu, Fe & Mn) act as prosthetic group and are responsible for the redox reactions. Different metal ions and compounds influence the micro environment of enzyme and thus influence the activity in

either way. Certain metal ions and compounds such as Al^{3+}, Zn^{2+}, Mg^{2+} and $NaNO_3$ inhibit SOD activity at both lower and higher concentration. Superoxide dismutase activity was found to be inhibited upto 50% in presence of 1mM Al^{3+} concentrations, confirming the initial results aluminium induced inhibiton of antioxidative enzyme.

Table 3.2.2 Effect of metal ions and compounds on the activity of purified Superoxide dismutase enzyme.

	Residual activity (%)	
Reagents	**0.1 mM**	**1.0 mM**
Blank	100	100
$MgCl_2$	93.21 ± 2.87	77.90 ± 3.50
$ZnSO_4$	86.43 ± 2.05	66.86 ± 3.25
$NaNO_3$	76.12 ± 4.62	43.92 ± 2.64
H_2O_2	91.43 ± 4.59	22.21 ± 3.07
$Al_2(SO_4)_3$	82.87±3.90	57.43 ± 1.01
EDTA.2Na	102.93 ± 4.30	93.83 ± 4.77
Gn-HCl*	78.21 ± 2.58	66.14 ± 5.24
Urea*	70.08 ± 2.84	59.57 ± 2.37
$HgCl_2$	94.15 ± 3.09	-
$CaCl_2$	109.36 ± 6.37	-

** Concentration of 1 mM and 10 mM was used to study enzyme activity. The data presented are the mean values of triplicate experiment ± standard error.*

3.2.8 N-terminal amino acid sequencing

N-terminal amino acids sequence of purified chickpea SOD shows conserved motifs (VXXXIFF), also found in other plant Cu-Zn SODs (Table 3.2.3) (*Voloudakis et al., 2006; wu et al., 2011*). Although the overall sequence homology was not very high, the amino acids of purified SOD at positions 6, 9 and 13-15 were highly conserved reflecting their importance. High molecular mass of purified SOD together with low sequence identity with other Cu/Zn SODs imparts novelty to this enzyme.

Table 3.2.3 The table shows amino terminal sequence of purified chickpea superoxide dismutase in comparison with other plant Cu/Zn superoxide dismutases. The bold letters shows the conserved residues and X in the sequence of chickpea superoxide dismutase indicates uncertain amino acid residue.

Enzyme	N- terminal sequence (first 15 amino acid residues of chickpea superoxide dismutase in comparison with superoxide dismutases from other sources)															
Chickpea (***Current studies***)	R	Q	I	X	D	**S**	D	**-**	**V**	X	P	I	**I**	**F**	**F**	E
*Zea mays (*Kernodle and Scandalios, 1996)	A	V	L	G	S	**S**	D	G	**V**	K	G	T	**I**	**F**	**F**	T
Rice II (Sakamoto et al., 1992)	A	V	L	A	S	**S**	E	G	**V**	K	G	T	**I**	**F**	**F**	S
Cabagge(Steffens et al., 1986)	A	V	L	N	S	**S**	E	G	**V**	K	G	T	**I**	**F**	**F**	T
Populus (Akkapeddi et al., 1999)	A	V	L	N	S	**S**	E	G	**V**	S	G	T	**I**	**F**	**F**	T
Soyabean (Arahira et al., 1998)	A	V	L	G	S	**S**	E	G	**V**	T	G	T	**I**	**F**	**F**	T
Ipomoea batatas (Lin et al., 1995)	A	V	L	S	S	**S**	E	G	**V**	S	G	T	**I**	**F**	**F**	S
Manihot esculenta (Shin et al., 2005)	A	V	L	N	S	**S**	E	Q	**V**	A	G	T	**I**	**F**	**F**	T
Gossypium hirsutum (Voloudakis et al., 2006	A	V	L	G	S	N	E	G	**V**	S	G	T	V	**F**	**F**	S
Allium sativum (Hadji et al., 2007)	A	V	L	N	S	A	E	G	**V**	K	G	H	V	**F**	**F**	T
Bambusa oldhamii (Wu et al., 2011)	A	V	L	A	S	**S**	E	Q	**V**	K	G	T	**I**	Y	**F**	V

3.3 SUPEROXIDE DISMUTASE-MULTIWALLED CARBON NANOTUBE BIOCATALYTIC CONJUGATE TOWARDS ALLEVIATING INDUCED OXIDATIVE STRESS

Exposure to adverse environmental conditions such as UV ionizing radiations, airborne gaseous environmental pollutants, unwanted xenobiotic compounds induce oxidative stress onto human skins which acts as protective boundary (*Athar, 2002*). Oxidative stress conditions are also induced under several diseased states further elevating its pathogenesis level; however reactive oxygen species (ROS) formation is also an inevitable part of aerobic metabolic reactions. Antioxidative defense system comprises of different antioxidative molecules and enzymes towards scavenging these oxidative radicals (*Sies, 1997).* However, the exogenous supplementation of antioxidant enzymes and molecules will enhance the therapeutic potential towards scavenging these increased levels of reactive oxygen species, maintaining the ionic homeostasis balance and reducing oxidative stress. Developing an efficient drug delivery system is the prime requisite for any therapeutic treatments. Significant advantages has been associated with nanoparticulate system of drug delivery including enhanced cellular and tissue uptake, increased stability in biological environment, sustained release and reduced toxicity being some of the key features (*Panyam & Labhasetwar, 2003*). Among the diverse nanomaterials synthesized, carbon nanotubes (CNTs) possess significant advantages. CNTs nanosize range, exceptional physical and chemical properties add numerous benefits towards its use as drug delivery vectors across the cellular membranes (*Bianco et al., 2005; Kostarelos et al., 2007).* Chemical modification of carbon nanotubes (CNTs) through different functionalization approaches is of key interests and is being explored for conjugating various therapeutic drugs and biomolecules (*Vashist et al., 2011; Pavlidis et al., 2012*). Enzymes have been conjugated through physical adsorption as well as covalent immobilization onto the functionalized CNTs. Covalent immobilization of drugs/ biomolecules and enzymes has certain advantages compared to other form of conjugations as it improve stability, accessibility, selectivity and reduces leaching overall. Diimide-activated amidation reaction chemistry is used for covalently immobilizing the amine group of enzyme used with carboxyl group of functionalized CNTs (*Jiang et al., 2004*). Superoxide dismutase (SOD) is an antioxidative enzyme with high scavenging activity for superoxide anions which are the initiators of oxidative stress. Here, an SOD enzyme purified from plant source in our laboratory *(Singh et al., 2013)*

was covalently immobilized on multiwalled carbon nanotubes (MwCNTs) using diimide activated amidation reaction chemistry. The synthsized MwCNT(SOD) biocatalytic conjugate was characterized and evaluated for its efficiency towards reducing the induced oxidative stress human skin HaCat cells.

3.3.1 Synthesis and characterization of MwCNT(SOD) biocatalytic conjugate

Superoxide dismutase (SOD) enzyme having high scavenging activity for superoxide anion was covalently immobilized onto oxidized multiwalled carbon nanotubes (MwCNTs), leading towards synthesis of MwCNT(SOD) biocatalytic conjugate. MwCNTs were selected as an ideal nanomaterial specifically for its nanosize range and ease in hydrophilic carboxylic acid group formation through acid oxidation, further adding easy aqueous dispersible nature (*Rosca et al., 2005*). Diimide activated amidation reaction was used for covalent immobilization leading to amide bond formation among carboxylic acid group of oxidized MwCNTs and amine group of SOD enzyme (*Jiang el at., 2004*). Figure 3.3.1.1.A shows schematic representation of steps of MwCNT(SOD) biocatalytic conjugate synthesis. Carboxylated MwCNTs were prepared thorough oxidation in mixture of sulfuric acid (H_2SO_4) and nitric acid (HNO_3) (3:1 v/v). Synthesizing carboxylic acid group in CNTs is an ideal choice as this functional group can undergo variety of reaction which can be applied for further conjugation with wide range of drugs/ enzymes and other biomolecules. The diimide activated amidation chemistry is a direct coupling reaction using N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and N-Hydroxysuccinimide (NHS) involving carboxylic acid groups and amine groups. In this two step process, EDAC in presence of NHS leads to formation of an active ester compound i.e succinimidyl intermediate. The active ester formed undergoes nucleophilic substitution reaction with the amine group of SOD enzyme and carboxylic acid group of oxidized MwCNTs, finally resulting in the formation of amide bond. The synthesized MwCNT(SOD) biocatalytic conjugates were found with easy aqueous dispersible nature and completely free of any flocculation even after week (Figure 3.3.1.1.B). The absorbance of MwCNT(SOD) conjugate were found to be varying linearly with increasing concentration at 500 nm and were quantified using extinction coefficient of 3.93×10^4 cm^2/g (*Asuri et al., 2006*) (Figure 3.3.1.1.C).

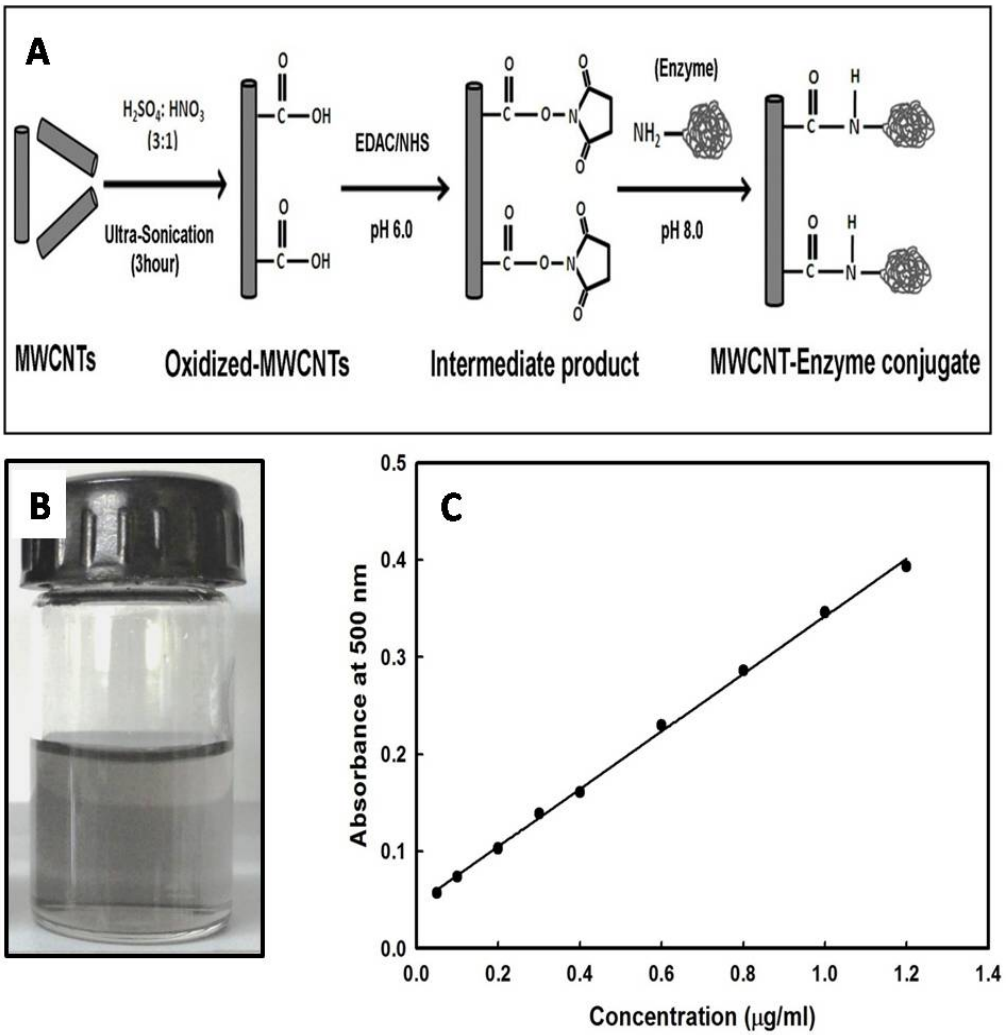

Figure 3.3.1.1 Scheme for MwCNTs-Enzyme conjugate synthesis using diimide activated amidation reaction. **(A)** Direct coupling reaction takes place using N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and N-Hydroxysuccinimide (NHS) involving carboxylic acid group of oxidized MwCNTs and amine groups of respective enzyme, leading further to amide bond formation. **(B)** Image of aqueous dispersible MwCNT(SOD) biocatalytic conjugate, without any flocculation even after week of storage. **(C)** Linear absorption of synthesized MwCNT(SOD) biocatalytic conjugate at 500 nm as a function of its increasing concentration and also indicating highly homogenous aqueous dispersible nature respectively.

FTIR analysis was performed at respective synthesis step to ensure the change in surface chemistry during the course of MwCNT(SOD) conjugate synthesis (Figure 3.3.1.2). The emergence of IR spectra in the region of 1690-1760 cm^{-1} indicates carboxyl group formation compared to the unrefined (raw) MwCNTs after acid oxidation (HNO_3:H_2SO_4; 3:1 v/v ratio) treatment (*Kim et al., 2005*). The EDAC/ NHS chemistry was also verified using IR spectroscopy; with carbonyl IR peak of ester group in the range of 1665-1710 cm^{-1}.The covalent immobilization of SOD onto EDAC/ NHS activated MwCNTs was confirmed with the amide region IR peak in the range of 1630-1690 cm^{-1}. Field-Emission Scanning Electron Microscopic (FE-SEM) analysis was also performed. The image of MwCNT(SOD) conjugate reveals change in surface morphology of the carbon nanotube against the unrefined MwCNTs which were found to be clustered together (Figure 3.3.1.3).

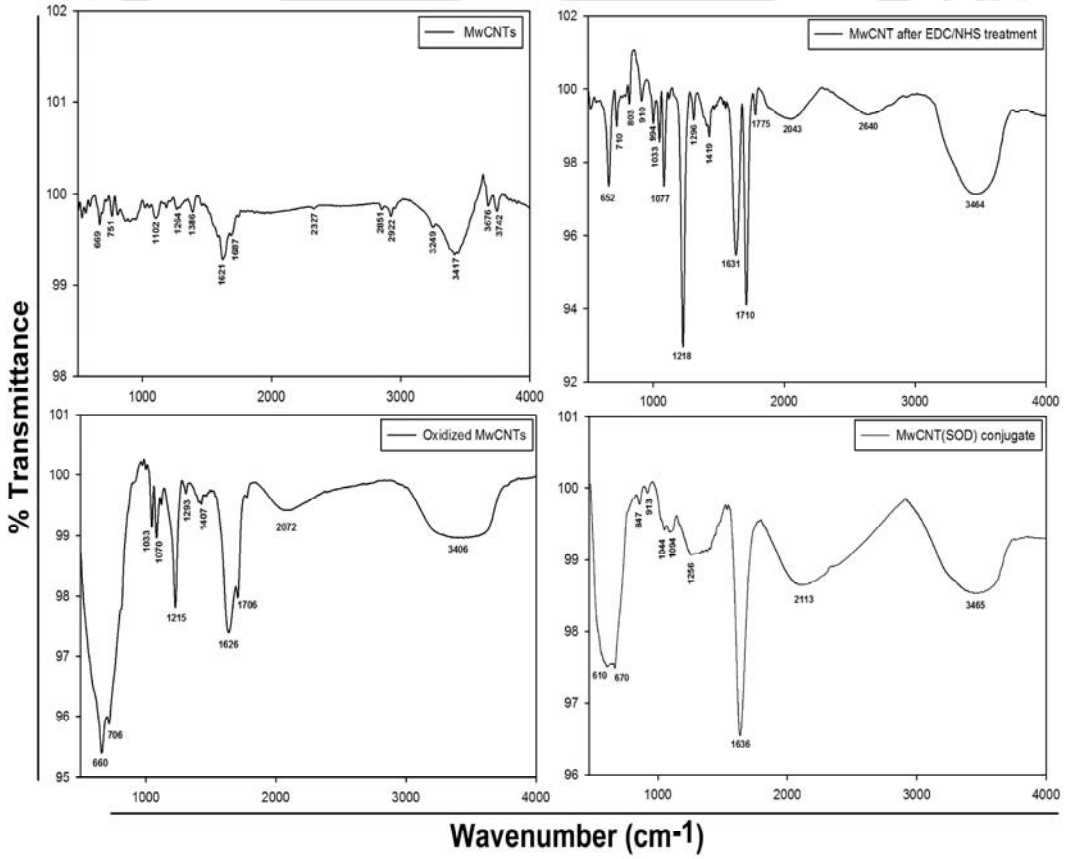

Figure 3.3.1.2 Fourier transform IR spectra of unrefined MwCNTs, oxidized MwCNTs, MwCNTs after EDAC/ NHS treatment and of MwCNT(SOD) conjugate. The samples were prepared by KBr disc method and scanning was done at 400 to 4,000 cm−1.

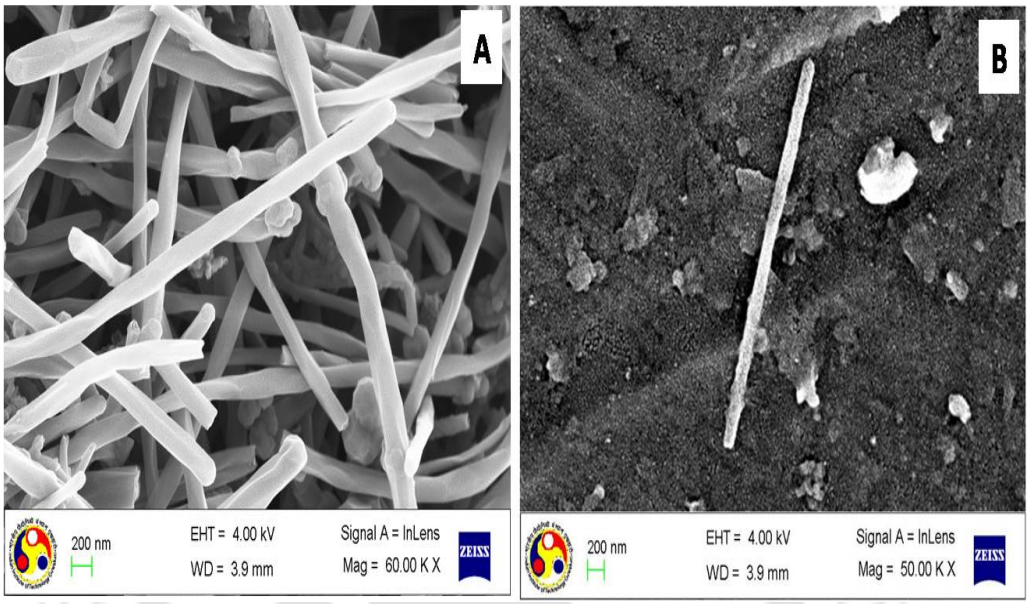

Figure 3.3.1.3 FE-SEM images of **(A)** unrefined MwCNTs and **(B)** synthesized MwCNT(SOD) biocatalytic conjugate. The respective sample was vacuum dried overnight onto a metal stab fitted with carbon tape, gold coated and analyzed further. FE-SEM image of a cluster of unrefined MwCNTs and aqueous dispersible, SOD conjugated MwCNTs.

3.3.2 Enzyme stability, temperature and pH characterization of synthesized MwCNT(SOD) biocatalytic conjugate

Antioxidative enzyme superoxide dismutase was covalently immobilized onto oxidized MwCNTs using diimide activated amidation reaction chemistry. The synthesized MwCNT(SOD) biocatalytic conjugates were subjected to superoxide dismutase enzyme stability using riboflavin mediated NBT reduction method (*Giannopolitis & Ries, 1977*). High SOD activity for MwCNT(SOD) biocatalytic conjugate was observed initially, however with respect to time loss in SOD activity were observed. The residual SOD activity obtained were 40% after 96 hours of incubation time period, indicating a significant amount of SOD enzyme stability to be retained with the synthesized MwCNT(SOD) conjugate (Figure 3.3.2.1). Decrease in SOD activity and stability might be due to the hindrance involving Cu^{+2} and Zn^{+1} metal ion while undergoing covalent immobilization, which are essential for enzyme activity and stability. MwCNT(SOD) conjugate were also subjected to temperature and pH characterization for its optimum activity and stability. Low temperature optima and stability were found to be

observed with the synthesized conjugate. Residual activity upto 60% was found to be retained up to 40 °C, however successive increase in temperature leads to decrease in SOD activity. MwCNT(SOD) biocatalytic conjugate was found to have wide pH optima and stability in the range of pH 6-9 with high activity and stability at pH 8, the optimum pH for native SOD enzyme in free form (*Singh et al., 2013*) (Figure 3.3.2.1).

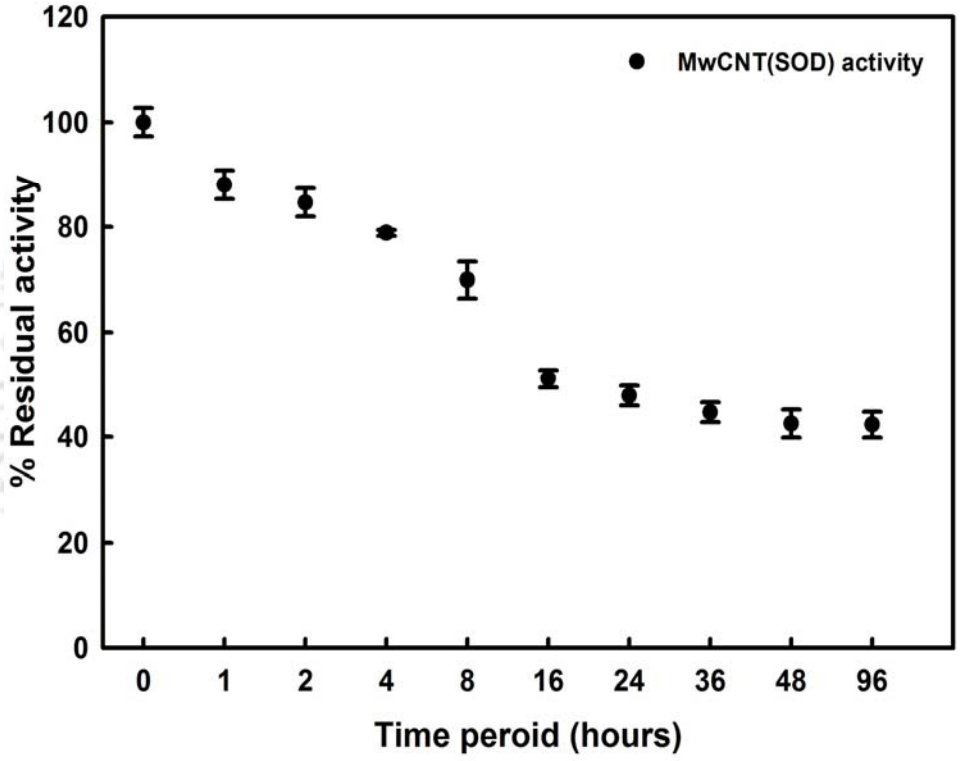

Figure 3.3.2.1 Superoxide dismutase activity of MwCNT(SOD) biocatalytic conjugate synthesized using diimide activated amidation reaction. SOD activity was determined using riboflavin mediated NBT reduction assay under different time period of incubation at 37 °C and 100 rpm. Data plotted in form of percentage residual activity and are the mean values of triplicate experiment performed individually±S.E.

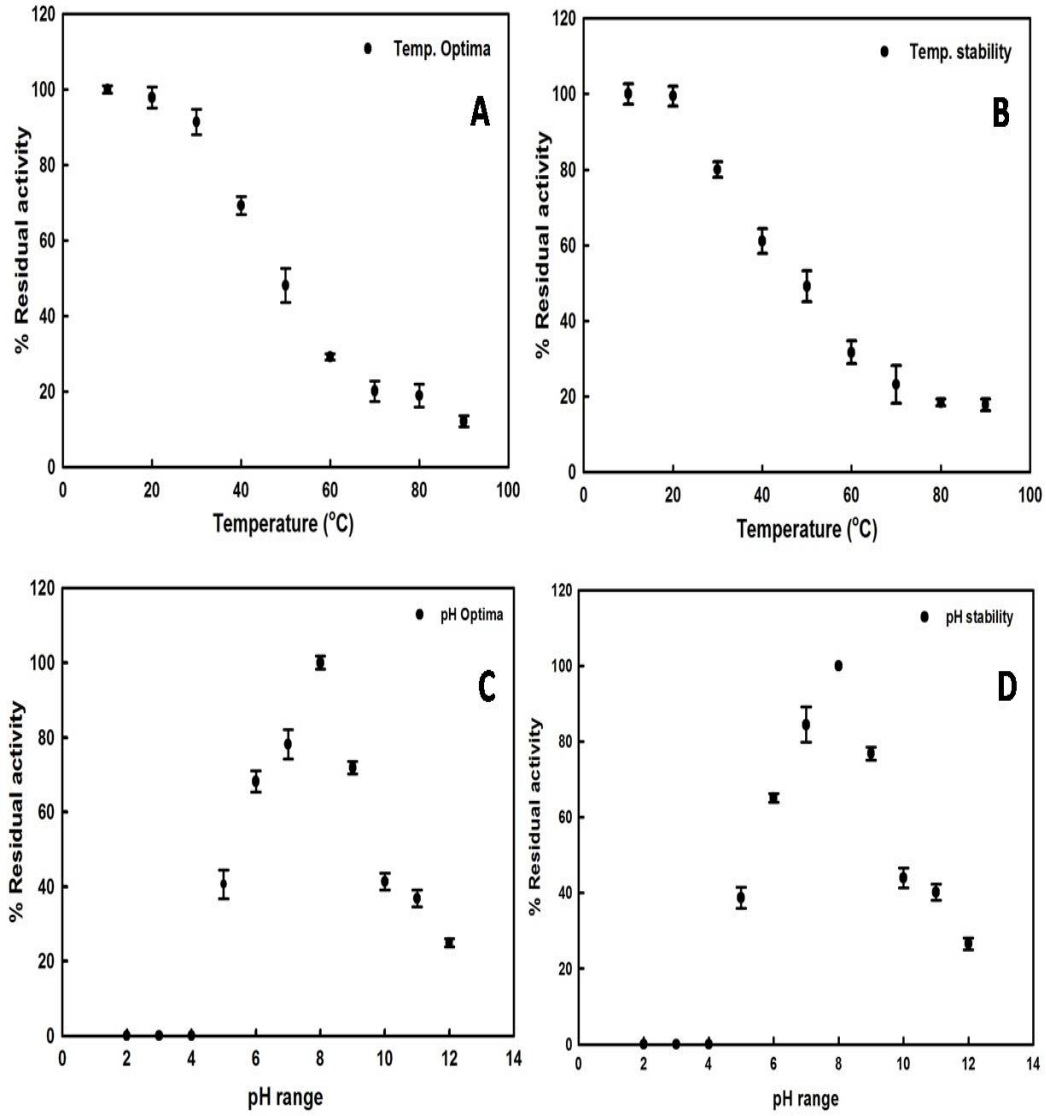

Figure 3.3.2.2 Temperature optima **(A)** and stability **(B)** of synthesized MwCNT(SOD) biocatalytic conjugate. Optimum pH **(C)** and pH stability **(D)** of synthesized MwCNT(SOD) biocatalytic conjugate. For temperature optima, the enzyme was incubated at different temperatures for 15 min and substrates pre-equilibrated at respective temperatures were added to carry out reaction at corresponding temperatures. Stability was determined by overnight incubating the enzyme at room temperature at different pH conditions and next day activity was taken as mentioned in method section For temperature stability measurements, enzyme was incubated at required temperature for 15 min and activity was measured as mentioned in method section.The data presented are mean values of three independent experiments±S.E.

3.3.3 Cytocompatibility of MwCNTs, oxidized MwCNTs and MwCNT(SOD) biocatalytic conjugates on human skin HaCat cell

MwCNTs are excellent nanomaterial synthesized and have been found to enhance the drug delivery techniques. In addition to exceptional physical and chemical properties, the nanosize range synthesis and its desired functionalization have enhanced the drug delivery approaches (*Kostarelos et al., 2007*). Human skin HaCat cell cytocompatibility analysis was done for unrefined (raw) MwCNTs, oxidized MwCNT, and SOD enzyme immobilized MwCNT(SOD) biocatalytic conjugate. Human skin HaCat cells ($2.5x10^4$ per well) incubated with unrefined (raw) MwCNTs and oxidized MwCNTs in concentration range of minimum 1 µg/ml to maximum 500 µg/ml were evaluated for cell survival (%) percentage under different incubation time period. Unrefined (raw) MwCNTs were found to induce toxicity in human skin cells. After 48 hours of incubation, the cell viability percent decreases up to 50 % at minimal concentration of 5 µg/ml, indicating high toxicity (Figure 3.3.3.A). In comparison to unrefined form, the oxidized MwCNTs were found to be comparatively less toxic as cell viability of 75 % was observed after 48 hours of incubation at similar concentration. However at much higher concentration *i.e.* 100 µg/ml for 48 hours incubation time period, toxicity was observed as cell viability decreases up to 50% (Figure 3.3.3.B). Impurities associated with unrefined MwCNTs during its synthesis are considered to impart cellular toxicity. However the acid oxidation step removes these impurities, making it less toxic and suitable for further applications. Before analyzing the antioxidative effect of MwCNT(SOD) conjugate, its cytotoxicity was examined on human skin HaCat cells. Similar experiment with HaCat cells ($2.5x10^4$ per well) was set up and incubated with synthesized MwCNT(SOD) biocatalytic conjugate in concentration range of 1 µg/ml to maximum 200 µg/ml. MTT analysis was done in order to analyze the percentage (%) cell viability. The synthesized MwCNT(SOD) conjugate were found to be cytocompatible, as indicated by more than 80% cell viability even after 48 hours of incubation at higher concentration of 200 µg/ml (Figure 3.3.3.C). Thus superoxide dismutase conjugated MwCNTs were found to be cytocompatible on human skin HaCat cells and was tested for its antioxidative effects in reducing oxidative stress.

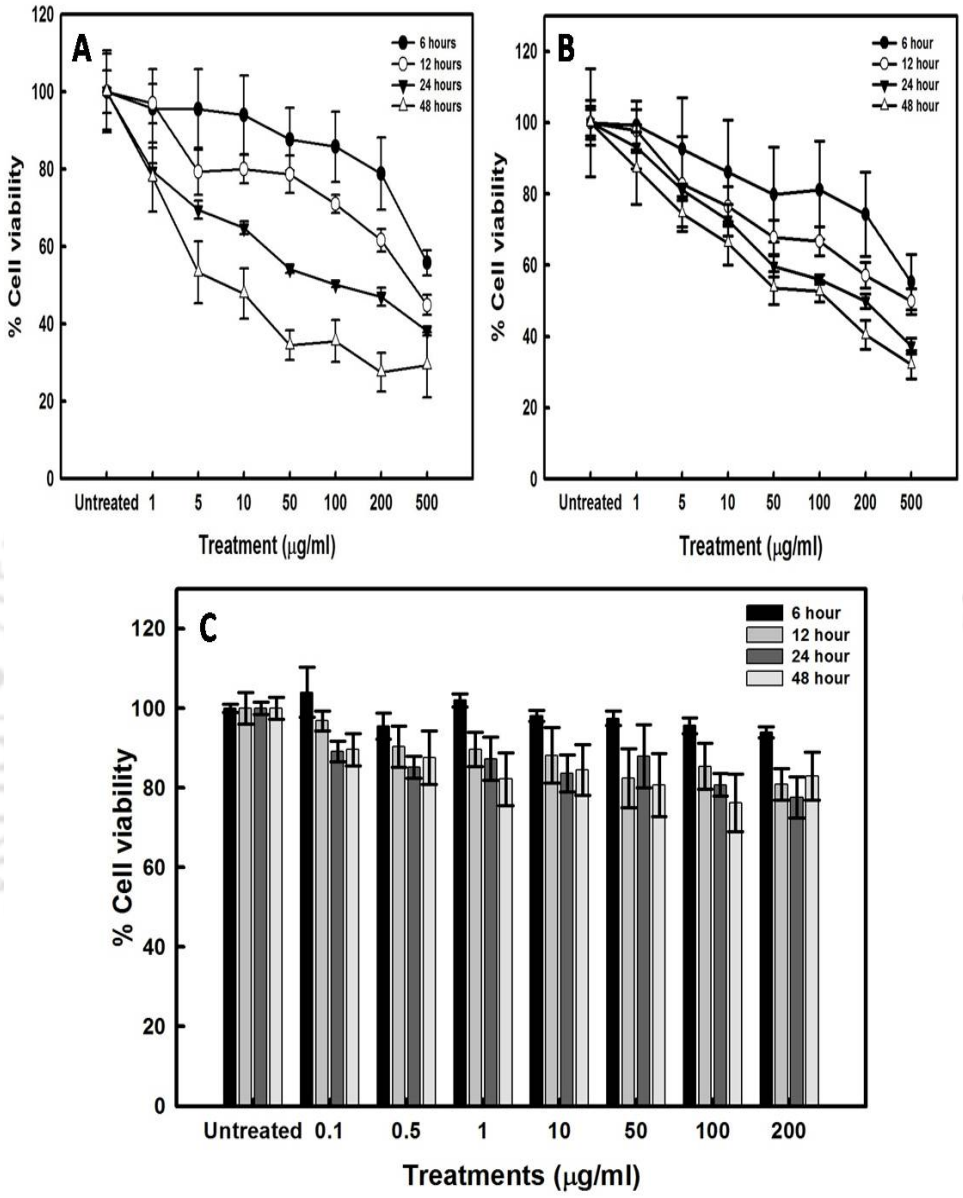

Figure 3.3.3 Cytocompatibility of **(A)** unrefined (raw) MwCNTs, **(B)** oxidized MwCNTs and **(C)** synthesized MwCNT(SOD) biocatalytic conjugates against human skin HaCat cells. Human skin HaCat cells (2.5×10^4/well) in 96-well micro plate were incubated with different concentration of respective MwCNTs. MTT viability assay was performed to determine percent cell survival. Data presented are the mean values of triplicate experiment performed individually±S.E.

3.3.4 H_2O_2 induced oxidative stress optimization in human skin HaCat cells.

H_2O_2 is one of the molecules which get generated in the redox metabolic system and induces oxidative stress. However it is also used extensively as an external source for inducing oxidative stress condition in human cells (*Reddy et al., 2008*). MTT analysis of HaCat cell post treatment with 1 mM concentration of H_2O_2 prepared in DMEM/ F12 media after 6 hours of incubation period indicates around 34 % cell viability (Figure 3.3.4). Earlier we have reported 1mM concentration of H_2O_2 and incubation time period of 5 hour, an average 75% of HaCat cells are under oxidative stress condition. This has also been analyzed using carboxy-H_2DCFDA, a ROS specific dye through FACS analysis (*Singh et al., 2013*). Thus, this induced oxidative stress, optimized treatment conditions of human skin HaCat cells has been used thereafter for analyzing the antioxidative response of synthesized MwCNT(SOD) biocatalytic conjugate.

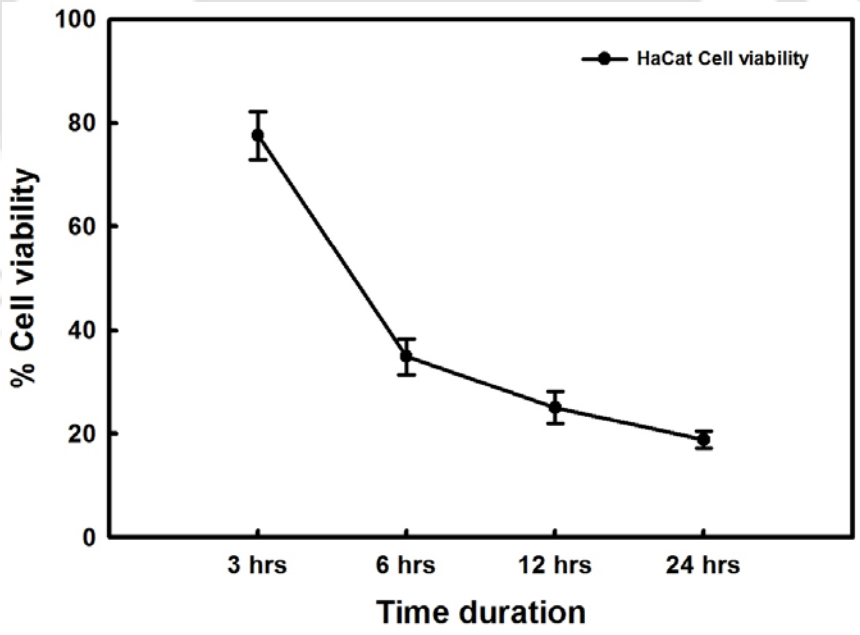

Figure 3.3.4 H_2O_2 is used as an external source of oxidative stress. Human skin HaCat cells (2.5×10^4/well) in 96 well microplate were exposed to 1 mM H_2O_2 and MTT assay was performed after respective time period of incubation. Percent Cell viability was determined with respect to untreated cell and data plotted are the mean values of triplicate experiment performed individually±S.E.

3.3.5 Antioxidative efficacy of MwCNT(SOD) biocatalytic conjugate on oxidative stress induced human skin HaCat cells

Advances in drug delivery have been observed with the use of carbon nanotubes, mainly due to its nanosize synthesis and easy functionalization approaches which have lead to its conjugation with variety of drugs/ proteins and therapeutic molecules (*Bianco et al., 2005*). Antioxidative enzyme, superoxide dismutase has very high scavenging property for superoxide anions which are the initiators of oxidative stress conditions. Here, synthesized MwCNT(SOD) biocatalytic conjugates were analyzed for its antioxidative efficacy towards reducing the induced oxidative stress in human skin HaCat cells. H_2O_2 induced oxidative stress optimized human skin HaCat cells ($2.5x10^4$ per well) were incubated with different concentration MwCNT(SOD) biocatalytic conjugates. MTT analysis was performed thereafter reveals increasing concentration of SOD conjugates leading to an increase in percentage cell viability indicating reduction of induced oxidative stress condition. After 6 hours of incubation, around 20 % increase in cell viability was observed at a concentration of 1 µg/ml with respect to oxidative stressed cells, indicating the efficient uptake of MwCNT(SOD) conjugate by human skin HaCat cells and enhanced antixodiative response towards reducing induced oxidative stress (Figure 3.3.5.A). Towards analyzing the antioxidative effect of MwCNT(SOD) biocatalytic conjugate, another similar experiment was performed at 1 µg/ml conjugate under different time duration of incubation. Increase in incubation time period leads to successive increase in cell viability percentage upto 70%, indicating efficient and enhanced uptake of MwCNT(SOD) conjugate and antioxidative defense respectively (Figure 3.3.5.B).

3.3.6 Reactive oxygen species and SOD activity analysis post MwCNT(SOD) conjugate treatment

Oxidative stress conditions develop due to increase in reactive oxygen species and antioxidative enzymes play scavenging role towards depleting these highly reactive molecules, avoiding the unwanted harmful effects. Reactive oxygen species (ROS) level was determined in H_2O_2 induced oxidative stress human skin HaCat cells and successively oxidative stress cells treated with MwCNT(SOD) conjugate in comparison to untreated normal cells. ROS level, analyzed after 24 hours of incubation with

MwCNT(SOD) conjugate in oxidative stress induced HaCat cells were found to be low and comparable to normal untreated cells (Figure 3.3.6.A).

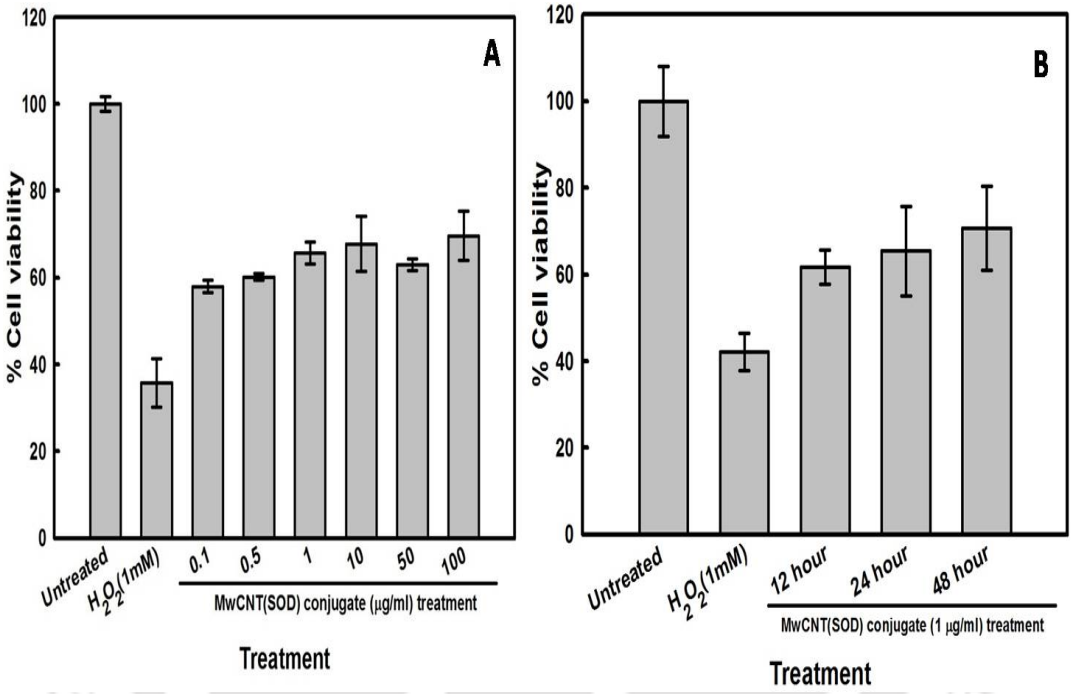

Figure 3.3.5 Antioxidative effect of synthesized MwCNT(SOD) biocatalytic conjugates were estimated following H_2O_2-induced oxidative stress condition in human skin HaCat cells. SOD enzyme, covalently immobilized on MwCNTs biocatalytic conjugates were incubated with H_2O_2 induced oxidative stress HaCat cells, **(A)** in different concentrations ranging from 0.1 to 100 µg/ml and **(B)** at a fixed concentration of 1 µg/mL for different time period. Percentage (%) cell viability was measured with respect to untreated cells where 100 % cell viability was considered. MTT assay was performed after respective incubation time period and the data plotted are the mean values of triplicate experiment performed independently ± S.E.

The low ROS level as determined by relative fluorescence unit in MwCNT(SOD) conjugate treated cells indicated enhanced uptake as well as efficient conjugated SOD enzyme activity towards reducing induced oxidative stress. High oxidative stress was observed in human skin HaCat cell, as indicated by its increased ROS level compared to untreated normal HaCat cells. ROS level were found to be comparable in MwCNT(SOD) conjugate treated HaCat cells in comparison to untreated normal cell indicating the

efficient scavenging of superoxide anion radicals and high reducing activity of oxidative stess overall. Superoxide dismutase activity was also estimated towards analyzing the increased antioxidative defense in combating the induced oxidative stress. Increase in SOD activity was observed with time duration of incubation in MwCNT(SOD) conjugate treated HaCat cells (Figure 3.3.6.B). Enhanced uptake of SOD conjugated MwCNTs leads to efficient removal of oxidative stress and successively increased SOD activity in comparison to normal untreated HaCat cells and H_2O_2 induced oxidative stress HaCat cells.

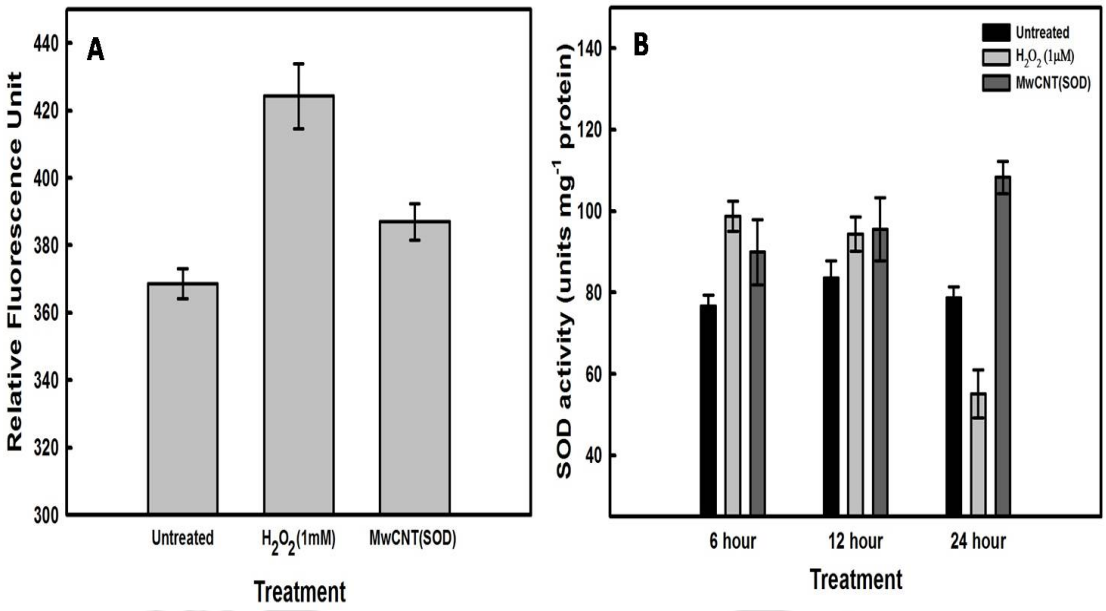

Figure 3.3.6. Analysis of reduction in oxidative stress condition was estimated using **(A)** Reactive oxygen species analysis under different condition of treatment i.e. untreated, after 1mM H_2O_2 treatment or with 1 µg/mL MwCNT(SOD) conjugate treatment following trement of 1mM H_2O_2. Reactive oxygen species was measured using increase in fluorescence level post carboxy-H_2DCFDA dye treatment. **(B)** Superoxide dismutase activity was also use as paramenter to analyze the increase in antioxidative defense under similar condition at different time interval. The data plotted are the mean values of triplicate experiment performed independently±S.E.

3.4 POLYCAPROLACTONE NANOSPHERE ENCAPSULATING SUPEROXIDE DISMUTASE AND CATALASE ENZYME TOWARDS ELEVATING ANTIOXIDATIVE DEFENSE AGAINST INDUCED OXIDATIVE STRESS

Human skin acts as a major boundary to the constantly changing external environment. As a result skin is permanently exposed to variety of adverse, physical as well as chemical environmental pollutants. Directly or indirectly, these environmental contaminants lead to the induction of oxidative stress through increase in reactive oxygen species (ROS) (*Brickers & Arthar, 2006; English et al., 2003*). ROS production is an inevitable part of normal aerobic metabolic system. A balance exists between ROS formation through sequential reduction of oxygen in redox metabolic reactions and its effective removal by protective endogenous antioxidative defense system. Enzymatic and non-enzymatic defense systems exists against oxidative stress which include superoxide dismutase (SOD), glutathione peroxidase, ascorbate peroxidase and catalase (CAT) as major antioxidative enzymes (*Sies, 1997*). Adverse environmental conditions such as exposure to ultraviolet (UV)-ionizing radiation and xenobiotics lead to uncontrolled production of ROS, which quickly overcomes the surrounding antioxidants and scavenging pathways inducing potential oxidative damage. The different agents like gaseous-airborne environment pollutants, UV radiations, and cosmetics products may also cause oxidative stress particularly in the skin (*Athar, 2002*). Higher induction of ROS also occurs in numerous diseased states, further elevating the pathogenesis level of many clinical symptoms (*Maier and Chan, 2002*). Exogenous supplementation of antioxidative enzymes demonstrates better therapeutic potentials towards depleting oxidative stress conditions and minimizing ROS-associated deleterious effects. Polymeric nano-encapsulation of antioxidative enzymes could be an effective delivery system because of their ability to release within intra- and extracellular compartments at a sustained rate. Nanoparticulate system had significant advantage towards overcoming drug deliveries as it can increase therapeutic index by several mechanisms such as sustained release of the drug, increasing its stability in biological environment, cellular and tissue uptake enhancement, and reducing the overall toxicity (*Panyam and Labhasetwar, 2003*). Here ROS-scavenging property of two different antioxidative enzymes has been explored. SOD of plant origin, isolated and characterized in our laboratory (*Singh et al., 2012*) has been used in combination with another commercially available bovine CAT. Both

enzymes play vital antioxidative role towards scavenging foremost ROS generated, i.e., SOD scavenge superoxide anions (O_2^-) and convert it to hydrogen peroxide (H_2O_2). Further CAT as H_2O_2- utilizing enzyme scavenges it to H_2O and O_2, thus diminishes the level of ROS and oxidative stress simultaneously (Figure 3.4). It has been hypothesized that protected and sustained delivery of SOD and CAT simultaneously by encapsulating it into biodegradable polycaprolactone (PCL) nanosphere would demonstrate better antioxidative efficacy in comparison to direct supplementation into the medium.

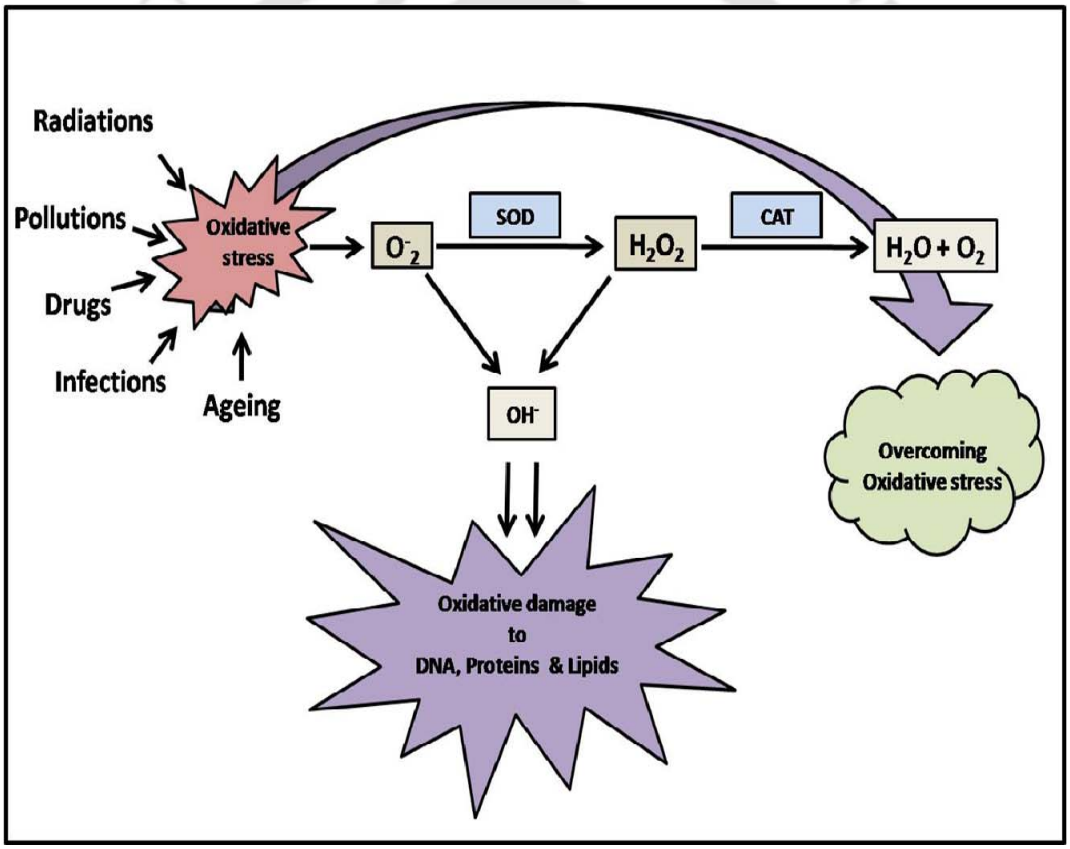

Figure 3.4 Scheme depicting induction of oxidative stress under adverse conditions and further consequence through reactive oxygen species (ROS) generation. Antioxidative enzymes superoxide dismutase (SOD) and catalase (CAT) activities in continuation leads to depletion of generated oxidative stress condition through scavenging of reactive oxygen species.

3.4.1 Synthesis and characterization of enzyme loaded polycaprolactone nanosphere

Biodegradable polymeric nanoencapsulation of antioxidative protein, SOD and CAT were successfully achieved using double emulsion (w/o/w) solvent evaporation technique (Figure 3.4.1.1). Different concentration of PCL as polymeric material and PVA as stabilizer was tested as this factor has been found to affect the synthesized nanosphere size and morphology. As reported in Table 3.4.1, the formulations include 3 % PCL, 2 % PVA in the inner aqueous phase while the external aqueous phase consisted of 1 % PVA which acts as a stabilizer during the synthesis. Higher PVA concentration in inner aqueous phase has been associated with homogenous distribution of cavities as well as influences the protein releases (*Coccoli et al., 2008*). DLS and FE scanning electron microscopic images reveal the size and spherical nature of synthesized PCL nanospheres (Figure 3.4.1.2A–C). The double emulsion methodology synthesizes PCL nanospheres in the mean range of 812±64 nm with an average PDI value of 0.34±0.02, indicating the synthesized nanosphere having homogenous distribution (*Nidhin et al., 2008*).

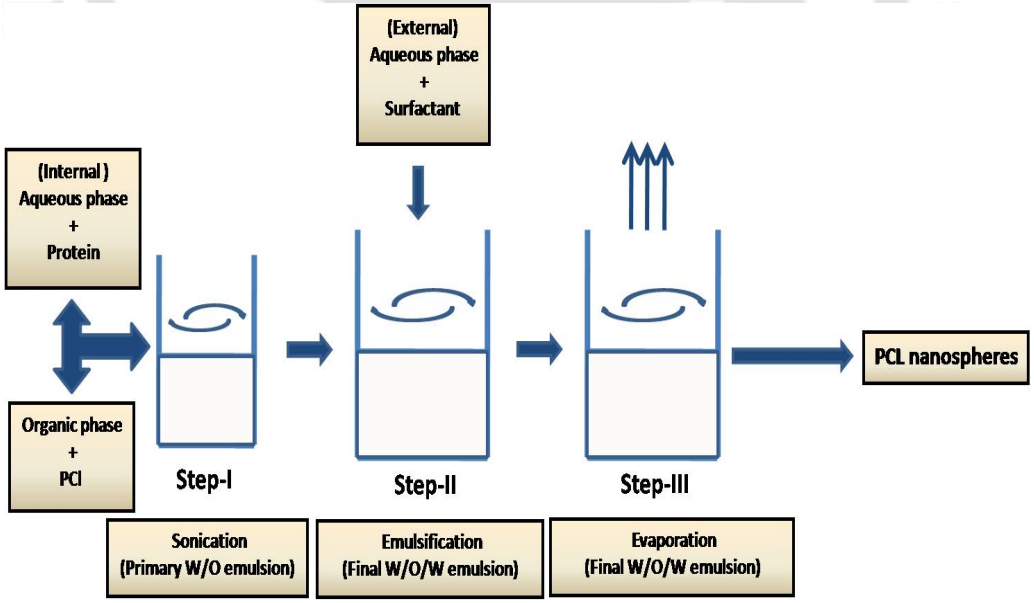

Figure 3.4.1.1 Synthesis of polycaprolactone (PCL) nanosphere using water oil water (W/O/W) emulsion solvent evaporation technique. Scheme depicts the synthesis consists of mixing three different phases, including protein sample and surfactant containing internal aqueous phase with polymer containing organic phase and finally surfactant containing external aqueous phase enhancing evaporation of organic solvent.

Table 3.4.1 Formulation followed and characteristic of polycaprolactone (PCL) nanosphere synthesized through double emulsion (w/o/w) solvent evaporation technique.

(Internal) Aqueous phase (% PVA)	Organic phase (% PCL)	(External) Aqueous phase (% PVA)	Nanosphere size range (nm)	Polydispersity index (PDI)	Encapsulation efficiency (%)
2%	3%	1%	812 ± 64	0.344 ± 0.027	55.42 ± 3.7%

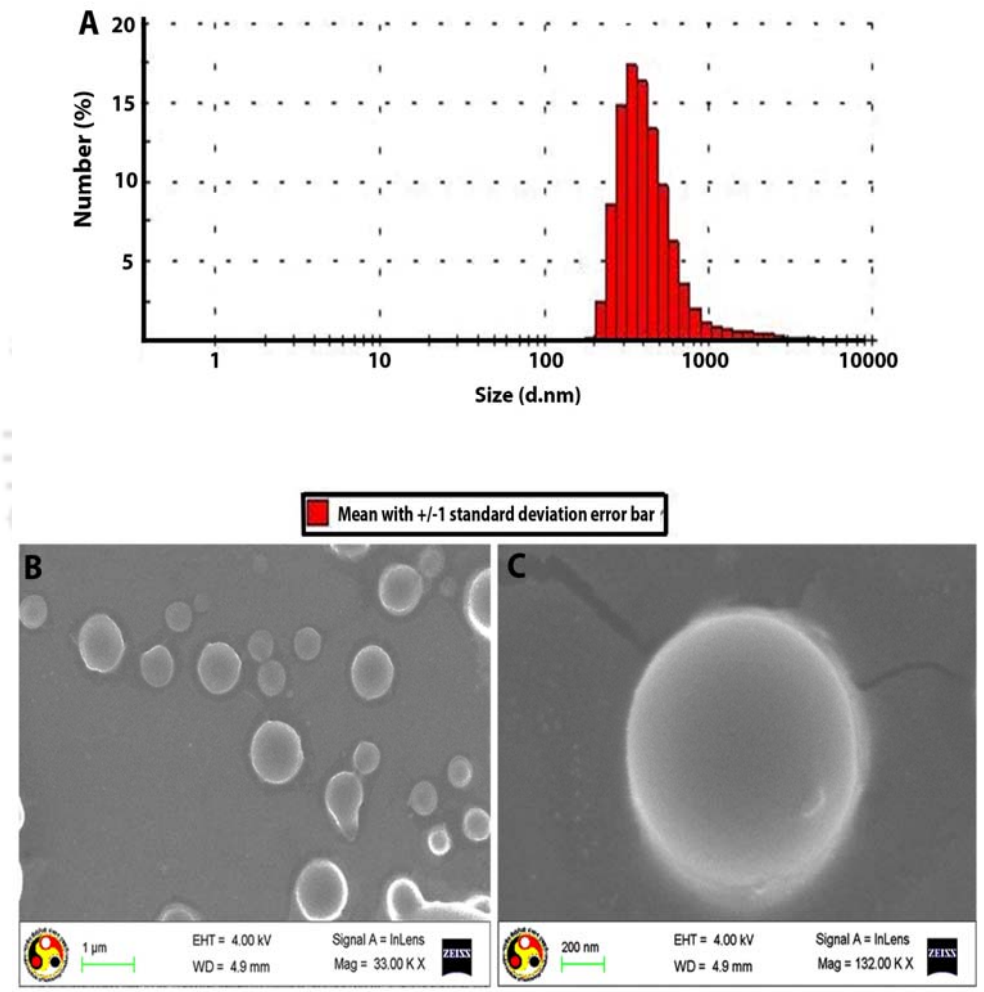

Figure 3.4.1.2 Physical characterization of PCL nanosphere synthesized through double emulsion (w/o/w) solvent evaporation technique. **(A)** Particle size distribution measured through DLS. Nanosphere was suitably diluted prior to particle size analysis. **(B)** FE-SEM images of synthesized nanosphere. A drop of nanosphere, vacuum dried overnight onto a metal stab fitted with carbon tape. The sample was gold coated and analyzed with magnification at ×32K and a scale bar of 1 µm. **(C)** FE-SEM image of single PCL nanosphere, observed at ×132K magnification and scale bar of 200 nm.

Post PCL nanosphere synthesis, FTIR characterization was done to confirm encapsulation of antioxidative proteins. FTIR spectrum of blank PCL nanosphere was analyzed in comparison to other protein encapsulated nanosphere, i.e., PCL(SOD), PCL(CAT), and PCL(SOD+CAT) synthesized nanosphere. The spectra of SOD and CAT proteins were also compared with PCL nanosphere (Figure 3.4.1.3). Infrared (IR) peak at 1,725–27 cm^{-1} corresponds to ester of PCL, whereas amines region peak around 1,628–41 cm^{-1} corresponds to SOD and CAT proteins.

The emergence of both peaks in PCL(SOD), PCL(CAT), and PCL(SOD+CAT) nanospheres indicates entrapment of protein into the polymeric nanosphere during the course of synthesis. Further entrapment of protein is confirmed by measuring encapsulation efficiency and monitoring the protein release kinetics. Encapsulation efficiency for PCL nanosphere-encapsulating SOD, CAT, and SOD+CAT was determined individually. PCL(SOD+CAT) nanosphere was found with the highest encapsulation efficiency of 61.42±1.36%. PCL(SOD) and PCL(CAT) nanospheres were found with 52.29±3.1 and 57.04±1.58% protein encapsulation. The formulation followed for PCL nanosphere synthesis has moderated protein encapsulation efficiency averaging to 55.42±3.7% which is in accordance with mostly polymeric protein encapsulation which lies in the range of 40–60% (*Cheng et al., 2010*). Encapsulation of antioxidative proteins into PCL nanosphere has another advantage as the semi crystalline nature of PCL polymer facilitates the release of encapsulated protein directly into the surrounding aqueous environment which takes place by simple diffusion (*Sinha et al., 2004*). Antioxidative protein released from synthesized PCL nanosphere as shown in Figure 3.4.1.4A indicates burst effect release. Encapsulated protein released up to 30% is observed within 8 h, whereas 90% release is observed within 24 h, indicating high release of protein in the surrounding environment. The burst release is aided mainly by the adsorbed protein on the surface and external pores of synthesized nanospheres and also because of the high aqueous solubility of these antioxidative proteins. Enzymatic activity of the released SOD and CAT protein was also checked and found to be minimally inhibited because of polymeric encapsulation. Active SOD and CAT proteins were found to be released up to 80% into the surrounding environment from the polymeric nanosphere within 24 h of incubation (Figure 3.4.1.4B).

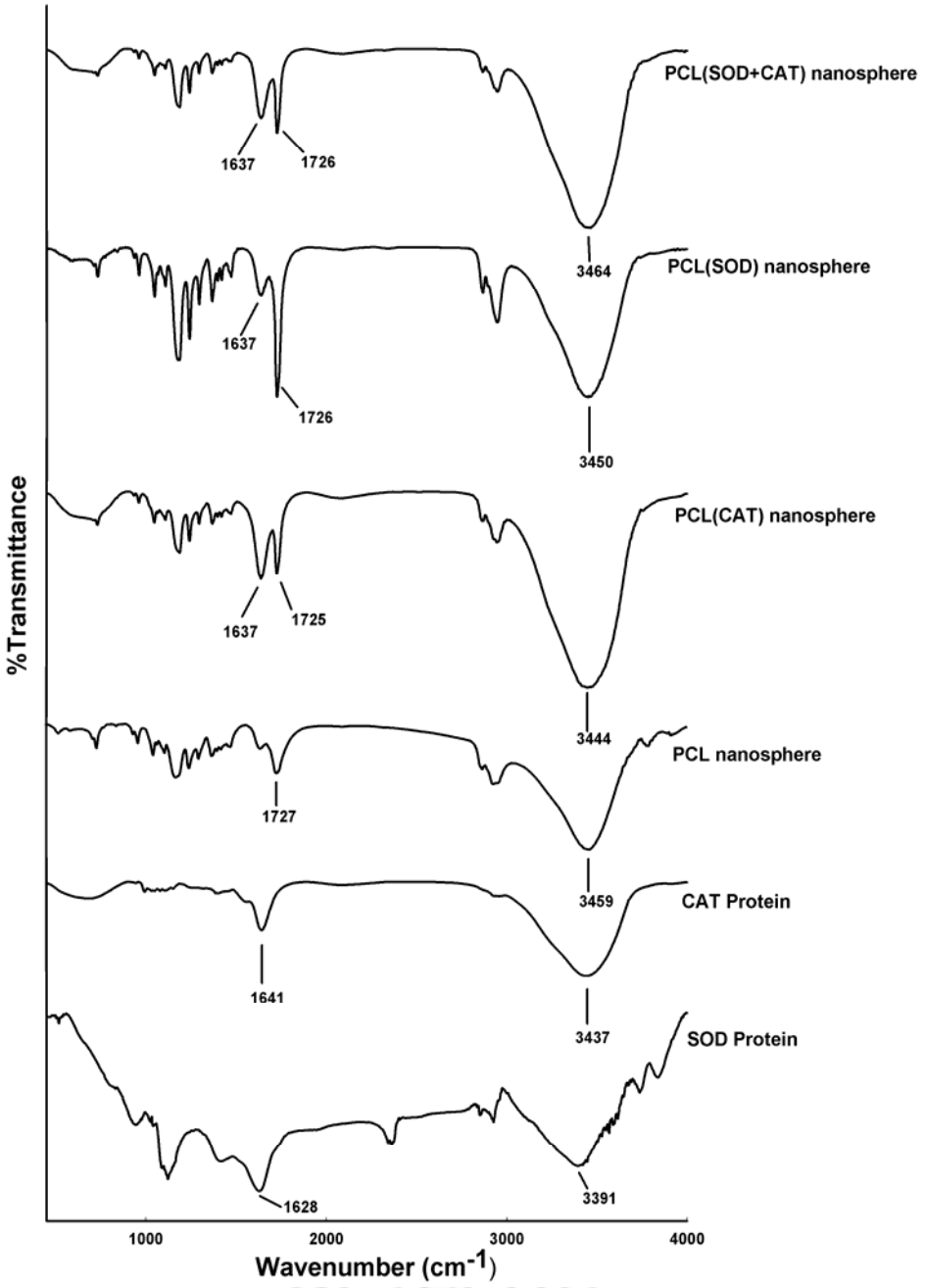

Figure 3.4.1.3 Fourier transform IR spectra of polycaprolactone (PCL), antioxidative protein superoxide dismutase (SOD), and catalase (CAT) in combination with PCL(SOD)-, PCL(CAT)-, and PCL(SOD+CAT)-synthesized nanosphere. The samples were prepared by KBr disc method and scanning was done at 400 to 4,000 cm^{-1}. IR peak at 1,725–27 cm^{-1} corresponds to ester of PCL, whereas amines peak around 1,628–41 cm^{-1} corresponds to SOD and CAT protein.

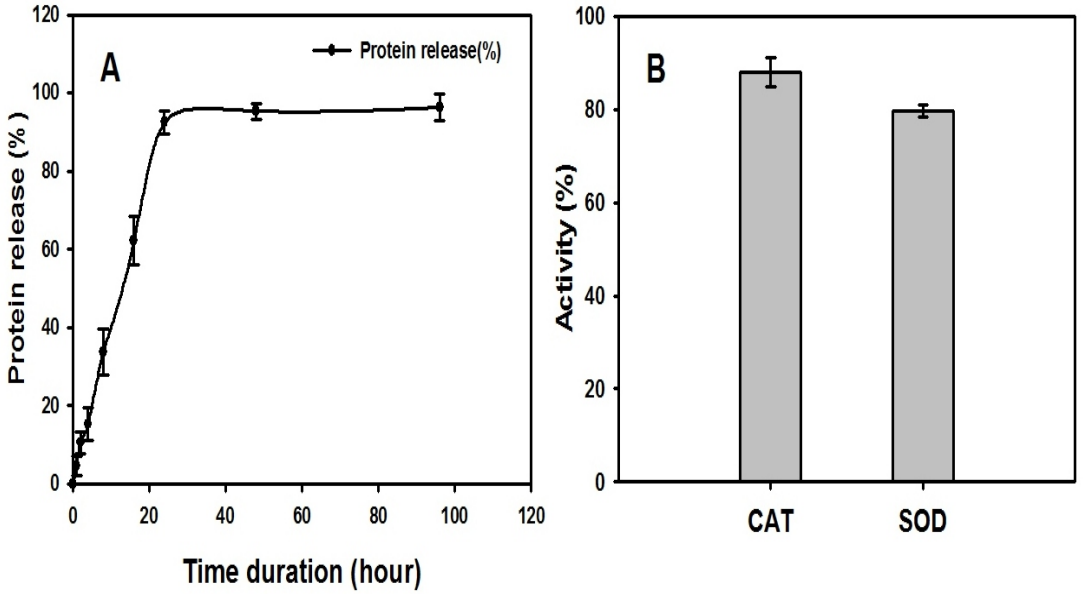

Figure 3.4.1.4 Efficient release of encapsulated protein from the nanospheres is an important criteria to be analyzed. **(A)** *In vitro* protein release from biodegradable PCL nanospheres synthesized through double emulsion solvent evaporation technique. The nanospheres encapsulating antioxidative protein were suitably diluted in PBS (pH 7.4) and kept at 37°C at 60 rpm in shaker incubator; 2 mL of sample was withdrawn at respective interval and replaced with PBS buffer. **(B)** Superoxide dismutase (SOD) and catalase (CAT) activity retention was analyzed after 24 h of incubation period and being released from biodegradable nanosphere. Data presented are the mean value of triplicate experiments ± SD.

3.4.2 Optimizing H_2O_2 induced oxidative stress in human skin HaCat cells

H_2O_2 is an inevitable part of oxidative conditions and has been extensively used as external source for oxidative stress induction. As no earlier data were available on the dose–response effect of H_2O_2 on human skin HaCat cells, first identifying the concentration of H_2O_2 required to induce optimal oxidative stress that would not cause excessive cell death (<75 %) was performed (*Reddy et al., 2008*). Varying concentration of H_2O_2 were tested ranging from lowest concentration of 1 μM to highest concentration of 5 mM; however, the optimum result was obtained with 1 mM concentration. Cell survival of 32% was observed with 1 mM of H_2O_2 after 6 h of incubation which further excessively decreases to 22 and 18% upon 12 and 24 h of incubation (Figure 3.4.2A). H_2O_2 at 1 mM concentration with exposure period of 5 h was used to induce optimal oxidative stress in human skin HaCat cells for all further experiments. H_2O_2-induced oxidative stress was further confirmed by ROS analysis using carboxy-H_2DCFDA (C400) dye studied through FACS analysis (Figure 3.4.2B). Carboxy-H_2DCFDA gets converted

to carboxy-H_2DCF by intracellular esterases and becomes impermeable to membrane. Further on reaction with intracellular ROS, the dye is converted into fluorescent derivative carboxy-DCF and the cells shows peak shift in fluorescence as compared with untreated cells indicating increase in ROS and simultaneously oxidative stress condition (*Robinson et al., 1998*). This similar condition of oxidative stress induction was used to evaluate the antioxidative efficacy of synthesized PCL nanosphere encapsulating SOD and CAT protein.

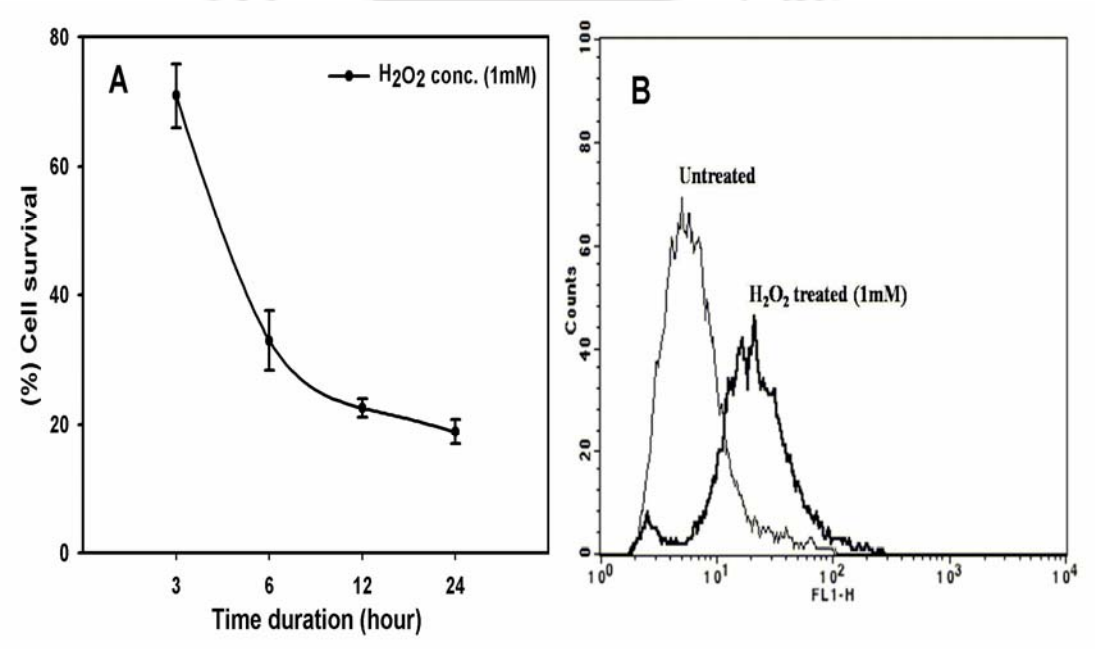

Figure 3.4.2 Oxidative stress condition was optimized using H_2O_2 in the externally supplemented media. **(A)** Human skin HaCat cells exposed to 1 mM H_2O_2 for optimal oxidative stress induction. MTT assay was performed after a respective period of incubation and percentage (%) cell survival was determined with respect to untreated cell. Data presented are the mean values of triplicate experiment ± SD. **(B)** H_2O_2 induced oxidative stress condition in HaCat cells was further verified through FACS approach. Carboxy-H_2DCFDA(C400) fluorescence dye was used which indicates oxidative stress condition through peak shift in fluorescence.

3.4.3 Synthesized polycaprolactone nanosphere cytocompatibility analysis

Biodegradable polymeric nanoparticulate system advances the modern drug delivery technology. The synthesized nanoparticles are found with enhanced

biocompatibility, superior encapsulation, and enhanced release properties for numerous therapeutic drugs and other biomolecules. Furthermore, new biodegradable polymers and advances in these polymer technologies have made the biomolecules, therapeutics, and drugs delivery system more effective (*Mahapatro & Singh, 2011; Kim et al., 2009*). PCL is biodegradable product, considered safe for use in combination with therapeutics and approved by Food and Drug Administration, USA (*Ghoroghchian et al., 2006*).

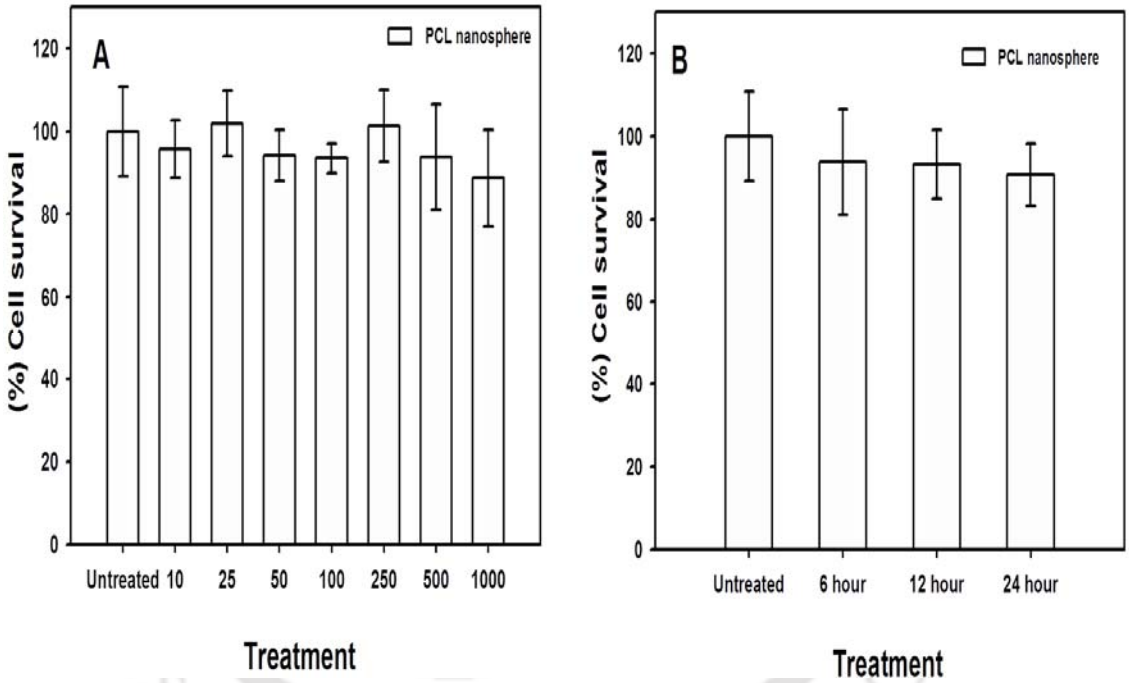

Figure 3.4.3 Biodegradable PCL nanospheres were checked for their cytocompatibility. **(A)** Human skin HaCat cells (2.5×10^4/well) in 96-well plates were treated with different concentration of blank PCL nanosphere. MTT assay was performed to determine percentage (%) cell survival after a respective period of incubation. **(B)** Cell cytocompatibility was also tested for different period at PCL nanosphere concentration of 500 µg/mL, and percentage (%) cell survival was determined in comparison to untreated cells using MTT assay. Data presented are the mean values of triplicate experiments performed individually ± SD.

PCL nanosphere synthesized through double emulsion (w/o/w) solvent evaporation technique was tested for cytocompatibility. Human skin HaCat cells treated with different concentration of synthesized blank PCL nanosphere were evaluated for (in

percent) cell survival after respective period of incubation. The initial 6 h of incubation period indicates cell survival of more than 85 % at concentration ranging from 10 to 1,000 µg/mL (Figure 3.4.3A). Also on further higher incubation for 12 and 24 h at higher concentration of 500 µg/mL nanosphere, HaCat cells does not show any toxic effects and percentage (%) cell survival was found to be unaffected (Figure 3.4.3B). This indicates non-toxic nature of synthesized PCL nanosphere and safer biocompatibility towards human skin HaCat cells, thus enabling the use of synthesized PCL nanosphere for encapsulation studies and further applications. It has been found that PCL degrades by ester linkage hydrolysis which takes place under normal physiological condition and thus completely safe for human body (*Kumari et al., 2010*).

3.4.4 *In-vitro* antioxidative evaluation of synthesized PCL(SOD+CAT) nanosphere

SOD reduces oxidative stress by scavenging superoxide radicals and converting it to H_2O_2 which is further diminished by H_2O_2 scavenging enzymes, i.e., CAT which overall reduces the oxidative stress completely. Thus, it is hypothesized that SOD and CAT on being encapsulated together will provide much better antioxidative defense compared with encapsulation of either SOD or CAT alone. Antioxidative effects of SOD, CAT, and (SOD+CAT) encapsulated PCL nanospheres were evaluated on H_2O_2 exposed oxidative stress human HaCat cell line. Percentage (%) cell survival of the treated sample indicates that antioxidative effect of PCL nanosphere encapsulating (SOD+CAT) had much better response as compared with the PCL nanosphere encapsulating either SOD or CAT protein alone. Oxidative stress-induced HaCat cells treated with PCL(SOD+CAT) nanosphere showed 20 % increase in cell survival (in percent), whereas PCL(SOD)-treated cells showed 10 % increase and PCL(CAT) nanosphere treated had 3% increase at higher nanosphere concentration of 500 µg/mL after initial 6 h incubation period (Figure 3.4.4A). Further increase in cell survival was found on successive higher duration of incubation for 12 and 24 h at 500 µg/mL concentration. PCL(SOD+CAT) treatment reported 24 and 44 % increase in cell survival (in percent), whereas in case of PCL(SOD) treatment, 13 and 15 % increase was reported. PCL(CAT)-treated cells shows 14 and 17% increase in cell survival percentage at 12 and 24 h incubation period after H_2O_2 induced oxidative stress (Figure 3.4.4B).

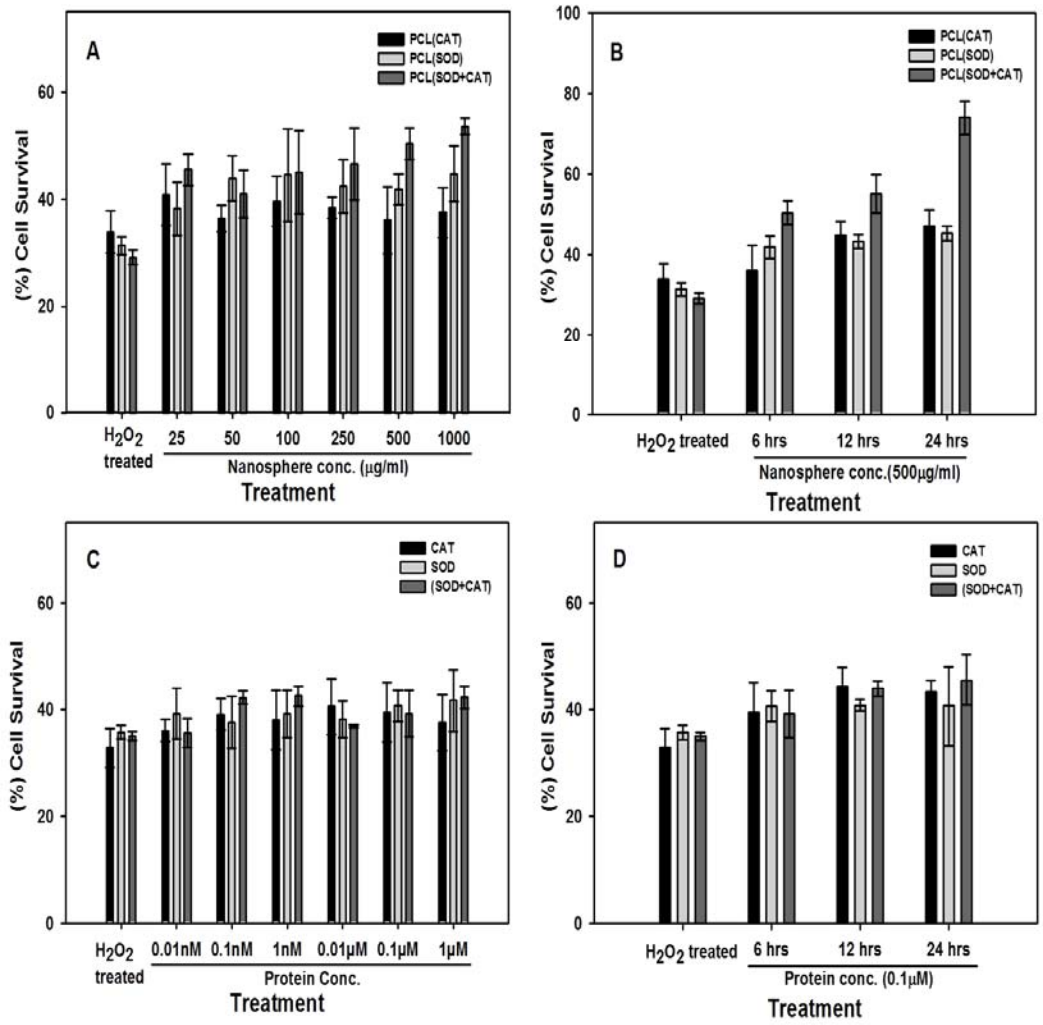

Figure 3.4.4 Antioxidative protective efficacy of PCL encapsulating SOD and CAT was determined following H_2O_2 induced oxidative stress condition in human skin HaCat cells. PCL nanospheres encapsulating antioxidative enzyme were incubated with H_2O_2 induced oxidative stress HaCat cells, **(A)** in different concentrations and **(B)** at a fixed concentration of 500 µg/mL for different period. Percentage (%) cell survival was measured with respect to untreated cells where survival was taken as 100 %. To ascertain the effects of antioxidative (SOD+CAT) encapsulated PCL nanosphere, similar experiments were repeated with **(C)** different concentration of SOD and CAT protein in solution form and **(D)** at a fixed concentration of 0.1 µM for different time period. MTT assay was performed after respective time duration of incubation and the data presented are the mean values of triplicate experiments performed separately ± SD.

To further analyze the effects of PCL(SOD+CAT) nanosphere, similar experiment was repeated with SOD and CAT enzyme without PCL encapsulation. Cell survival (in percent) indicates that using direct antioxidative protein into the solution was not as effective as PCL encapsulated form although minor increase in cell survival was recorded with higher dose of SOD+CAT protein at 0.1 and 1 µM concentration. Higher incubation

period of 12 and 24 h at SOD+CAT protein concentration of 0.1 μM also remains ineffective (Figure 3.4.4C-D). Polymeric nanoencapsulated SOD has been found to reduced H_2O_2 induced oxidative stress in human neuron cells (*Reddy et al., 2002*) and also individual encapsulation of SOD and CAT when applied together provided improved antioxidative response to pancreatic porcine cell clusters against generated oxidative stress (*Giovagnoli et al., 2005*). These observed results indicate that sustained and continuous release of antioxidative SOD and CAT from polymeric nanosphere has deleterious effect on reactive oxygen radicals and efficiently overcomes the induced oxidative stress in HaCat cells.

3.4.5 Analyzing SOD and CAT activities of oxidative stress-induced HaCat cells after PCL(SOD+CAT) nanosphere treatment

SOD and CAT are the key antioxidative enzymes which help in reducing the generated oxidative stress by scavenging superoxide radicals and H_2O_2, converting it finally to oxygen and water molecule. Human skin HaCat cells SOD and CAT activities were determined to further analyze the effect of PCL(SOD+CAT) nanosphere after externally supplying H_2O_2 induced oxidative stress condition. Increased SOD and CAT activities were observed in oxidative stress-induced HaCat cells treated with PCL(SOD+CAT) nanosphere as compared with untreated cells. Increase in incubation period of up to 24 h with PCL(SOD+CAT) nanosphere leads to a 3 fold higher increase in SOD and CAT activities of HaCat cells (Figure 3.4.5A-B). The increase in antioxidative activities of HaCat cells indicate close association with synthesized nanospheres as well as uptake of PCL(SOD+CAT) nanosphere. The increase in SOD and CAT activity of HaCat cells enhances the overall antioxidative defense and protect against induced oxidative stress. SOD and CAT simultaneous scavenging activities deplete the induced oxidative stress.

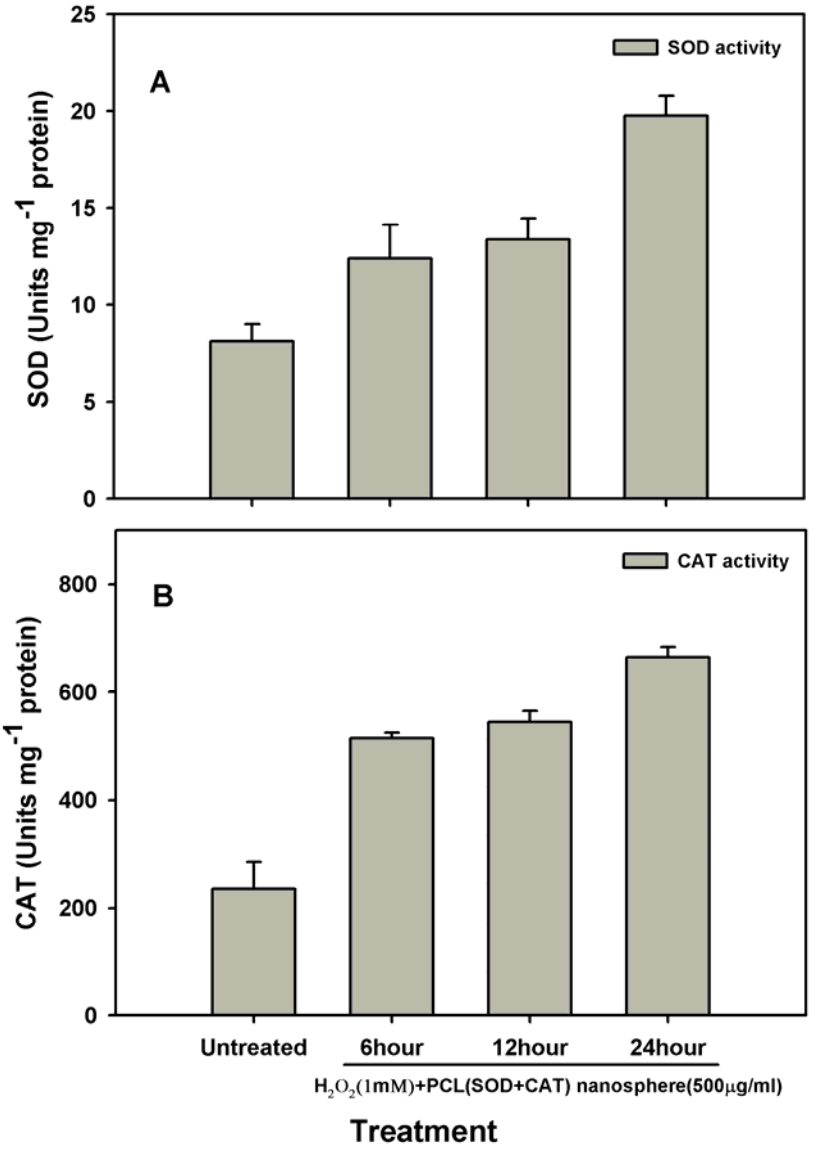

Figure 3.4.5 Superoxide dismutase and catalase enzymes analysis post treatment with PCL(SOD+CAT) nanospheres. H_2O_2 exposed (1 mM) oxidative stress HaCat cells were treated with PCL(SOD+CAT) nanosphere for a different period. **(A)** Superoxide dismutase (SOD) and **(B)** catalase (CAT) activities were determined from human skin HaCat cells. The activities of SOD and CAT represent the mean values of triplicate experiment performed individually ± SD.

Further encapsulating these antioxidative enzymes together into a biodegradable polymeric nanosphere enhances its stability and sustained releases into the surrounding environment. Antioxidative compounds in biodegradable polymeric material such as ethyl

cellulose, methyl cellulose, and PCL (*Suwannateep et al., 2012; Filho et al., 2013*) have been reported towards scavenging oxidative radicals. Encapsulating SOD and CAT individually into poly(D,L- lactide-co-glycolide), another polymeric material have been reported and studied for its antioxidative properties in human neurons and neonatal porcine pancreatic cell clusters (*Reddy et al., 2002*; *Giovagnoli et al., 2005*). However, this is the first report on co-encapsulation of SOD and CAT together into a biodegradable PCL nanosphere synthesized through double emulsion solvent evaporation technique. The synthesized PCL(SOD+CAT) nanosphere was found to efficiently reduce H_2O_2 induced oxidative stress in human skin HaCat cells indicating its sustained efficiency toward depleting oxidative stress conditions.

Chapter IV

Summary

Abstract

We explore physiological and biochemical response under increasing aluminium stress at different time intervals in chickpea seedlings (*Cicer arietinum*). The data obtained indicated that high concentration and long exposure of aluminium increases oxidative stress. As a response to increased oxidative stress, plant induces antioxidative enzyme as a defense mechanism. Further, a novel superoxide dismutase enzyme was isolated and was biochemically characterized in detail. The purified enzyme was found to be a Cu/Zn category of SOD with high molecular weight and distinct properties. The purified novel superoxide dismutase was explored for its application in pathological conditions that results from pollutions. MwCNT(SOD) conjugate and PCL(SOD+CAT) nanosphere were found to be effectively reducing the induced oxidative stress in human cell lines.

4.1 ALUMINUM METAL STRESS INDUCED ANTIOXIDATIVE ENZYMATIC ANALYSIS IN GERMINATED *CICER ARIETINUM* SEEDLINGS

Environmental pollution towards agricultural land has turned out to be a key issue affecting the normal crop development. Increased industrialization, inappropriate sewage sludge disposal, excessive usages of insecticides, pesticides and fertilizers onto the agricultural land has increased its metal toxicity level. These increased agricultural soil pollution due to high concentration of metal ions induces oxidative damage onto the germinating crop plants and deregulates its normal metabolic processes, overall leading to poor growth and survival of the seedlings. Analyzing the antioxidative responses of germinating crop plants against these different stress factors is of utmost importance. Here we have explored the physiological and enzymatic changes under increasing aluminium metal stress at different time intervals in chickpea (*Cicer arietinum*) seedlings. Normal physiological responses were found to be highly reduced under increasing external aluminium metal stress factor. Aluminium metal stress induced oxidative condition led to fluctuations in the antioxidative enzymatic responses. Low concentration of aluminium induces higher superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities. Malondialdehyde concentration indicated higher oxidative damage in roots as compared to shoots. Results obtained indicated increased oxidative stress and impaired antioxidative defense system, overall leading to poor growth and survival of seedlings. As a whole, the report presents an overview of antioxidative enzymatic response under increasing aluminium stress in germinating chickpea seeds. Under low concentration of external aluminium metal stress, the antioxidative defense is induced efficiently. However, long exposition and higher concentration deregulates its antioxidative defense system. A relation observed between SOD and peroxidases, APX and GPX indicates synchronous fluctuation of its antioxidative activities.

4.2 ISOLATION, PURIFICATION AND CHARACTERIZATION OF ALUMINIUM METAL STRESS INDUCED, A NOVEL SUPEROXIDE DISMUTASE PROTEIN FROM *CICER ARIETINUM* SEEDLINGS

Superoxide dismutase (SOD) is an important antioxidative enzyme as it forms the very first line of defense against induced oxidative stress into the aerobic redox system. Search of a new source and SOD protein with novel properties is always of much

significance. Here, we have reported the purification and biochemical characterization of a novel copper zinc superoxide dismutase (Cu-Zn SOD) obtained from Chickpea (*Cicer arietinum*) seedlings germinated under aluminium (Al^{3+}) metal as external stress factor. The SOD enzyme was purified upto 28 fold with specific activity of 158 units applying anion exchange and size exclusion chromatography techniques. The purified enzyme was found to be of homodimeric nature with the subunit molecular weight of 33.27 kDa, as determined using Rf vs Log Mw graph obtained from SDS-Gel electrophoresis. Molecular weight was also determined using MALDI-TOF analysis. The purified SOD was characterized. It belonged to the category of Cu-Zn SODs based on its native NBT-Zymography staining. Upon biochemical characterization the purified SOD was found to have broad pH optima for activity as well as stability, ranging from 6.5-8.5 with highest activity and stability at pH 8. The purified SOD was found to have low temperature optima for activity and stability (upto 40°C) where more than 60% of activity was retained indicating the purified SOD to be useful in low temperature stress tolerance mechanism. The K_m value for purified SOD was found to be 10.16 ± 2.5 μM using riboflavin as substrate, indicating high efficiency for balancing the oxidative stress generated due to increased superoxide radicals. SOD was found to retain its activity in presence of low concentrations of different metal ions and compounds while at higher concentration of metal ions the enzyme was inactive. The purified SOD was also subjected to N-terminal amino acid sequencing and was found to have highly conserved motifs upon alignment with other reported plant Cu-Zn SODs. High molecular weight together with low sequence identity with other Cu/Zn SODs and broad pH and temperature characteristic imparts novelty to this enzyme.

4.3 SUPEROXIDE DISMUTASE-MULTIWALLED CARBON NANOTUBE BIOCATALYTIC CONJUGATE TOWARDS ALLEVIATING INDUCED OXIDATIVE STRESS

Developing an efficient drug delivery system is the prime requisite for any therapeutic treatments. Significant advantages had been associated with nanoparticulate system of drug delivery including enhanced cellular and tissue uptake, increased stability in biological environment, sustained release and reduced toxicity being some of the key features. Among the diverse nanomaterials synthesized, carbon nanotubes (CNTs) possess significant advantages. Multiwalled carbon nanotubes (MwCNTs) are exceptional

nanomaterial, based on its physical and chemical properties; it has numerous applications of which usages as vector for drug deliveries is of much significance. Superoxide dismutase, the antioxidative enzyme was covalently immobilized onto oxidized MwCNTs using two step process of diimide activated amidation reaction. The amine groups of enzyme forms amide bonds on reacting with the highly reactive intermediate ester group onto oxidized MwCNTs. The MwCNT(SOD) conjugates obtained were highly aqueous dispersible and were also characterized using field emission scanning electron microscopy and Fourier transformed infrared spectroscopy. MwCNT(SOD) conjugates were found to retain significant superoxide anion scavenging activity even after 96 hours time period. Characterization for temperature reveals its optimum activity and stability up to 40 °C with more than 60% residual activity, while wide pH optima and stability range was obtained with highest activity and stability at pH 8. Unrefined (raw) and oxidized MwCNTs were found to induce significant toxicity on human skin HaCat cell line, however MwCNT(SOD) conjugate was found to be comparatively nontoxic when applied on human skin HaCat cells with more than 80% cell viability. Oxidative stress condition on human skin HaCat cells was optimized using H_2O_2 (1mM) as an external stress factor for analyzing antioxidative response of synthesized SOD conjugates. Upon incubation with oxidative stress HaCat cells, MwCNT(SOD) conjugates were found to alleviate the increased oxidative stress, indicating its enhanced uptake and efficient antioxidative activity. Low level of reactive oxygen species and increased SOD activity in HaCat cells, post treatment with MwCNT(SOD) conjugate further confirms the antioxidative response on SOD enzyme in conjugation with MwCNTs which acts as intracellular drug delivery vector.

4.4 POLYCAPROLACTONE NANOSPHERE ENCAPSULATING SUPEROXIDE DISMUTASE AND CATALASE ENZYME TOWARDS ELEVATING ANTIOXIDATIVE DEFENSE AGAINST INDUCED OXIDATIVE STRESS

Nanoparticulate system increases therapeutic index by several mechanisms and has significant advantages in overcoming drug deliveries. Biodegradable nanoparticulate system encompasses various advantages for drugs, different biomolecules, and therapeutic agent encapsulation towards targeted deliveries. Biodegradable Polycaprolactone (PCL) was explored as a polymeric material for synthesizing a

biocompatible nanosphere encapsulating SOD and CAT antioxidative enzymes. Superoxide dismutase (SOD) and Catalase are the foremost antioxidative enzymes which are highly specific in scavenging reactive oxygen species. SOD scavenges superoxide radicals into hydrogen peroxide while CAT further reduces hydrogen peroxide into oxygen and water. Polycaprolactone (PCL) nanospheres were synthesized using double emulsion (w/o/w) solvent evaporation technique encapsulating SOD and CAT. The synthesized nanospheres were subjected to characterization which revealed its spherical-shaped nature, nanometer size range, homogenous distribution throughout synthesis and with high *in vitro* protein release into the medium. Oxidative stress alleviating property of SOD/CAT encapsulated biodegradable PCL nanosphere was analyzed using Human skin HaCat cells. Human skin is constantly exposed to adverse physical as well as chemical environmental pollutants. Oxidative stress induction takes plane into the directly and indirectly into the skin thorough increase in reactive oxygen species (ROS). Hydrogen peroxide, as external stress factor was employed to induce oxidative stress condition with analysis using ROS specific H_2DCFDA dye. SOD/CAT encapsulated PCL nanosphere was found to impart better antioxidative defense in H_2O_2 induced oxidative stress in Human skin HaCat cells as compared to PCL encapsulating either SOD or CAT alone as well as in comparison to direct supplementation of free SOD and CAT enzyme into the medium. The *in vitro* supplementation of SOD and CAT encapsulated into biodegradable PCL, i.e., PCL(SOD+CAT) nanosphere has shown significant advantages towards reducing H_2O_2 induced oxidative stress on human skin HaCat cells in comparison to direct protein supplementation in solution form. The results show that antioxidative protein in encapsulated form with its effective release has better efficacy in overcoming oxidative stress condition.

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List of Publications and Conferences

Publications in peer reviewed international journals

From PhD work:

1. **Sushant Singh**, Anil Verma and Vikash Kumar Dubey. Superoxide Dismutase-Multiwalled Carbon nanotube biocatalytic conjugate towards alleviating induced oxidative stress. (Manuscript under preparation)

2. **Sushant Singh**, Abhay Narayan Singh, Anil Verma and Vikash Kumar Dubey. Biodegradable polycaprolactone (PCL) nanosphere encapsulating superoxide dismutase and catalase enzymes. *Applied Biochemistry and Biotechnology*. 171: 1545-1558, 2013.

3. **Sushant Singh**, Abhay Narayan Singh, Anil Verma and Vikash Kumar Dubey. A novel superoxide dismutase from *Cicer arietinum* L. seedlings: purification and characterization. *Protein and Peptide Letters*. 20: 741-748, 2013.

4. **Sushant Singh**, Anil Verma and Vikash Kumar Dubey. Effectivity of antioxidative enzymatic system on diminishing the oxidative stress induced by aluminium in chickpea (*Cicer arietinum* L.) seedlings. *Brazilian Journal of Plant Physiology*, 24: 47-54, 2012.

From collaborative work:

5. Abhay Narayan Singh, **Sushant Singh** and Vikash Kumar Dubey. Immobilization of Procerain B, a Cystein Endopeptidase, on Amberlite MB-150 Beads. *PLOS ONE*, 8(6): e66000 doi: 10.1371/journal.pone.0066000, 2012.

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7. B. Praveen Kumar, **Sushant Singh** and Vikash Kumar Dubey*. Effect of Arsenic stress on *Vigna radiate*: A Biochemical studies. *International Journal of Environmental Science and Engineering Research*. 1: 1-4, 2010.

Publications in Conferences/Workshops

1. **Sushant Singh**, Anil Verma and Vikash Kumar Dubey. Polycaprolactone (PCL) nanoencapsulation of Superoxide Dismutase/ Catalase towards elevating antioxidative defense against generated oxidative stress. 3rd International conference on Advance Nanomaterials and Nanotechnology (ICANN-2013). IIT Guwahati, Guwahati, Assam. Dec 1st -3rd, 2013.

2. **Sushant Singh**, Anil Verma and Vikash Kumar Dubey. Environmental stress induced Superoxide Dismutase: A Novel Antioxidative protein against Oxidative Stress conditions. TechEvince 1.0, The Annual Research Exhibition. IIT Guwahati, Guwahati. November 9-10, 2013.

3. **Sushant Singh** and Vikash Kumar Dubey. Biomarkers: Effect of pollutants on biological systems. Quality improvement programme (QIP) on "Green Chemistry and Clean Technology". Organized by Centre for Environment, IIT Guwahati. November 26-28, 2012.

4. **Sushant Singh**, Abhay Narayan Singh, Anil Verma and Vikash Kumar Dubey. Nanoencapsulating superoxide dismutase/catalase towards enhancing antioxidative defense against generated oxidative stress. 81st Annual Meeting of the Society of Biological Chemists (India) & Symposium on Chemistry and Biology: Two Weapons against Diseases. Science city, Kolkata (W.B). November 8-11, 2012. *(Poster presentation)*

5. Attended International Symposium on "BIOENGINEERING 2012" (ISBE 2012), Indian Institute of Technology Guwahati, Assam. India 10th December, 2012.

6. **Sushant Singh**, Abhay Narayan Singh, Anil Verma and Vikash Kumar Dubey. Response to metal stress in plant: Superoxide dismutase, its purification and characterization from *Cicer arietinum* L.seedlings. One day symposium on "Environment and Us". IIT Guwahati, Guwahati. June 05, 2012. *(Poster presentation)*

7. Attended Young Ecologist Talk and Interact (YETI) - 2011, IIT Guwahati, Guwahati, Assam. December 13-15. 2011.

8. **Sushant Singh**, Abhay Narayan Singh, Anil Verma and Vikash Kumar Dubey. Heavy metal stress induced Superoxide dimutase: Purification and characterization from *Cicer arietinum* L. seedlings. 80th Annual Meeting of the Society of Biological Chemists (SBC). Central Institute of Medicinal and Aromatic Plants (CIMAP). Lucknow (U.P). November 12-15, 2011. *(Poster presentation)*

9. **Sushant Singh**, Anil Verma and Vikash Kumar Dubey. Effects of increasing Aluminium metal stress in germinating chickpea (*Cicer arietinum*) seeds. International Conference on 'Climate Change and Water: Assessing vulnerability, impact, and adaptation in the Eastern Himalayas''. IIT Guwahati, Guwahati, Assam. January 3-5, 2011. *(Poster presentation)*

10. **Sushant Singh**, Anil Verma and Vikash Kumar Dubey. Oxidative stress analysis in germinated Chickpea (*Cicer arietinum*) seeds under Copper ions. International

Conference on Frontiers on Biological Sciences (INCOFIBS). NIT,Rourkela, Rourkela, Odisha. October 1-3, 2010. *(Oral presentation)*

11. Two day National Workshop on Bioinformatics, Organized by M.I.E.T, Meerut and Mascon Global Ltd. November 16-18, 2007.