CHAPTER 1: INTRODUCTION

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of the Sub-Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Antioxidants</td>
<td>7</td>
</tr>
<tr>
<td>1.2</td>
<td>Super oxide Dismutase</td>
<td>8</td>
</tr>
<tr>
<td>1.3</td>
<td>Aim and scope of the study</td>
<td>14</td>
</tr>
<tr>
<td>1.4</td>
<td>The objective of this research is</td>
<td>16</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Scholars and scientists are of the opinion that Micro organisms in nature are man’s most best friends and sometimes worst enemies. Scientific history reveals that since 6000 B.C Microorganisms have been exploited for many useful purposes, and the application part of microorganisms are very useful in the production of desirable Metabolites. One of the most fascinating aspects of man’s scientific and technological developments is the harnessing of the activities of microorganisms. From the point of Industrial Microbiology, it is believed and proved that, Microorganisms are considered as chemical factories in miniature. Microorganisms are becoming more attractive as commercial exploitation is concerned, because they have potential to produce new therapeutic agents and to convert relatively inexpensive raw materials into high value products of human value. During this present century, 2 important and dramatic experiments are conducted, they are the discovery of antibiotics and development of Recombinant DNA technology. The significant natural sources of therapeutically active molecules are found in Microorganisms and plants. As a result of diversity of pathways that exist in the Metabolism of Microorganisms and the rich variety of bioactive molecules that they produce such as organic acids and antibiotics, Microbes can be exploited to get various Bioactive substances. Bioactive substances are low molecular weight compounds (generally molecular weight is < 3000 K.D) produced in low concentrations which exhibit various activities (Good fellow; et al. 1984, Schatz. et al,

Amylases are the most useful enzymes used since many decades in various fields such as food industries, Textile industries, detergent industries and paper industries. In food industry amylases is used to convert starch into sugar syrups, and in fermentation of food, feed. Since amylases are widely used in many industries, the production of amylases from microbial origin has started in 1960s. Most commonly B.subtilis and Aspergillus niger were used in industrial scale and produced glucoamylase. Glucoamylase replaced acid catalysis during the production of cyclodextrins from starch. Amylases are produced both by Bacteria and Fungi. But bacterial amylases are more preferred than fungal amylases because of their characteristic advantages.

Fungi are able to survive successfully in harsh or extreme environmental conditions and also they are able to synthesize rare enzymes which are able to carry out difficult chemical reactions. Industrially these biocatalysts are employed to carry out processing in timber industry, plastic industry, paints industry, polythene industry
and jet fuel purification. Many fungal enzymes are already used in the processing of paper and pulp industry and the process is known as biobleaching and biopulping. Many fungal enzymes are found to convert broad range pollutants (bioremediation and biodegradation) into potentially useful substances.  

Like other enzymes the target enzyme plays a significant part in cleaning up other chemical wastes. Respiration in aerobic organisms naturally promotes the synthesis and accumulation of ROS in cells. The ROS species are considered as toxins as they seriously damage the cells integrity. In cells ROS produces due to various reasons such as partial reduction of oxygen during aerobic respiration or due to oxidation of fatty acids or due to environmental process. Cells have got the defense mechanism to destroy ROS. Unfortunately all the produced ROS could not be eliminated completely by the cells own defense mechanism. When detoxification is incomplete, ROS remains within the cells and this results in oxidative stress. Due to partial detoxification, ROS may disturb the molecular events and finally causes damage to DNA and can also lead to senescence, ontogenesis, and neurological diseases. To overcome these cellular damages all aerobic organisms naturally possess mechanisms to neutralize or eliminate ROS and a part of this mechanism is known as the ‘Reprogramming of gene expression’. Cells have got several types of antioxidant enzymes such as super oxide dismutase, peroxidase and catalase. These group of antioxidants limits the levels of ROS. Singlet oxygen (O2·⁻) does not combine with many compounds because of its
unpaired electrons. By accepting one or two or three electrons. Activity of oxygen increases and become superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and Hydroxyl radicals (OH·) respectively. These are known as mediators of oxidative stress. And these are collectively known as ROS. ROS is a term that includes all highly reactive oxygen containing molecules including free radicals. Various types of ROS are known, they include super oxide anion radicals (O$_2^-$), Hydrogen peroxide (H$_2$O$_2$), Singlet oxygen, Nitric oxide radical, Hypochlorite radical and various lipid peroxides. All of these ROS are found to combine or react with membrane lipids, Nucleic acids, proteins, enzymes and other small molecules and finally results in cellular damage$^{17}$. When individuals got exposed to radiations, heavy metals and redox active compounds, ROS are generated and accumulated in cells. ROS are also synthesized under normal metabolic processess. ROS induces oxidative stress trough a number of enzymatic cascades and pathological processess$^{18}$. When cells defense mechanism could not completely detoxify oxidative stress, then Oxygen toxicity increases and damages DNA, RNA, proteins and lipids. It has been evidenced by various researchers that ROS induces apoptosis (the process of programmed cell death: PCD) $^{19}$. It is been experimentally proved that free radicals bring various physical, Biochemical and pathological changes as the age proceeds$^{20}$. Animal experiments have revealed that free radicals are involved in cellular injury that is observed in neurodegenerative diseases such And there is aggrandize evidence that free radicals are involved in the
initiation of cellular injury observed in neurodegenerative diseases such as, Parkinson’s disease (PD), Huntington’s disease (HD), Alzheimer’s disease (AD), \(^{21}\). It has been proved that ROS is involved in all stages of oncogenesis. Free radicals are also important in the development of diabetes\(^{22}\).

1.1 **ANTIOXIDANTS:**

“Antioxidants” can be defined as group of enzymatic molecules which are able to neutralize or deactivate oxygen free radicals before they oxidize or damage the cellular or molecular system. Human beings cell system have got clusters antioxidant mechanism (enzymatic and non-enzymatic), that functions sometimes singly or in combination with one another. In this systematic manner antioxidants protect cellular and organ system from free oxygen radical damage. These antioxidant molecules exist endogenously or obtained exogenously through the food we consume or as nutritional supplements. It has been found that few dietary antioxidant components do not deactivate free radicals directly, but work with endogenous antioxidants, such molecules are also been included and classified as antioxidants\(^{23}\). Living organisms possess Non-enzymatic antioxidants such as glutathione, uric acid, bilirubin and thiols, such as thioredoxins, glutathiones, and lipoic acids; melatonin, albumin, carotenoids, natural flavonoids and other nutritional compounds such as phenols, vitamin E and vitamins C and also enzymatic antioxidants. Enzymatic antioxidants are superoxide dismutases, catalase, and glutathione peroxidases [GSHPx]\(^{24}\). Few of the
antioxidant’s sources are obtained by nutrition diet (Exogenous antioxidant); Such exogenous antioxidants fall into phenol family. These Nutritional antioxidants are primarily known as scavengers of free radicals, which are significant in many molecular events, such as

1) Directly neutralize free radicals
2) Reduces the levels of Hydrogen peroxide and carry out repairing of oxidized membranes
3) Production of reactive oxygen species will be lowered by Quenching iron
4) short-chain fatty acids and cholesteryl esters undergo lipid metabolism, finally free oxygen species will be neutralized

1.2. SUPER OXIDE DISMUTASE

Everyone requires oxygen to stay alive, and the same oxygen contributes to the formation of deleterious oxygen free radicals. Many published research articles reveal that free radicals leads to the initiation of degenerative disease and also accelerated aging. Superoxide dismutase (SOD), catalase, and glutathione peroxidase are produce naturally by the cells of young human beings to protect against free radical damage during normal physiological conditions. production of SOD and other antioxidant enzymes decrease with age, and leads to age related degenerative diseases. Recent researches in nutritional science now advice adults to compensate the loss by powerful exogenous antioxidant enzymes nutritionally.

The deleterious effects Oxygen was first noticed during 1878, when the experimental models come across pure oxygen. Later in
1899, during World War II, while in Battle of Britain, pilots were breathing pure oxygen for many hours’ everyday showed the development of emphysema, scarred lungs, and greatly induced aging. during those days, the pilots were seemed to be aged three times more than their actual age. Similar incidents of oxygen toxicity was further noticed in the 1940s, neonatal when kept in oxygen incubators. Few of those neonatal became blind since their optical tissues had not been accumulated with dietary antioxidant carotenoids to protect from the deleterious effects of higher oxygen concentrations\textsuperscript{27}.

When lungs of animals exposed to increased concentration of oxygen have shown massive tissue damage, and this condition is known as hyperoxia. Hence oxygen at higher-than-normal concentrations oxygen is found to be toxic element. Oxygen consumptions by humans is associated with the synthesis of powerful free radicals\textsuperscript{28}. Oxygen in its ground state is called molecular oxygen or dioxygen is relatively unreactive, but it is capable of giving rise to lethal reactive excited states as free radicals and derivatives. Utilization of oxygen proceeds most readily via a complete stepwise pathway, in which 4 electrons get reduced to water, during which partially reduced reactive intermediates are generated. The reactive species of reduced dioxygen include the superoxide radical (\textit{O}_2\textsuperscript{-}), Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and the hydroxyl radical (\textit{OH})\textsuperscript{29}.
Superoxide dismutases (SOD, EC 1.15.1.1) was first discovered by Irwin Fridovich, an American biochemist along with Joe McCord, a graduate student of Irwin Fridovich in 1969. His research paper on SOD was published 39 times by the year 2009; PubMed has revealed that approximately 49,000 papers are published on superoxide dismutase from the date of its discovery. Since SOD is found in all most all organisms, both aerobic and anaerobic organisms and also SOD plays a major part in reducing the damage caused by oxidative stress conditions. Initially SODs were believed as metalloproteinase and their function was unknown. All SODs irrespective of source are multimeric metalloproteins that are very efficient at scavenging the superoxide radical. The Cu/Zn SODs as well as most prokaryotic Mn SODs and Fe SODs are dimeric, whereas Mn SODs from mitochondria and certain thermophilic bacteria are tetrameric. Previously Cu,Zn SOD was termed as Erythrocuprien. Due to immense research on SOD, now it is been regarded as antioxidant that mainly catalyses detoxification of ROS, as a result of which hydrogen peroxide is generated. Finally, hydrogen peroxide will be converted into water by the enzymatic action of catalase or peroxidase. Superoxide ions undergo sequential oxidative and reductive reaction by SOD's neutralization activities. Richard cutler during 1980s, conducted research at Gerontology Research Center, the National Institute of Health, revealed that the longer living mammals had produced higher concentrations of tissue SOD and serum SOD than those with lower life span mammalian SOD levels.
Cutler’s research also emphasized that rodents have been shown to produce lowest SOD levels among mammals, and this SOD concentration is highest when compared to more highly evolved mammals. In case of humans the relative concentration of SOD is quite highest\textsuperscript{35}.

Humans beings who live nearly 80 years had produced an approximate of 90 $\mu$g/ml of SOD. Our closest primates, chimpanzees, live for about 40 years had produced 40 $\mu$g/ml of SOD. Cutler’s investigations on experimental cross-species emphasize that in mammals SOD is a primary determinant of longevity\textsuperscript{36}. Higher levels of SOD production in mammals is playing a key role in evolution from shorter to longer life spans.

Most of the higher organisms, plants, microorganisms, and animals possess at least one type of antioxidant mechanisms or enzymes. The research has revealed that vegetarian diet rich such as fruits, vegetables, and green leaves decreases the problems of degenerative diseases and other disorders by inhibiting oxidative stress\textsuperscript{37}. Not only the higher organisms, microbes are also producing SOD efficiently. As microbes also get exposed to oxidative stress during aerobic respiration and metabolic activities. To destroy oxidative stress conditions microbes have evolved antioxidative defense mechanism. Hence microbes could be the significant source for the production of SOD industrial scale\textsuperscript{38}. Most commonly \textit{Corynebacterium glutamicum} is presently used for the production pharmaceutical grade amino acids, vitamins successfully in large
quantities\textsuperscript{39}. \textit{Corynebacterium glutamicum} during the production of amino acids will get exposed to high oxidative stress as a consequence of this SOD is produced abundantly. Corynebacterial genes have been cloned successfully, hence cloning strategies could be used to enhance SOD production\textsuperscript{40}. SOD by scavenging free radicals, reduces oxidative stress and finally reduces the risk of diseases\textsuperscript{41}.

Numerous studies on efficiency and safety of SOD has been conducted on animal models and human individuals. It has shown that SOD is a potent anti-inflammatory agent\textsuperscript{42}. Therapeutically SOD is a significant drug and various researchers have described many therapeutic applications. These therapeutic applications are prevention of tumor promotion, prevention of carcinogenesis, protection of tissues and traumatic of burn injuries and in the treatment of arthritis and inflammatory diseases \textsuperscript{43}. SOD's are efficient in the treatment of inflammatory diseases, as well as in the treatment of skin ulcers, caused due to burns and wounds. For such conditions SODs were administered in the form of injections encapsulated with liposoms and it was found to be effective\textsuperscript{44}. It is found that destruction of joint tissues will be inhibited or suppressed directly by SOD. SOD decreases arthritic inflammation by limiting positive feedback between secretion of ROS and inflammatory cytokine production\textsuperscript{45}.

"Orgotein" is a pharmaceutical form of SOD approved in many countries as an efficient anti-inflammatory agent. Orgotein is recommended for the treatment of degenerative diseases and aslo in
the general treatment inflammations and degenerative diseases. Research is constantly being conducted on SOD's and other antioxidant agents efficiency and safety on experimental models. During the research scholars and scientists have noticed significant changes such as promotion of hair growth, reduction in hair loss, amazing reduction in side effects. Such as radiation induced and fibrosis (radiofibrosis), and sclerosis. Also found effective in irradiation for breast cancer. These changes have been observed when only Cu,Zn superoxide dismutase therapy is administered. SOD could also be proposed as effective antifibrotic drug for hepatitis C related fibrosis. Besides these applications, SOD can also be used as one of the component in many drugs that are used in the treatment of myocardial ischemia, Peyronie's Disease, multiple sclerosis, colitis. It is also been evident that SOD influences suppression of diabetic retinopathy. Amazing improvements seen when SOD is administered on Behcet's syndrome.

"Tempol" an another pharmaceutical preparation is observed to be beneficial in reducing acute kidney injury and hypertension developed in experimental animal models. SOD's cream preparation is recommended for topical applications for conditions such as burns, systemic lupus erythematosus (SLE), progressive systemic sclerosis (PSS), Behcet's disease, and herpes simplex. Topical applications are beneficial when symptoms are stabilized. For the same cases wound
excision is done when burn or wound is sever. In such conditions SOD’s are suggested for management of pathogenesis.

1.3 AIM AND SCOPE OF THE STUDY:

Mankind has been greatly benefited by the technological advancement and man has used science as an organ of enquiry to explore nature. The torrent of development is science and technology is a reason for the tumult in the world.

Twenty first century has seen biology emerging as one of the priority in science with biotechnology gaining a new status.

The most important research includes finding most beneficial microbial metabolites from rare, slowly growing and simply neglected microbes from various habitats, where the different ecological conditions and requirements may have produced different types of contrastive organisms, occurring in environment. During the past 20 to 30 years, several compounds have been isolated from eukaryotic yeasts and yeast like fungi and many of this has been reported to have biological catalytic activities. Some of which are of interest from the viewpoint of potential drug development.

So far, more than hundreds of new unique enzyme molecules isolated from fungi. The baker’s yeast is remaining as an unexploited source for many enzymes and pharmacologically active substances as well as for enzymes for industrial usage. Before the advent of modern therapy, the treatment of disease in general and of neurological degenerative disorders in particular by means of extracted enzymes was arbitrary and fragmentary in nature. The isolation of industrially
useful amylases opened a new era in the field of medicine for their potential therapeutic applications\textsuperscript{11}. The introduction of SOD in the treatment of various neurological degenerative disorders completely revolutionized medical sciences and medical practice.

Ever since the isolation of SOD in 1969 by Irwin Fridovich and his graduate student Joe McCord\textsuperscript{30}, the SOD received attention of the scientist all over the world. The yeasts like \textit{fungi} are prolific SOD enzyme producers in the laboratory and factory.

The wide range of usefulness of SOD produced by genetically modified \textit{saccharomyces} makes them the most important of all the SOD enzymes produced microorganisms.

Scientists have proved that genetically modified \textit{saccharomyces} is the most potential sources of SOD enzyme. Of all available fungi, the \textit{Saccharomyces} merits special consideration in view of their proven biosynthetic capabilities. The wide spread search for new enzyme producing \textit{saccharomyces} unleashed during the last two decades brought with it the problem of their classification. In the early attempts of species differentiation exclusively use was made highly valuable culture and physiological properties as taxonomic criteria and too little attention was paid to the more stable morphological characters\textsuperscript{58}.

\textit{Saccharomyces} is recommended for the industrial production of pharmaceutical grade SOD due to its diversity and proven ability. The above account indicates that intensive studies on various \textit{saccharomyces} species are still likely to yield purposeful results
towards isolation of SOD enzyme with high degree of specificity. As such it was felt that optimization and strain modification of *saccharomyces* is very much necessary. As a step in this direction, various *saccharomyces* were studied with their strain improvements for high productivity of SOD enzyme with great catalytic activity and high degree of specificity\(^59\).

1.4. THE OBJECTIVE OF THIS RESEARCH IS

- To study the fermentation profile isolation, purification and characterization of the super oxide dismutase from five wild type yeasts namely, *Kluyveromyces lactis* (1024), *Pichia fermentans* (1052), *Saccharomycodes ludwiggi* (1184), *Saccharomyces mellis* (1148) and Baker's Yeast.

- To screen 5 wild type yeasts for maximum Biomass yield on YPD with altered carbon and Nitrogen source at various pH, Temperature values.

- To extract and assess the super oxide Dismutase (SOD) productivity in 5 wild type yeasts, which were grown on YPD broth with altered carbon sources and Nitrogen sources at optimal pH and Temperature values.

- Optimization of fermentation parameters to improve the yield for SOD enzyme production from selected *saccharomyces* species.

- Carrying out strain improvement for high productivity with great degree of specificity of SOD enzyme from selected *saccharomyces* species.