CHAPTER - 3

THEORETICAL ANALYSIS
### Chapter - 3. Theoretical Analysis

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3. THEORETICAL ANALYSIS

3.1 YEAST

Yeasts are classified under kingdom Fungi, over 1,500 species currently recognized as Yeast species\textsuperscript{60}. Yeasts are Eukaryotic organisms some are unicellular, and many are multicellular budding cells that possess false hyphae or pseudohyphae\textsuperscript{61}. This characteristic feature is seen in most molds as well. Size of yeasts varies from species to species. Some yeasts measure 3-4 µm in diameter few could measure up to 40 µm \textsuperscript{62,63}. Both sexual and asexual mode of reproduction is observed among yeasts. Asexually yeasts multiply by budding. Since many centuries yeasts are being used in baking and in the preparation of alcohol and alcoholic beverages\textsuperscript{64}.

As yeasts carry out fermentation of carbohydrates into carbon dioxide and alcohol\textsuperscript{65}. Yeasts are the most deeply researched eukaryotic microorganisms. Hence yeasts have become model organisms in the field of Modern cell biology and Genetic engineering\textsuperscript{66}. Some species of yeasts, such as \textit{Candida albicans}, causes infectious diseases in human individuals and are opportunistic pathogens\textsuperscript{67}. Recently Yeasts are suggested for electricity generation and for bio fuel industry\textsuperscript{68}.

3.2 NUTRITION AND GROWTH

Nutritionally all Yeasts are chemoorganotrophic, as they utilize organic compounds as energy source. They are not phototrophs. They use fructose and glucose which are hexose sugars,
and maltose, sucrose, which are disaccharides as carbon source. Few species are found to grow best on pentose sugars like ribose sugars. They can also metabolise and grow profusely on alcohol and organic acids. Yeast are both obligate aerobes and facultative anaerobes. But Obligate anaerobes are never observed. Yeasts favour to grow in neutral to slightly acidic pH range.

Yeasts grow in a wide temperature ranges. Some of the examples are; favorable temperature for Candida slooffi is 28 to 45°C, similarly, for Leucosporidium frigidum grows between -2 to 20°C, and Saccharomyces telluris grows between 5 to 35°C. Reduced viability is observed in certain species that survive in freezing temperature.

Under laboratory conditions, yeasts can be grown on liquid and solid media. Yeasts could be cultivated well on yeast mould agar or broth (YM), Wallerstein Laboratories nutrient (WLN) agar, potato dextrose agar (PDA) or potato dextrose broth, yeast peptone dextrose agar (YPD). Particularly brewer's yeast are commonly cultivated on Dried malt extract (DME) agar growth medium. While selectively wild or indigenous yeasts has to be grown, antibiotic cycloheximide is added to media to avoid contamination. by Saccharomyces yeasts.

3.3 Ecology

Naturally Yeasts are known to grow on skin of fruits, particularly the citrus fruits such as peaches, apples, grapes. also commonly isolated from sugar-rich material. They are also found to grow on plant exudates such as cacti or plant saps. yeasts are known
to grow in association with other microbes and on insects in soil as well \(^73\). However, ecologically yeasts are known to occur on a diversified habitats. Some species of Yeasts such as *Torulopsis*, *Rhodotorularubra*, *Candidaalbicans*, and *Trichosporon cutaneum*, are the common skin micro flora in human beings. They lie between toes and sometimes become opportunistic pathogens too. Some species of Yeasts are common micro flora in gut of mammals and in gut of some insects\(^74\). Some of the Halophillic yeasts are found in deep-sea environments\(^75\).

45 species of yeasts from 16 genera have been found to colonize in stomach of 7 Honey bees species and also in nectarines of 9 flowering plant species. Among these 45 species most were belonging to the Candida genera. The most common yeast species found in stomach of honey bee is *Dekkera intermedia* and *Candida blankii* in flower nectaries\(^76\). Yeasts by occupying nectarines increases the temperature of flowers as a result of which volatile organic compounds will get evaporated and attracts the pollinators\(^77\). Many yeast species are found in mutualistic relationship with ants and some other fungus. Few yeasts are known to be antagonistic with other bacteria\(^78\).

### 3.4 CLASSIFICATION

The term *yeast* is often taken as a synonym for *Saccharomyces cerevisiae*, \(^79\) but the phylogenetic diversity of yeasts is shown by their placement in two separate phyla. The *Ascomycota* and
the Basidiomycota. The budding yeasts ("true yeasts") are classified in the order Saccharomycetales. Table: 3.1

<table>
<thead>
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<th>Scientific classification</th>
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<td>Domain:</td>
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<td>Kingdom:</td>
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<td>Division:</td>
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<td>Subdivision:</td>
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<td>Class:</td>
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<td>Order:</td>
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<td>Family:</td>
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<td>Genus:</td>
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3.4.1. Species

Saccharomyces bulderi, Saccharomyces cariocanus, Saccharomyces cariocus, Saccharomyces bayanus, Saccharomyces boulardii, Saccharomyces exiguus, Saccharomyces florentinus, Saccharomyces martiniae, Saccharomyces monacensis, Saccharomyces uvarum, Saccharomyces zonatus, Saccharomyces cerevisiae, Saccharomyces chevalieri, Saccharomyces dairenensis, Saccharomyces ellipsoideus, Saccharomyces eubayanus, Saccharomyces kluyveri, Saccharomyces pastorianus, Saccharomyces spencerorum, Saccharomyces turicensis, Saccharomyces unisporus, Saccharomyces paradoxus, Saccharomyces norbensis.
3.5. MORPHOLOGY

Some of the yeasts are unicellular and many are multicellular. Some yeasts are dimorphic that exhibit both Yeast phase (unicellular) and filamentous phase (multicellular). Multicellular yeasts have pseudohyphae. Hyphae are filamentous structures with cross walls called septa between other cells. A network of hyphen spreads to acres in soil or can cover an orange peel is called Mycelium. Mycelium as a part of its metabolic activity absorbs nutrients, secretes digestive enzymes and participates in sexual mode of reproduction. Mycelia of some rare fungal species capture live animals for prey. Mycelia organise into various shapes and sizes. Some yeasts grow like plants with rooting and shooting, such fungi are known as Rhizomorphs. Such rhizomorphs grow quickly over plants and trees or on forest floor. Some fungi grow into tough, rock like mosses, such mycelia are called sclerotia. Since sclerotia are tough structures they exist in extreme environmental conditions such as freezing and drying out\(^{82-85}\). During sexual reproduction, mycelia forms sporangia, that gives rise to spores. These sporangia forms during asexual reproduction also. Sexual spores are known as Meiospores and asexual spores are known as Mitospores or conidia. The morphology of sporangia plays a major role in identification and classification of Fungi. As morphology sporangia varies from one fungi to another\(^{86,87}\).

3.6. YEAST IDENTIFICATION

Yeasts are identified on the basis of morphology and by biochemical tests. Sometimes a combination of both are used to
identify the yeasts. Morphological criteria is primarily used to establish genera. Whereas, biochemical tests are conducted to differentiate among various species. Based on the following criteria yeasts are identified.

1. Culture characteristics - shape, Size, Colour of the Colony, and texture are considered.

2. Based on asexual structures;
   a. Size and shape of reproductive cells are noticed.
   b. Yeasts are differentiated based on asexual structures or activities such as budding, fission, bipolar, multiple or unipolar cells.
   c. Based on the presence or absence of ballistoconidia, arthroconidia, blastoconidia and clamp connections, germ tubes, endoconidia, sporangia, sporgangiospores, hyphae, pseudohyphae,

3. Sexual structures ;
   a. Presence or absence of ascospores or basidiospores are observed.
   b. Shape and size of ascospores or basidiospores are considered and also their number, cell wall pattern and arrangement are considered

4. Physiological studies are considered.
   a. Assimilation capabilities are considered.
   b. Resistance to Cycloheximide are considered
   c. Fermentation criteria
   d. Nitrogen utilization tests are considered
e. Urea hydrolysis tests are considered

f. Temperature and pH studies are performed\textsuperscript{89-91}.

\textbf{3.7. YEAST METABOLISM}

Metabolism includes assimilation pathways or anabolism and catabolic pathways or dissimilation of nutrients by the cells. All these metabolic pathways are mediated and regulated by enzymic reactions. Assimilation pathways are cellular material production process with a series of reductive reactions. Whereas dissimilative pathways are oxidative processes that remove electrons utilizing NADP or NAD as co-factors. Yeasts have exhibited diversified mechanisms to generate and consume energy needs in the form of ATP by breaking down organic compounds\textsuperscript{92,93}.

Thorough understanding of complete metabolic processes is of great importance to the field of Biotechnology and Pharmaceutical sciences. And also it is important to exploit the new metabolic capabilities of particular yeasts. It is well known that majority of yeasts utilize sugars as their source of carbon and energy needs. But there are certain yeast species which can metabolise non-conventional carbon sources. Yeasts synthesise amino acids and proteins by breaking down simple nitrogenous sources. Metabolism of phosphorous, sulphur and other inorganic compounds are also well studied in yeasts predominantly in \textit{Saccharomyces cerevisiae}\textsuperscript{94,95}. 
3.7.1. REGULATION OF BIOCHEMICAL PATHWAYS:

3.7.1.1. Biochemical pathways in yeasts are regulated at various levels:

By various enzymic reaction biochemical pathways are regulated in yeasts at various levels

(i) Enzyme synthesis - involves induction, repression and derepression of gene expression;

(ii) Enzyme activity - involves mechanisms such as allosteric activation, inhibition, or interconversion of isoenzymes;

(iii) Cellular compartmentalization - involves localization of particular metabolic pathways in the cytosol, mitochondria, peroxisomes, or the vacuole;

(iv) Transport mechanisms - involves sequence of activities such as internalization, secretion, trafficking of compounds between the various cellular compartments96-98.

3.8. RESPIRATION VERSUS FERMENTATION

Yeasts undergo either fermentation or respiration for energy production. Based on this certain yeasts can be categorized variously. These processes are regulated by environmental factors and enzymic reactions. Yeasts to the varying surrounding growth environments, adopt variously. The existing pathways in yeasts depends on growth conditions. The utilization of glucose and oxygen is best documented in yeasts. *S. cerevisiae* metabolize glucose depending on presence of oxygen and other carbon sources. When glucose or an initial product of glucose metabolism makes catabolite repression by repressing
synthesis of gluconeogenic enzymes and various respiratory enzymes. Hence on rapid addition of glucose catabolite repression results. Available glucose or other sugars sends signals to not to synthesize new enzymes and it is a part of gene repression. Glucose will get metabolized to carbon dioxide and ethanol. Hence fermentation process will take place when oxygen is available and glucose is available in bulk concentration\(^99,100\). In batch cultures when glucose concentration declines, cells also get deprived, hence cells will switch over to respiratory mechanism and respiratory enzymes synthesis is induced. This is known as diauxic shift as the second phase of growth has been initiated\(^101\). Catabolite repression and certain enzymes such as fructose 1,6-bisphosphate which is a key enzyme will get limited. cAMP works as a second messenger in regulatory catabolic repression\(^102\).

**3.9. ENZYMES FROM FUNGI:**

Fungi have the ability to survive in extreme environmental conditions and are found to produce rare and unusual enzymes. These enzymes can catalyze amazing chemical reactions. Hence they have the ability to act on tough substances like paints, plastic, jet fuel, and wood. And convert them into useful simple nutrients. Such enzymes are already employed in pulp and paper industries and the process is known as biopulping and biobleaching respectively. Fungal enzymes are also suggested for breaking down persistent pollutants into non-harming simpler substances, and the process is known as bioremediation and biodegradation\(^103-105\).
Table 3.2 Applications of Enzymes in Industrial and Environmental Processes\textsuperscript{106-110}.

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Name of the Enzymes</th>
<th>Substrates it acts on</th>
<th>Applications</th>
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<tbody>
<tr>
<td>1</td>
<td>Lactases, cellulases, pectinases, xylanases, mannases, esterases, lipases, lignin peroxidases, manganese peroxidases</td>
<td>cellulose, pitch, lignin, hemicellulose,</td>
<td>manufacturing of pulp and paper</td>
</tr>
<tr>
<td>2</td>
<td>Laccases, cytochrome P450, monoxygenases, esterases, lipases, lignin peroxidases, manganese peroxidases</td>
<td>DDT, polyaromatic hydrocarbons (anthracene, fluoranthene, benzopyrene), azo and heterocyclic dyes, tropaeolin, azure B, nitrotoluene, creosote, diesel oil, plastics, chloroaniline, lindane, chloro-dibenzo-p-dioxines, chlorobiphenyls, chlorophenols,</td>
<td>waste treatment and Bioremediation</td>
</tr>
<tr>
<td>3</td>
<td>Esterases, peroxidases, laccases</td>
<td>Coal</td>
<td>coal liquefaction</td>
</tr>
<tr>
<td>4</td>
<td>Proteases, lipases, amylases, cellulases</td>
<td>cleaning laundry and dishes at a wide range of temperatures</td>
<td>Manufacturing of household and industrial detergents</td>
</tr>
<tr>
<td>5</td>
<td>Pectinases, glucomylases, cellulases, xylanases, proteases, invertases, alpha-amylases, lactases, glucose isomerases</td>
<td>clarification and extraction of juice, flavor enhancement, meat extraction, arome production, production of sugar, lactose modification, texture of pasta and noodles, production of baby formula</td>
<td>food processing</td>
</tr>
<tr>
<td>6</td>
<td>Proteases and lipases</td>
<td>removing fat and hair from skin and hide</td>
<td>leather processing</td>
</tr>
<tr>
<td>7</td>
<td>Amylases, catalases, cellulases, proteases</td>
<td>degradation of hydrogen peroxide, degumming, dye removal, de-sizing of starch, improve color brightness and smoothness,</td>
<td>Textile</td>
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### 3.10. OXYGEN TOXICITY:

Activated oxygen species such as singlet oxygen, hydrogen peroxide and hydroxyl radical are extremely reactive and cytotoxic in all organisms. These highly reactive species can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in the plasmalemma or intercellular organelles. Peroxidation damage of the plasmalemma leads to leakage of cellular contents, rapid desiccation and cell death. Intracellular membrane damage can affect respiratory activity in mitochondria, cause pigment breakdown and loss of carbon fixing ability in chloroplasts.

### 3.11. SUPEROXIDE DISMUTASES

Irwin Fridovich and his graduate student Joe McCord discovered superoxide dismutase. Initially SOD’s were regarded as metalloproteins but the function was not known. After enormous research on this wonderful enzyme, structure was...
elucidated. Pharmaceutical applications were understood as researchers started creating awareness regarding the SOD’s physiology, Biochemistry and other activities\textsuperscript{113}. SOD’s are metalloenzymes that function as scavengers whose active centers are occupied by metal motifs such as copper and Zinc or Manganese or either by iron. Based on the metal motifs, they are named as CuZn-SOD, Mn-SOD, and Fe-SOD respectively\textsuperscript{114}. The Fe SODs and Mn SODs are closely related because they share a high degree of amino acid sequence and structural homologies, but are unrelated to Cu/Zn SODs. The available data suggest that the 2 families of SODs must have evolved independently \textsuperscript{115}. SOD plays significant role in protecting aerobic life systems in all types of organisms starting from unicellular prokaryote to Human beings\textsuperscript{116}. SOD has been postulated as a reactant in the formation of highly reactive hydroxyl radical, SODs may play a central role in protecting chloroplast proteins and membranes against damage from reactive oxygen species \textsuperscript{117}. SOD neutralizes oxidative stress by converting singlet oxygen to Dioxygen and Hydrogen peroxide\textsuperscript{118}. Later Hydrogen peroxide will be neutralized by peroxidase. SOD is an intracellular enzyme found in all the cells of body of all organisms. In nature, 3 main forms of SOD is been determined\textsuperscript{119}, some of them listed below;

1. SOD enzyme with divalent copper and divalent zinc are found in erythrocytes of Human beings
2. SOD with trivalent manganese is found in mitochondria of chicken liver
3. SOD with trivalent iron is found in *E. coli*\(^{120}\).

The Cu,Zn-SOD is a dimer with 32,500 KD molecular weight. Disulfide bonds join Cu and Zn motifs. In cells of Human beings 2 types of SOD are identified. Human Mitochondria possess Mn-SOD, similarly, Cu,Zn-SOD is found in cytosol of human cells\(^{121-123}\).

The Cu/Zn SOD is the most prevalent SOD in plant tissue. In leaves the bulk of Cu/Zn SOD activity is contained in chloroplasts\(^{124}\).

Various metallic groups are found to associate with SOD as prosthetic group. Among the various types of SOD, the most prevalent enzyme is CuZn-SOD, followed by Mn-SOD, both the enzymes break down reactive oxygen species\(^{125,126}\). SOD is found to protect retina of eye from free radicals\(^{127}\). All organisms that undergo aerobic respiration generate ROS throughout their lives\(^{128}\).

These ROS molecules bind to DNA, proteins and lipids and causes permanent damage. Among the Superoxide radicals, hydroxyl radicals are the most dangerous species. To scavenge such radicals, cells have been provided with antioxidant enzymes\(^{129}\). Various oncogenic cells have been investigated and they will be lacking one or the other type of antioxidant enzyme. Hence cells will be triggered to cancerous and leads to mortality. re-expression of SOD cluster genes makes the cells immortal. Accumulation of various types of ROS in cells are could be one of the reasons for cancerous cells\(^{130-131}\).

SOD enzyme was given to the individuals with osteoarthritis of the knee to know the beneficial role of SOD. Treatment constituted 2
mg SOD three times per day, that amazingly reduced the pain. As a result of which pain was not noticed during walking and climbing stairs. SOD functions by decreasing inflammation. Ab-Ag complexes also trigger phagocytes to dump free radicals. These events signal WBCs to raise and cause swelling and inflammation etc. SOD clean up the ROS.

A crippling neuromuscular disease Amyotrophic Lateral Sclerosis (ALS), or Lou Gehrig’s Disease, is seen in usually age-old people. However, rarely because of change in life style, such symptoms are reported by teen age to middle age people. Prolonged research says that Mutations the SOD1 gene is the cause for Amyotrophic Lateral Sclerosis. SOD1 gene is responsible for the formation of Cu, Zn SOD. This enzyme detoxifies and neutralises oxygen free radicals, and also avoids cell and tissue damage. Is the SOD1 undergoes mutations, Cu,Zn-SOD enzyme will also gets mutated. Antioxidant property of the enzyme will also be lost, and it begins to exhibit peroxidaseal properties. Hence research suggests that, mutations to SOD1 gene may lead to nerve cell degeneration. In ALS patients, nerve cells begins to degrade and finally muscle control disrupts. An investigation about the mutations to Cu,Zn-SOD and inhibitors of Cu,Zn-SOD gene would be beneficial in studying the cause and treatment of ALS and other related diseases too.

SOD scavenges ROS and reduces oxidative stress which are the lethal substances in nature. SOD is found in majority of green plants,
Brussels’ sprouts, wheat grass and broccoli, which could be termed as Dietary antioxidants\textsuperscript{142}.

There are various types of SODs, metal co-factors are conjugated to the SOD. The three major types of SODs are,
1. Cu,Zn-SOD
2. Mn-SOD
3. Ni-Cu,Zn-SOD: Commonly found in all aerobic eukaryotes\textsuperscript{143}. It is an 8 stranded “Greek key” beta barrel, with the active site held between the barrel and two surface loops\textsuperscript{144}. The two subunits are tightly linked back-to-back, primarily by hydrophobic and some electrostatic interactions. The ligands of the copper and zinc are six histidine and one aspartate side chains; one histidine is shared between copper and zinc units\textsuperscript{145,146}.

As we know the cytosol of all aerobic organisms contain Cu,Zn-SOD, pharmaceutical grade Cu,Zn-SOD has been recovered and purified from the cytoplasm of bovine erythrocytes\textsuperscript{147}. The Bovine Cu,Zn-SOD is having a molecular weight of 32,500 daltons, and it is a Homodimer\textsuperscript{148}. Bovine Cu,Zn-SOD is the 1\textsuperscript{st} sequenced structure in 1975 among the family of SOD group of enzymes\textsuperscript{149}.

3.11.1. Iron or manganese used by prokaryotes and protists, and in mitochondria

\textit{E. coli} and many other prokariotic bacteria and protists possess iron as the metal sub unit called Fe-SOD. some bacteria contain Fe SOD, others possess Mn SOD, and some bacteria may contain both
Fe-SOD and Mn-SOD. Fe-SOD is found to occur in plastids of plants\textsuperscript{150,151}. The 3D structures of the homologous Mn and Fe SOD have the same arrangement of alpha helix, and their active sites contain the same type and arrangement of amino acid side chains\textsuperscript{152}.

Manganese SOD: Found in Chicken liver mitochondria, and many bacteria like \textit{E. coli}. Mn-SOD also occur in mitochondria of Human beings. The ligands of the manganese ions are 3 histidine side-chains, an aspartate side chain and a water molecule or hydroxy ligand\textsuperscript{154}. In plants Mn SOD are located in mitochondrial matrix. In maize and yeasts Mn SOD subunits are synthesized in the cytoplasm as larger molecular mass precursors, which are post translationally processed upon import into mitochondria\textsuperscript{155}.

Nickel SOD: This has a hexametric structure built from right handed 4 helix bundles, each containing N-terminal hooks that chelate a Ni ion. The Ni hook contains the amino aci motif His-Cys-X-X-Pro-Cys-Gly-X-Tyr it provides most of the interactions critical for metal binding and catalysis\textsuperscript{156,157}.

In higher plants, SOD isoenzymes occur different cell organelle. Mn SOD exists in mitochondria and peroxisomes. Fe SOD occurs mainly in chloroplasts, and also found in peroxisomes. Cu, Zn SOD exists in cytosol, chloroplasts, peroxisomes, and apoplast\textsuperscript{158,159}. In chloroplasts, the superoxide radical is produced by the univalent reduction of dioxygen during photosynthetic electron transport, particularly under conditions where carbon dioxide is limited and light intensity is high\textsuperscript{160}. 


3.11.2. SOD in Humans

Three types of superoxide dismutase are found in humans beings, other mammals, and in chordates. SOD1 gene codes for Cu,Zn-SOD and it is located in cytoplasm. SOD2 gene is responsible for Mn-SOD and it is located in the mitochondria, and SOD3 is extracellular. The Cu,Zn-SOD is a dimer (consists of two units), the other 2 are tetramers (four subunits). These genes are located on chromosomes 21, 6, and 4, respectively (21q22.1, 6q25.3 and 4p15.3-p15.1)\textsuperscript{161-164}.

3.11.3. Biochemistry

SOD protects the cell system by scavenging the reactions of superoxide. The superoxide anion radical (O$_2^-$) spontaneously dismutase's O$_2$ and hydrogen peroxide (H$_2$O$_2$) ($\sim$10$^5$ M$^{-1}$s$^{-1}$ at pH 7). The SOD dismutase's oxygen radical in second order with respect to initial superoxide concentration. Thus, the half-life of superoxide, although very short at high concentrations (e.g., 0.05 seconds at 0.1mM) is actually quite long at low concentrations (e.g., 14 hours at 0.1 nM). In contrast, the reaction of superoxide with SOD is first order with respect to superoxide concentration. Moreover, superoxide dismutase has the largest k$_{cat}$/K$_M$ (an approximation of catalytic efficiency) of any known enzyme ($\sim$7 x 10$^9$ M$^{-1}$s$^{-1}$),\textsuperscript{110} this reaction being only limited by the frequency of collision between itself and superoxide. That is, the reaction rate is "diffusion limited". Even at the sub nanomolar concentrations achieved by the high levels of SOD within cells, superoxide deactivates aconitase, the citric acid cycle
enzyme, and it can poison energy metabolism, and releases potentially toxic iron. Aconitase is one of several iron sulphur containing (de)hydratases in metabolic pathways shown to be inactivated by superoxide\textsuperscript{165-168}.

**3.11.4. Physiology:**

The main reactive oxygen species in the cell is superoxide. And this superoxide is efficiently discarded by SOD. To understand the catalytic activity of SOD in pathological aspect, genetically engineered Mice was developed which is lacking SOD enzyme internally. It was observed that mice lacking SOD2 gene died just few days after birth as a result of massive oxidative stress\textsuperscript{169}. Mice lacking SOD1 gene, developed series of pathological Consequences like, Hepatocellular carcinoma\textsuperscript{170}, age related muscle mass loss, cataract, and reduced lifespan\textsuperscript{171}. Mice lacking SOD3 was normal and did not show any pathological consequences and lifespan was also normal. But that mice was sensitive to Hyperoxic injury\textsuperscript{172}. Mice lacking any of these SODs genes, was more sensitive to drugs such paraquat and diquat\textsuperscript{173}. Similar findings were also reported from other investigations also. SOD1 gene lacking Drosophila showed reduced lifespan. Flies lacking SOD2 gene died before birth within the egg. In contrast *C elegans* lacking SOD did not show any major physiological disruptions. In yeast *Sacchormyces cerevisiae* null mutations did for SOD1 gene, this was detrimental to aerobic growth and post diauxic lifespan\textsuperscript{173-175}. 
3.11.5. *Role in disease*

Investigations shown that Mutations to SOD1 gene could be the primary cause for ALS, a form of motor neuron disease\textsuperscript{176}. The most commonly studied mutations are in the U.S. is A4V and G93A\textsuperscript{177}. The Mn-SOD and Fe-SOD are not linked to any disease in humans. But in mice SOD1 and SOD2 mutations causes perinatal lethality\textsuperscript{178} and hepato cellular carcinoma\textsuperscript{179}. It is noticed that ALS progresses sporadically with mutated SOD1 and SOD2, it is observed in 90\% of patients\textsuperscript{176,180}. In contrast over expression of SOD1 gene is linked to neural disorders seen in down’s Syndrome\textsuperscript{181}.

If Extracellular superoxide dismutase (SOD3, ecSOD) in mice diminishes, hypertension development is observed\textsuperscript{182}. Other investigations also highlights that suppression of SOD3 activity also leads to lung diseases such as Chronic obstructive pulmonary disease (COPD) or Acute Respiratory Distress Syndrome (ARDS)\textsuperscript{183-185}.

Normally SOD genes do not express in neural crest cells of developing foetus. Hence increased concentrations of free radicals in mother’s somatic cells can cause damage to the foetus and induce dysraptic anomalies (neural tube defects)\textsuperscript{186}.

3.11.6. *Pharmacological activity*

SOD has antioxy and anti inflammatory property. Experimental evidences states that inflammation of colitis could be reduced efficiently with SOD. SOD detoxifies reactive oxygen species by lowering the formation of oxygen free radicals and decreases oxidative
stress. SOD suppresses endothelial activation and modulates the factors that controls adhesion molecule expression and leukocyte endothelial interactions. Hence, antioxidants are significant in the treatment of inflammatory bowel disease\textsuperscript{187}.

Similar multiple pharmacological activities of SOD are also observed in various investigations conducted till date. SOD ameliorates cis-platinum-induced nephrotoxicity in rodents\textsuperscript{188}. Pharmacologically active purified bovine liver SOD is marketed as "Orgotein" or "ontosein", is used in the treatment of urinary tract disease in human individuals\textsuperscript{189}.

Several european countries have regulatory approval to use bovine liver SOD in the treatment of inflammatory disorders. TEMPO\textsubscript{L} a commercially available pharmaceutical grade is to be proposed for the treatment of hair loss induced by radiotherapy and also for radioprotection. Hence, clinical trials are going on in this regard\textsuperscript{190}.

3.12. FERMENTATION

Fermentation is the most common mechanism in many types of industries such as Food industries and pharmaceutical industries. Microbes easily available low cost substances into useful products during fermentation. Literally fermentation is a biochemical process that occurs within the microorganism during metabolism on carbon. Microbial fermentation occurs synergistically with a sequence of reactions such as oxidations, reductions, polymerizations, hydrolysis and also biosynthesis and the formation of cells. Some fermentations also occur in presence of air (aerobic) some do not require
Air (anaerobic). Industrially fermentation process is carried out mechanically in various sizes of bioreactors or fomenters under controlled parameters such as inoculum size, substrate concentration, temperature and pH, agitation, airflow\(^{191}\).

Batch fermentation is one of the traditional concepts of fermentation. In batch process the reactor is provided with suitable nutrient media which is a substrate and desired microorganism is inoculated in a suitable concentration. In between fermentation, only oxygen is provided in the form of air, but nutrients will not be added in-between. To control foaming a chemical antifoaming agent is added and to maintain content pH balance acid or base added. The inoculated microbial culture is allowed to grow until the preinvestigated stage by which stage the desired product is being accumulated. At this stage fermentation process will be stopped, product is harvested and cleaned out for another batch of fermentation. 4 growth phases seen, they are; 1. Lag phase (organism adapts to surroundings and get prepared for reproduction); 2. Exponential phase (cell multiplication occurs); 3. Stationary phase (Multiplication stops, desired metabolites gets accumulated); 4. Death phase\(^{192}\).

Another conventional method of fermentation is fed batch fermentation, which is also known as closed batch process. This method is usually used to produce products such as penicillin. In this process substrate will be fed in to the bioreactor often when the
substrate gets over. The inoculated microorganisms will be continuously growing until the nutrients availability.

In this process, fermentation is started batch wise with initial small concentrations of substrate. When process starts, the nutrient media will be consumed, then calculated amount of sterile substrate must be added aseptically in such a concentration that should not inhibit the process in any ways. While adding the substrate, the fermented product is also removed off for further purification.

Normally it is difficult to calculate the amount of substrate to be added continuously as and when the previous substrate gets over. Hence we should rely on the indirect parameters which are related to the metabolism of inoculated microorganism. For example, during organic acid production, the $\text{pH}$ value will be considered to add glucose feed. Similarly, in ethanol production depending upon the rate of production of $\text{CO}_2$ of glucose feed to be added in increments to the bioreactor will be decided, and it is represented in the below equation

$$C_6H_{12}O_6 \rightarrow 2 \text{C}_2\text{H}_5\text{OH} + 2 \text{CO}_2$$

In fermentations with critical osmotic values, feeding can be regulated by monitoring the $\text{CO}_2$ content released out in the exhaust air. Along with the continuous addition of the substrate many other parameters must also be regulated such as, to avoid microbial contamination, concentration of the medium, air flow, and equipment must be sterilized. Foam is controlled by either mechanical means or chemical means. Along with these parameters, air pressure,
temperature, agitator, shaft power and viscosity must also be considered. Another significant factor to be considered is "Scale up". It includes conversion of all parameters from laboratory scale to industrial scale. It is well investigated and observed that in industrial microbiology the laboratory scale parameters works poorly in 1st attempt of large scale production. however, constant power consumption per unit broth and the maintenance of constant volumetric transfer rate are calculated based on the product\textsuperscript{194}.

3.13. BIOREACTOR

A bioreactor refers to an engineered device or system that supports for biological growth mechanism by providing biologically active environment. Typically a bioreactor is a vessel in which Micro organisms or biochemically active substances derived from such organisms or even tissues carry out chemical and biological process. These processes will be carried out under controlled conditions. Such processes are termed as fermentation. This process can be either aerobic or anaerobic. These bioreactors are also known as fomenters. Usually Bioreactors are cylindrical in shape, size varies from several litres to cubic metres, and are often made of stainless steel. Bioreactors or fomenters are being developed for use in tissue engineering or biochemical engineering. Pharmaceutical industries, Food and beverage industries and also in biotechnological processes. On the basis of mode of operation, a bioreactor may be classified as batch, fed batch or continuous (e.g. a continuous stirred tank reactor model). An example of a continuous bioreactor the chemo stat.
Either activated culture of microbial cells or immobilised cell could be grown in bioreactors may. Large scale bioreactors are of following types\textsuperscript{195,196};

\begin{itemize}
  \item packed bed bioreactors
  \item fibrous bed bioreactors
  \item membrane bioreactors
  \item moving media, also known as Moving Bed Biofilm Reactor (MBBR)
\end{itemize}

3. 14. ENZYME ASSAYS:

Enzyme assays are laboratory methods for measuring enzymatic activity. They are vital for the study of enzyme kinetics and enzyme inhibition.

3. 14. 1. Enzyme activity

Enzyme activity is equal to moles of substrate converted per unit time which is equal to the rate of reaction volume. Enzyme activity is a measure of the quantity of active enzyme present and is thus dependent on conditions, which should be specified. The SI unit is the katal, 1 katal = 1 mol s\(^{-1}\), but this is an excessively large unit. A more practical and commonly used value is 1 enzyme unit (U) = 1 μmol min\(^{-1}\). 1 U corresponds to 16.67 nanokatals\textsuperscript{197}.

Enzyme activity as given in katal generally refers to that of the assumed natural target substrate of the enzyme. Enzyme activity can also be given as that of certain standardized substrates, such as gelatine, then measured in gelatine digesting units (GDU), or milk proteins, and then measured in milk clotting units (MCU). The units
GDU and MCU are based on how fast one gram of the enzyme will digest gelatine or milk proteins, respectively. 1 GDU equals approximately 1.5 MCU\textsuperscript{198}.

### 3.15. PURIFICATION OF ENZYMES

Enzyme purification is a complex process. The main steps of purification are: (i) preparation of concentrated solution by vacuum evaporation at low temperature or by ultra filtration, (ii) clarification of concentrated enzyme by a polishing filtration to remove other microbe, (iii) addition of preservatives or stabilizers, for example, calcium salts, proteins, starch, sugar, alcohols, sodium chloride (18-20 %), sodium benzoate, etc. (iv) precipitation of enzymes with acetone, alcohols or organic salts, e.g. ammonium sulfate or sodium sulfate, (v) drying the precipitate by free drying, vacuum drying or spray drying, and (vi) packaging for commercial supply (Aunstrup \textit{et al}, 1979)\textsuperscript{199}.

#### 3.15.1 Precipitation:

The first method of enzyme purification is using the precipitation technique based on salt concentration. Ammonium sulphate is a common solution used in this method. Usually the protein or enzyme solution is brought to a 50% saturation with a saturated ammonium sulphate solution. Due to the salt balance, the proteins will coagulate and precipitate to the bottom when centrifuged at a high speed for approximately 15 minutes.
3.15.2. Chromatography

Chromatography methods include ion exchange, bio-affinity and hydrophobicity. Ion exchange uses molecular charge, and bio-affinity uses biomolecular interaction. Initial preparation for such methods includes the lysing of cells and centrifugation for a pure supernatant. Supernatants contain the appropriate isolated enzymes and can be further purified by one of the above chromatography methods.

3.15.3. Gel Filtration

Gel filtration is also a method of chromatography based on the molecular weight of the desired enzyme sample. The method uses beads that come in various pore sizes. The pores of these beads hold the appropriate proteins that are intended to be filtered. Once all of a sample is run through, the beads are extracted for the purified enzymes.