Chapter – 1

General introduction to enzymes

SECTION 1.1 GENERAL INTRODUCTIONS

1.1.1. Enzyme introduction

Enzymes are nature's sustainable catalysts. They are biocompatible, biodegradable and are derived from renewable resources [1]. Enzymes constitute a large biological globular protein molecule responsible for thousands of metabolic processes that sustain life [2], and function as catalysts to facilitate specific chemical reactions within the cell. These reactions are essential for the life of the organism. The living cell is the site of tremendous biochemical activity called metabolism. This is the process of chemical and physical changes which go on continually in the living organism; enzyme facilitate life processes in essentially all life-forms from viruses to man.

Enzymes have been naturally tailored to perform under different physiological conditions. Build-up of new tissues [3], replacement of old tissues [4], conversion of food into energy [5], disposal of toxic materials [6] , reproduction [7] almost all the activities that can be characterized as "life." Enzymes act as life catalysts, substances that accelerate the rate of a chemical reaction. By reducing the activation energy (E_a) necessary to initiate the reaction, thus dramatically increasing the rate of reaction. Enzymes do not initiate reactions that would not naturally occur but they accelerate any reaction that is already underway. Enzymes enable the reaction to take place more rapidly at a safer, relatively low temperature that is consistent with living systems. During an enzyme-mediated reaction, the substrate physically attaches to the enzyme at its active site, allowing the substrate(s) to be converted to new product molecule(s). Most enzyme reaction rates are millions of times faster than those of comparable un-catalyzed reactions. Enzymes are neither consumed by the reactions they catalyze, nor

do they alter the equilibrium of these reactions. Enzymes are known to catalyze about 4,000 biochemical reactions [8].

1.1.2. History of evolution of enzymes

The existence of enzymes has been known for well over a century**.** Biological catalysis was first recognized and described in the early 1800s, in studies of the digestion of meat by secretions of the stomach and the conversion of starch into sugar by saliva and various plant extracts [9]. In 1835, Swedish chemist Jon Jakob Berzelius termed their chemical action as catalytic in nature. In 1860 Louis Pasteur recognized that enzymes were essential to fermentation but assumed that their catalytic action was inextricably linked with the structure and life of the yeast cell. Not until 1897 was it shown by German chemist Edward Buchner that cell-free extracts of yeast could ferment sugars to alcohol and carbon dioxide, Buchner denoted his preparation as *zymase*. The term enzyme comes from *zymosis,* the Greek word for fermentation, a process accomplished by yeast cells and long known to the brewing industry [10].

In 1876, William Kuhne proposed that the name 'enzyme' be used as the new term to denote phenomena previously known as 'unorganised ferments', that is, ferments isolated from the viable organisms in which they were formed. The word itself means 'in yeast' and is derived from the Greek 'en' meaning 'in', and 'zyme' meaning 'yeast' or 'leaven'. This important achievement was the first indication that enzymes could function independently of the cell. It was not until 1926, however, that the first enzyme was obtained in pure form, a feat accomplished by American biochemist James B. Sumner of Cornell University. Sumner was able to isolate and crystallize the enzyme urease from the jack bean. His work was to earn him the 1947 Nobel Prize. John H. Northrop and Wendell M. Stanley of the Rockefeller Institute for Medical Research shared the 1947 Nobel Prize with Sumner [11]. They

discovered a complex procedure for isolating pepsin. This precipitation technique devised by Northrop and Stanley has been used to crystallize several enzymes [12].

1.1.3. Proteinaceous nature of enzymes

Enzymes are proteins and are nature's own biocatalyst and their function is determined by their complex structure. With the exception of a small group of catalytic RNA molecules, all enzymes are proteins which are made up of amino acids linked together by peptide bonds [13]. By the early 1800s, the proteinaceous nature of enzymes had been recognized. Knowledge of the chemistry of proteins drew heavily on improving techniques and concepts of organic chemistry in the second half of the 1800s; it culminated in the peptide theory of protein structure, usually credited to Fischer and Hofmeister. However, methods that had permitted the separation and synthesis of small peptides were unequal to the task of purifying enzymes. Indeed, there was no consensus that enzymes were proteins. After isolation of a series of crystalline proteolytic enzymes beginning with pepsin by Northrop et al., in 1930, the proteinaceous nature of enzymes was established. They are high molecular weight compounds made up principally of chains of amino acids linked together by peptide bonds. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into its subunits, catalytic activity is usually lost. Thus the primary, secondary, tertiary, and quaternary structures of protein enzymes are essential to their catalytic activity [14].

1.1.4. Structure of enzymes

Enzymes are proteins and, are agreeable to structural analysis by the methods of protein chemistry, molecular biology, and molecular biophysics. Like all proteins, enzymes are composed mainly of the 20 naturally occurring amino acids. The structures of enzymes can be elucidating by the physical methods such as

Spectroscopic methods [15], x-ray crystallography [16], and more recently, multidimensional NMR methods [17]. On the basis of arrangement of amino acids enzyme structure can be classified into following types,

1.1.4.1. Primary structure

The structure and reactivity of a protein are defined by the identity of the amino acids that make up its polypeptide chain, this amino acid sequence of the peptide chains is the primary structure of the enzyme.

1.1.4.2. Secondary structure

Secondary structure is due to the interaction of amino acids with each other in the same chain of protein. As a result the protein chain can fold up on itself in two ways, namely α-helix or β-sheet resulting secondary structures.

1.1.4.3. Tertiary structure

The arrangement of secondary structure elements and amino acid side chain interactions that define the three-dimensional structure of the folded protein. So that specific contacts are made between amino acid side chains and between backbone groups. The resulting folded structure of the protein is referred to as its tertiary structure.

1.1.4.4. Quaternary structure and domains

Many enzymes consist of more than one polypeptide chain (or subunit) that aggregate to confer catalytic activity. In some enzymes the subunits are identical, in others they differ in sequence and structure. This description of subunit arrangement in such enzymes is called the quaternary structure. A typical enzyme is not an entity completely folded as a whole, but may consist of apparently autonomous or semi-autonomous folding units called domains.

Chemical nature of enzyme

Enzymes are proteins that are produced by all living organisms. They are high molecular weight compounds made up principally of chains of amino acids linked together by peptide bonds. They have molecular weights ranging from 10,000 to 2,000,000. The important terminologies related to enzymes are,

Cofactor: A non-protein chemical component required for proteins biological activity are called co-factor.

Apoenzyme: The protein part of an active enzyme is called apoenzyme.

Holoenzyme: the active enzyme composed of Apoenzyme and a co-factor is termed as holoenzyme.

Coenzyme: coenzyme is a non –protein compound or substance that is necessary for an enzyme to initiate the function of the enzyme.

Prosthetic group: A coenzyme or metal ion that is very tightly or even covalently bound to the protein component of the enzyme is called a prosthetic group.

1.1.5. Specificity of enzymes

Enzymes are the most remarkable and highly specialized proteins, they have a high degree of specificity for their substrates, and they accelerate chemical reactions tremendously. In general, four types of behavior can be described:

1.1.5.1. Absolute specificity - Catalyze only one reaction.

1.1.5.2. Group specificity – catalyses a particular type of functional group, which can occur in a variety of substrate.

1.1.5.3. Linkage specificity - Catalyses a particular type of chemical bond regardless of the rest of the molecular structure.

1.1.5.4. Stereochemical specificity - the enzyme will act on a particular steric or optical isomer.

1.1.6. Sources of enzyme

Enzymes occur in all living organisms and catalyze biochemical reactions necessary to support life [18]. A wide array of enzymes are extracted from plant sources; they have many advantages including cost of production and stability of products [19]. An ample range of sources are used for commercial enzyme production from a broad spectrum of plant species. Non-microbial sources provide a larger proportion of these, at the present time. Microbes are preferred to plants and animals as sources of enzymes because [20]:

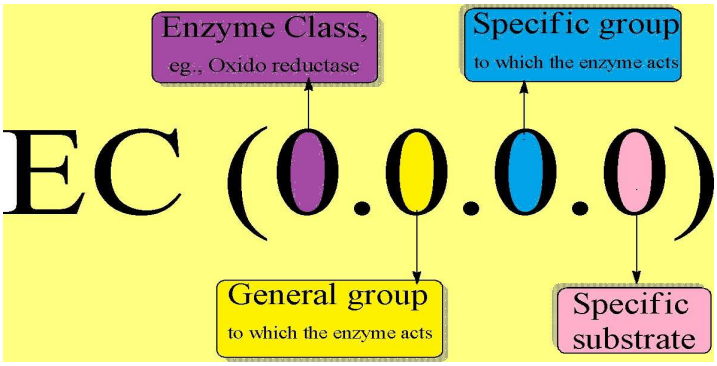
- They are generally cheaper to produce.
- Their enzyme contents are more predictable and controllable,
- Regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media.
- Plant and animal tissues contain more potentially harmful materials than microbes, including phenolic compounds (from plants), endogenous enzyme inhibitors and proteases.
- Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer

About fifty years ago, enzymes were being extracted strictly from animals like pig and cow from their pancreases [21]. Animal enzymes were multifold, they were not very stable at the low pH environment so that the enzyme product was destroyed before doing the job. To overcome this problem plant enzymes were discovered, most important one is extraction of peroxidase from horseradish roots occurs on a relatively large scale because of the commercial uses of the enzyme [22]. Peroxidase can also be extracted from soybean, it is also having the common features with horseradish peroxidase [23]. Some plants like *Cruciferous* vegetables, including *broccoli*,

cabbage, kale and collard and turnip greens and papaya are rich in catalase [24-26]. Wheat sprouts contain high levels of catalase [27] and vegetarian sources of catalase include apricots, avocados, carrots [28]. Catalase is also present in some microbes and bacteria [29], *Aspergillus niger* culture also produces catalase enzyme [30].

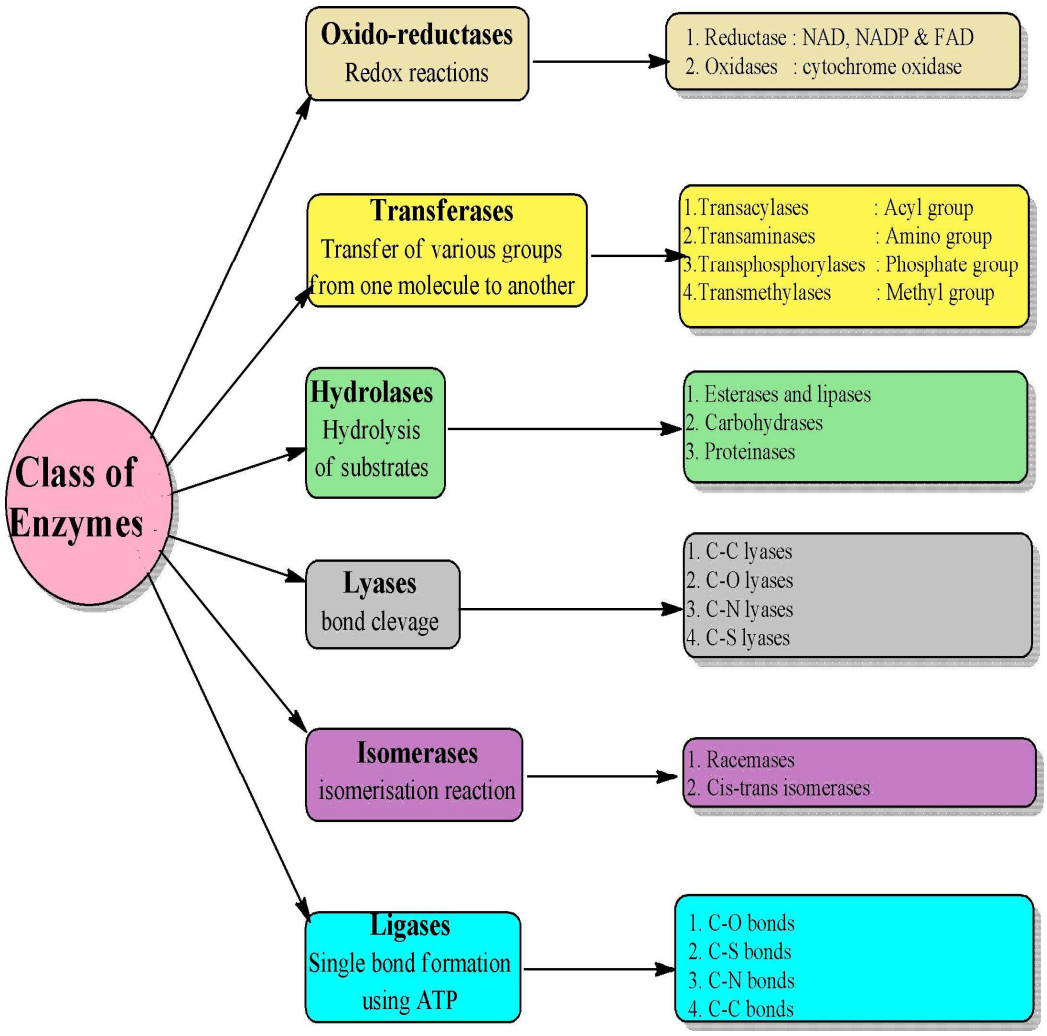
1.1.7 Naming and enzyme classification

In general many enzymes have been named by adding the suffix "-ase" to the name of their substrate or to a word or phrase describing their activity. In 1961, according to the report of the first Enzyme Commission (EC) of International Union of Pure and Applied Chemistry (IUPAC), Enzymes are classified in to six types on the basis of reaction they catalyze [31]. They were assigned code numbers, prefixed by E.C., which contain four elements separated by points and have the following meaning as shown in **Scheme 1.1.**



Scheme 1.1: Naming of enzyme according to 'International Union of Biochemistry' formed a 'Commission on Enzyme Nomenclature

The enzyme can be classified in to six main classes as shown in the **scheme 1.2**,



Scheme 1.2: Classification of enzyme and examples of related enzyme classes

1.1.8 Mechanism of enzyme action

Enzymes are macromolecules that help to accelerate (catalyze) chemical reactions in biological systems. Some biological reactions in the absence of enzymes may be as much as a million times slower [32]. Any chemical reaction converts one or more molecules, called the **substrate,** into different molecule(s), called the **product**. Most of the reactions in biochemical processes require chemical events that are unfavorable or unlikely in the cellular environment, such as the transient formation of unstable charged intermediates or the collision of two or more molecules in the precise orientation required for reaction. In some of the Reactions like, digestion of food [33], send nerve signals [34], or contract a muscle simply do not occur at a useful rate without catalysis[35]. Enzyme overcomes these problems by providing a specific environment within which a given reaction can occur more rapidly. Enzymes are usually proteins – each has a very specific shape or conformation. Within this large molecule is a region called an **active site**, which has properties allowing it to bind tightly to the substrate molecule(s). The active site of the enzyme is shown in **Figure 1.1.**

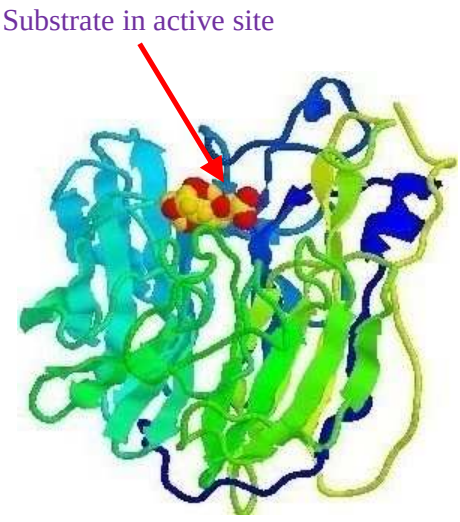


Figure. 1.1: Structure of enzyme showing the active state

As proposed by Charles-Adolphe Wurtz, an active site is a three dimensional cleft or crevice formed by groups that come from different parts of the amino acid sequence - residues far apart in the amino acid sequence may interact more strongly than adjacent residues in the sequence. The active site encloses a substrate and catalyzes its chemical transformation. The enzyme substrate complex was first discovered in 1880, is central to the action of enzymes. The enzyme–substrate interactions can be explained by the following theories.

1.1.8.1 Lock and key model

In "lock and key" model the active site of the enzyme is complementary in shape to that of the substrate. The substrate is held in such a way that its conversion to the reaction products is more favorable. It was thought that the substrate exactly fitted into the active site of the enzyme molecule like a key fitting into a lock. In the Figure 1.2 "lock" refers to enzyme and "key" refers to its complementary substrate [36].

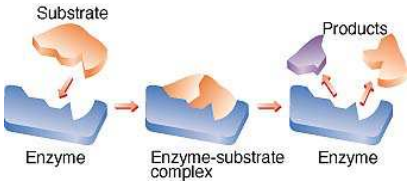


Figure. 1.2 Lock and key model for enzyme – substarte

1.1.8.2 Induced fit

Lock and key model does not explain the stability of the transition state for it would require more energy to reach the transition state complex. To explain this concept Koshland in 1958, first proposed the induced-fit model, this suggests that the enzyme active site is conformationally fluid. Enzyme itself usually undergoes a change in conformation when the substrate binds, induced by multiple weak interactions and hydrophobic characteristics on the enzyme surface mold into a precise formation [37].

1.1.8.3 Transition state theory

According to this theory when an enzyme catalysis, the enzyme binds more strongly to its *"transition state complex rather than its ground state reactants."* This indicates, the transition state is more stable [38]. A simple enzymatic reaction can be written as,

$$E + S \leftrightarrow ES \leftrightarrow EP \leftrightarrow E + P$$

Where E, S, and P represent the enzyme, substrate, and product respectively; ES and EP are transient complexes of the enzyme with the substrate and with the product respectively.

The transition state is not a chemical species with any significant stability and should not be confused with a reaction intermediate (such as ES or EP). It is simply a fleeting molecular moment in which events such as bond breakage, bond formation, and charge development have proceeded to the precise point at which decay to either substrate or product is equally likely. The difference between the energy levels of the ground state and the transition state is the activation energy; the rate of a reaction reflects this activation energy: higher activation energy corresponds to slower reaction. Reaction rates can be increased by raising the temperature, thereby increasing the number of molecules with sufficient energy to overcome the energy barrier. Alternatively, the activation energy can be lowered by adding a catalyst.

SECTION 1.2 ENZYME ASSAY

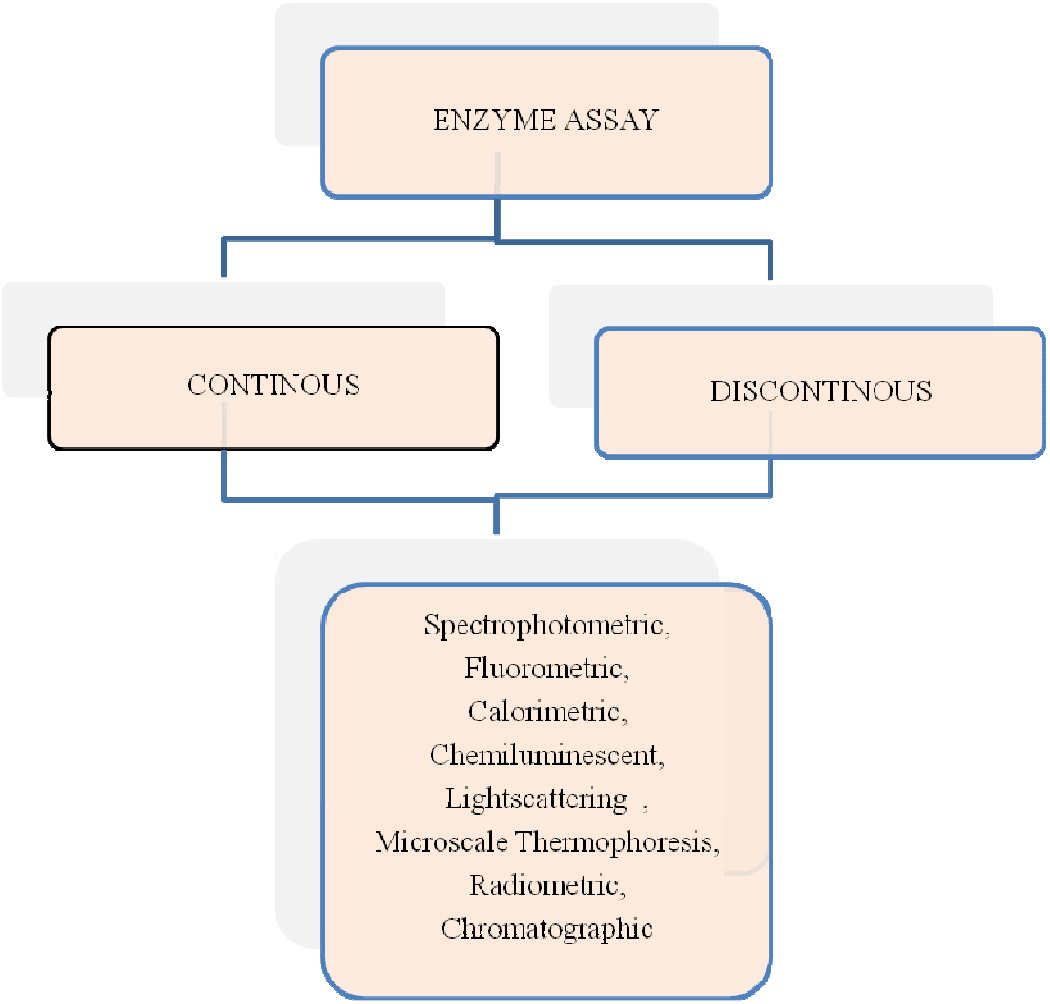
1.2.1. Introduction

In recent years enzyme assays have greatly advanced in their scope and in the diversity of detection principles employed. Enzyme assays are laboratory methods to visualize enzyme activities. An enzyme assay consists of mixing the enzyme with a substrate in a solution of controlled pH with any additional substance whose effect is to be tested, incubating the reaction mixture at an appropriate temperature for the required time, stopping the reaction precisely, and then measuring the amount of reaction that has occurred. The amount of reaction that has taken place may be quantified by two ways, in terms of the disappearance of substrate or the appearance of product. In recent years a large variety of enzyme assays have been developed to assist the discovery and optimization of enzymes, in particular for "white biotechnology'' where selective enzymes are used with great success for economically viable, mild and environmentally benign production processes [39, 40].

Enzyme assays can be classified into two types namely,

1.2.1.1 Continuous assay: Continuous assay gives a continuous reading of activity.

1.2.1.1 Discontinuous assay: In discontinuous assay the reaction is stopped and then the concentration of substrates or product is determined. The flow chart of enzyme assay and different analytical techniques available are shown in **Scheme 1.3**



Scheme. 1.3: Flow chart of enzyme assay classification.

Enzyme assays leads to the development of new analytical enzyme experiments in different fields like enzyme fingerprinting, cocktail fingerprinting, microarray experiments, enzyme coupled reactions, Bio- and nano sensors, ELISA and isotopic labeling studies. The immense knowledge of genetic information that has been accumulated over the past decade has further claimed the importance of enzyme.

1.2.2. The nature and origin of diversity in enzyme assay:

The major consideration in the design of an enzyme assay method is that the amount of enzyme present should be the only variable which affects the reaction rate. This condition is more likely to be met if the assay procedure ensures optimal

substrate concentration, pH, temperature, etc, and thus many of the modifications that have from time to time been made to assay methods have been motivated by the desire to achieve optimal conditions. Unfortunately, it has not always been appreciated that such modifications almost inevitably result in an alteration in the enzyme activity as measured by the method.

1.2.3. Measures of enzyme activity

In order to express the activity of an enzyme in absolute terms it is necessary to ensure that the assay procedure used is measuring the true initial velocity and that this is proportional to the enzyme concentration. Under these conditions the ratio will be a constant that can be used to express the activity of an enzyme quantitatively. This can be valuable for comparing data obtained with the enzyme from different laboratories, assessing the effects of physiological and pharmaceutical challenges on cell or tissues, monitoring the extent of purification of enzymes and comparing the activities of different enzymes, or of the same enzyme from different sources or with different substrates.

1.2.4. Units of enzyme and Specific activity:

The activity of an enzyme may be in any convenient units, such as absorbance change per unit time per mg of enzyme protein, but it is preferable to have a more standardized unit in order to facilitate comparisons. The most commonly used quantity is the units, sometime referred to as the international unit or Enzyme unit. One unit of an enzyme activity is defined as that catalyzing the conversion of 1 µM substrate in 1 minute. The specific activity of an enzyme preparation is the number of units per mg of protein.

If the relative molecular mass of an enzyme is known it is possible to express the activity as the molecular activity, which is defined as the number of units per

µmol of enzyme, in other words the number of mol of product formed, substrate used per mol of enzyme per minute. This may not correspond to the number of mol substrate converted per enzyme active site per minute since an enzyme molecule may contain more than one active site. If the number of active site per mol is known the activity may be expressed as the catalytic centre activity, which corresponds to mol substrate used, or product formed per minute per catalytic center. The nomenclature commission of the International Union of Biochemistry recommended ***katal*** as the enzyme unit which is abbreviated as kat, in this the unit of time is expressed in terms of second rather than minute with international system of unit (SI Units).

One katal corresponds to the conversion of 1 mol of substrate per second. It is larger quantity than the enzyme Unit.

1 kat = 60 mol min^{-1} = 6×10^7 Units

1 Unit = 1 µmol min^{-1} = 16.67 nkat

1.2.5. Related terminology

1.2.5.1 Maximum velocity V_{max} of the reaction:

In enzyme kinetics, V_{max} is defined as the maximum initial velocity of the enzyme catalysed reaction under the given conditions or highest possible rate when the enzyme is saturated with the substrate [51]. It is the limiting value that V_o approaches as the substrate concentration approaches infinity.

1.2.5.2 Turnover number

Turnover number (also termed k_{cat} and abbreviated as TN) is defined as the maximum number of molecules of substrate that an enzyme can convert to product per catalytic site per unit of time (a turnover rate) or it is also defined as the number of moles of product formed per mole of cofactor per unit time [41] and can be calculated as follows,

$$K_{cat} = \frac{V_{\max}}{[E]_T}$$

Where, $V_{\max}$ is the maximum rate or velocity of the reaction and $[E]_T$ is the total enzyme concentration.

For example, carbonic anhydrase has a turnover number of 400,000 to 600,000 s^{-1}, which means that each carbonic anhydrase molecule can produce up to 600,000 molecules of product *per second* [42].

1.2.5.3 Specific activity

Specific activity is a term used in measuring enzyme kinetics. It is defined as the amount of substrate the enzyme converts (reactions catalyzed), per mg protein in the enzyme preparation [43], per unit of time or in other words the enzymatic activity per unit mass of enzyme [44]. Specific activity is a measure of enzyme purity. The value becomes larger as an enzyme preparation becomes more pure, since the amount of protein (mg) is typically less, but the rate of reaction stays the same.

1.2.5.4 Michaelis–Menten equation (K_M)

The Michaelis-Menten equation is the elementary equation of enzyme kinetics, even though it is originally derived for the simplest case of an irreversible enzyme reaction. A relationship between the value of Michaelis – Menten constant (K_M) for an enzyme and the physiological concentration of substrate was mentioned over two decades ago. Cleland stated a general rule that enzyme will function with reactant concentrations in the region of their apparent Michaelis constants or above, when at the pathway they are a part of is operating at full capacity; otherwise the catalytic potential of the enzyme is wasted. Apart from this Fersht explained that the tight binding of substrate implies a stable, and therefore unreactive substrate. He

argued that K_M should be high, even as high as 10 times the concentration of substrate under physiological conditions.

The Michaelis –Menten constants can be evaluated by four different methods namely,

i. Plot of V_0 *versus* $[S]_0$ wherein the constants are determined from a graph of initial rate *versus* initial substrate concentration [45].

ii. Eadie - Hofstee transformation: It is the plot of initial rate (V_0) *versus* ratio of initial rate to initial substrate concentration $\frac{V_0}{[S]_0}$ which will give a straight line with an intercept of $V_{\max}$ and slope of *m*[46, 47].

iii. Lineweaver-Burke plot: it is also called a double reciprocal plot. It is obtained by plotting $\frac{1}{V}$ *versus* $\frac{1}{[S]}$, where V is the rate of the reaction, [S] is the concentration of the substrate.[48].

iv. Hanes-Woolf plot is constructed by plotting the ratio of product of substrate and co-substrate concentration to initial rate of reaction *versus* substrate or co-substrate concentration [49].

$$\text{i.e} \quad \left[\frac{H_0 D_0}{V_0}\right] \text{ v/s } H_0 \text{ or } D_0$$

Where, D_0 and H_0 are initial concentrations of any phenol or other aromatic co-substrates and substrate (H_2O_2).

Significances of K_m: When

$[S] = K_m$, then

$V = \frac{V_{\max}}{2}$.

Hence K_m is equal to the substrate concentration at which the reaction rate is half its maximum value. In other words, if an enzyme has small value of K_m, it achieves its maximum catalytic efficiency at low substrate concentrations. Hence, the smaller the value of K_m, the more efficient is the catalyst. The value of K_m for an enzyme depends on the particular substrate. It also depends on the pH of the solution and the temperature at which the reaction is carried out. For most enzymes K_m lies between 10^{-1} and 10^{-7} M.

1.2.5.5 Catalytic power (K_{Pow})

Catalytic power is the rate of an enzyme catalyzed reaction divided by the rate of the uncatalyzed reaction. Or catalytic power is the degree to which enzymes increase the rate of a chemical reaction [50]. The catalytic power of enzymes is due to the precise molecular interactions that occur at the active site, which lower the energy barrier and enable formation of the transition state.

$$K_{Pow} = \frac{V_{\max}}{K_m}$$

Where, $V_{\max}$ is the maximum velocity of the reaction and K_M is the Michaelis – Menten constant.

1.2.5.6 Catalytic efficiency

Enzymes are important for a variety of reasons, most significantly because they are involved in many chemical reactions that help us to maintain our daily lives. Increasing the reaction rate of a chemical reaction allows the reaction to become more efficient, and hence more products are generated at a faster rate. This is known as catalytic efficiency of enzymes, which, by increasing the rates, results in a more efficient chemical reaction within a biological system. Enzyme efficiency is measured by,

$$k_{cat} = k_2 = \frac{V_{\max}}{[E_0]}$$

Some time enzyme efficiency is referred to as specificity constant' $\frac{k_{cat}}{K_M}$. It is an useful indicator for comparing the relative rates of an enzyme acting on alternative, competing substrates.

1.2.6. Factors affecting the enzyme activity:

The catalytic activity of an enzyme is measured in terms of the rate of the reaction catalyzed. The reaction conditions for the extent of activities of an enzyme should be optimal, so that maximum possible rate of reaction is measured. The optimum reaction conditions refer to the type of buffer and its concentration, the pH, effect of inhibitors on enzyme activity, activators and substrate concentration(s).

1.2.6.1 Enzyme concentration

An enzyme molecule binds its substrate (s), catalyzes a reaction, and releases the product (s). Each step in this process requires time—time to receive the raw materials, do what needs to be done to them, and release the product. So each enzyme molecule requires x amount of time to produce one unit of product. The more the enzyme molecules are available, however, the more will be the product that can be produced in x time. Thus the more enzyme is available, the more quickly the substrate can be converted into product. In general, as enzyme concentration increases, there is a proportional increase in reaction rate.

1.2.6.2 Substrate concentration

The rate of an enzyme reaction depends on the substrate concentration. Coenzymes behave as substrates from the point of view of reaction kinetics. With increasing coenzyme concentration, the rate of the reaction becomes greater until it reaches the limiting value [52]. If the optimum reaction rate *V* of an enzyme reaction

is to be attained, the Michaelis constant K_m must be negligibly small in comparison with the concentration of the substrate. In ideal condition according to Michaelis – Menten equation,

$$v = \frac{V[S]}{K_m + [S]},$$

If $[S] >> K_m$ then

$v = V$, is probably rare, since practical factors often prevent it.

1.2.6.3 Effect of inhibitors on enzyme activity

Enzyme inhibitors are substances which alter the catalytic action of the enzyme, as a result either it slows down, or it stops catalysis [53]. Mainly there are three types of enzyme inhibition namely, competitive, non-competitive and substrate inhibition. In competitive inhibition the substrate and a substance resembling the substrate are both added to the enzyme. Whereas in non-competitive inhibition substances which when added to the enzyme alter the enzyme in a way that it cannot accept the substrate, if the substrate concentration becomes more than that causes the substrate inhibition.

1.2.6.4 Temperature effects

Chemical reactions are sensitive to the temperature; in the same way enzyme catalyzed reactions are sensitive to temperature changes. Many enzymes show unusual relationship between reaction rate and temperature. Although over much of the range of temperatures which biological organisms experience there is increased enzyme activity with increased in temperature, however there is often decrease in reaction rates at very high temperatures. Due to higher temperature the shape of the active site will begin to distort, and the enzyme will lose its ability to bind substrate

and catalyze the reaction. Thus the decrease in reaction rate is due to the inability of the enzyme to function as a catalyst when it is denatured by heat [54].

1.2.6.5 Effect of cofactor / coenzyme concentration

Many enzymes require certain additional substances to be bound to them in order to function as catalysts [55]. These substances are often referred to as cofactors and coenzymes. These auxiliary substances may need to be bound to the enzyme so that the enzyme will have proper shape to its active site or may be the actual catalytic agent used to facilitate the reaction taking place, whereas the enzyme merely binds the substrate and holds it in proper orientation. For those enzymes that require a cofactor or coenzyme, enzyme activity is dependent upon the concentration of that cofactor [56]. If the cofactor is at very low concentrations, few enzyme molecules will have the necessary cofactor bound, thus few will be able to catalyze the reaction, and reaction rates will be low. As cofactor concentration increases, more and more enzyme molecules will get bound to cofactor and thus be catalytically active. However, as cofactor concentration increases, there will be progressively smaller and smaller increase in reaction rate because majority of enzyme molecules will already have the cofactor they need. Indeed, above a certain limit, when all enzyme molecules have got the cofactor they need, further increasing cofactor concentration will have no influence on reaction rate.

1.2.6.6 Effect of pH

pH is known to alter the activity of enzymes as it affects ionization state of side chains of enzymatic proteins. The each enzyme has an optimum pH range for its activity, this range is often very narrow [57]. Therefore while selecting the buffer, care must be taken to see that the pH – optimum of the reaction is as close as possible to the pK of the buffer. The type and concentration of the buffer also affect the

activity of enzyme. As for example leucine amino peptidase shows higher activity in tris buffer than in phosphate buffer [58].

1.2.7. Limits of enzymatic analysis:

1.2.7.1 Methodological limits

The enzymatic analysis is highly sensitive and it varies on the method of analysis [59]. Micromolar quantity of enzyme can be determined by photometry and nanomolar quantity in catalytic assays. With the assist of fluorimetry, the sensitivity can be enhanced by two to three powers of ten. In the enzymatic cycling methods as little as 1×10^{-16} mol of substance can be determined quantitavely. The accuracy of enzymatic analysis is high because of the generally marked specificity of the enzymes. The precision of enzymatic analysis depends on the complexity of the assay system. Metabolites can be determined with greater precision by end point procedures than the catalytic activities of enzyme. If errors due to the measuring and reagent-dispensing apparatus are minimized, coefficient of variation of 1% or even less can readily be attainable in metabolite assays. The precision of determination of the catalytic activity of an enzyme has a coefficient of variation of 2 – 4 % in the case of simple reactions, and higher in the case of coupled or multiply-coupled reactions. As for all analytical methods, there are also limits to enzymatic analysis when it is transferred to automatic analyzers. Frequently, such instruments do not permit the simultaneous determination of one or more blank values, and it is often impossible to let a certain preliminary reaction take place. Occasionally, moreover the method has to be modified,

1.2.7.2 Economic limits

The term highly purified enzyme as reagents is still associated with the idea of expensive reagents. It is overlooked that only microorganism or at the most, milligram amounts of enzymes are used for an analysis. Nevertheless, for some analysis of interest in food chemistry, for example the costs of reagents for the enzymatic procedure are generally higher than for the conventional one. At the same time, in contrast to enzymatic analysis, the total cost of a chemical analysis is generally substantially higher than the cost of reagents [60].

SECTION 1.3 SCOPE OF ENZYME ASSAY

Enzymes are biocatalysts widely used in several fields due to their extensive applicability.

1.3.1. Food chemistry

Enzymes are being used to an increasing extent in the determination and production of alcohol [61], carbohydrates [62], organic acids [63], nitrogen compounds [64], in beverages, baked products, chocolate, sugar and sugar confectionary [65]. In meat products there is the determination of pyrophosphate [66], creatine and creatinine [67] as well as gluconate and in egg containing products and fats, the determination of cholesterol. The number of parameters that can be determined using enzymes is increasing steadily.

1.3.2. Chemistry of cosmetics

The foodstuff legislation of many countries also includes cosmetics. Again the determination of such substances as glycerol, glucose, fructose, cholesterol, lactate, citrate and ethanol in skin creams or face lotions [68], by means of enzymatic analysis has got many advantages.

1.3.3. Botany and agricultural chemistry

Enzymatic methods are becoming more and more application oriented in the investigation of the physiology of plant metabolism in the normal state [69], in parasitic [70] and non parasitic [71] plant diseases, and for evaluation of quality of plant products with respect to their suitability for storage and technological processing [72]. This also applies to the investigation of soil biology and characterization of its biological activity.

1.3.4. Microbiology

Enzymatic analysis is used for monitoring the growth and metabolism of microorganisms [73]. In the cultivation of microorganisms for the production of enzymes, the amount of substrate in the nutrient medium is determined in relation to the amount of enzyme in the microorganism. In the fermentation process in the food sector, faulty fermentation can readily be discovered by determining certain parameters. The latest developments have made it possible to monitor fermentation process continuously by enzymatic analysis.

1.3.5. Pharmacology

Enzymatic methods are being used increasingly in biochemical pharmacology. Summ and Christ have investigated different inhibitory effects of various tetracycline derivatives in systems of cell free protein biosynthesis [74]. With this experimental arrangement it is possible to screen various antineoplastic agents for their action on tissue samples.

1.3.6. Clinical chemistry

For the whole of human medicine, enzymology and hence enzymatic analysis have become so important as a diagnostic aid and also in the monitoring of diseases during treatment that this activity is now a large specialty by itself. This is the domain of the determination of the catalytic activities of enzymes. The classical metabolites determinable by enzymatic analysis are glucose [75], triglycerides [76], cholesterol [77], uric acid [78], urea [79] and many others. Here also, the parameters of thyroid gland function, steroid hormones, insulin, immunoglobulins, viral antigens etc., are determined by means of enzyme immunoassays.

SECTION 1.4: NEED FOR THE DEVELOPMENT OF NEW ANALYTICAL METHODS

Due to the wide scope of enzyme assay in majority of fields like Bio-Technology, Bio- chemistry, Chemistry, Clinical laboratories, Food technology and environmental chemistry they create more demand for new and rapid methods of analysis of enzymes, whose number is also increasing in multiple folds, is forcing the analysts to develop sensitive chromogens for their assays.

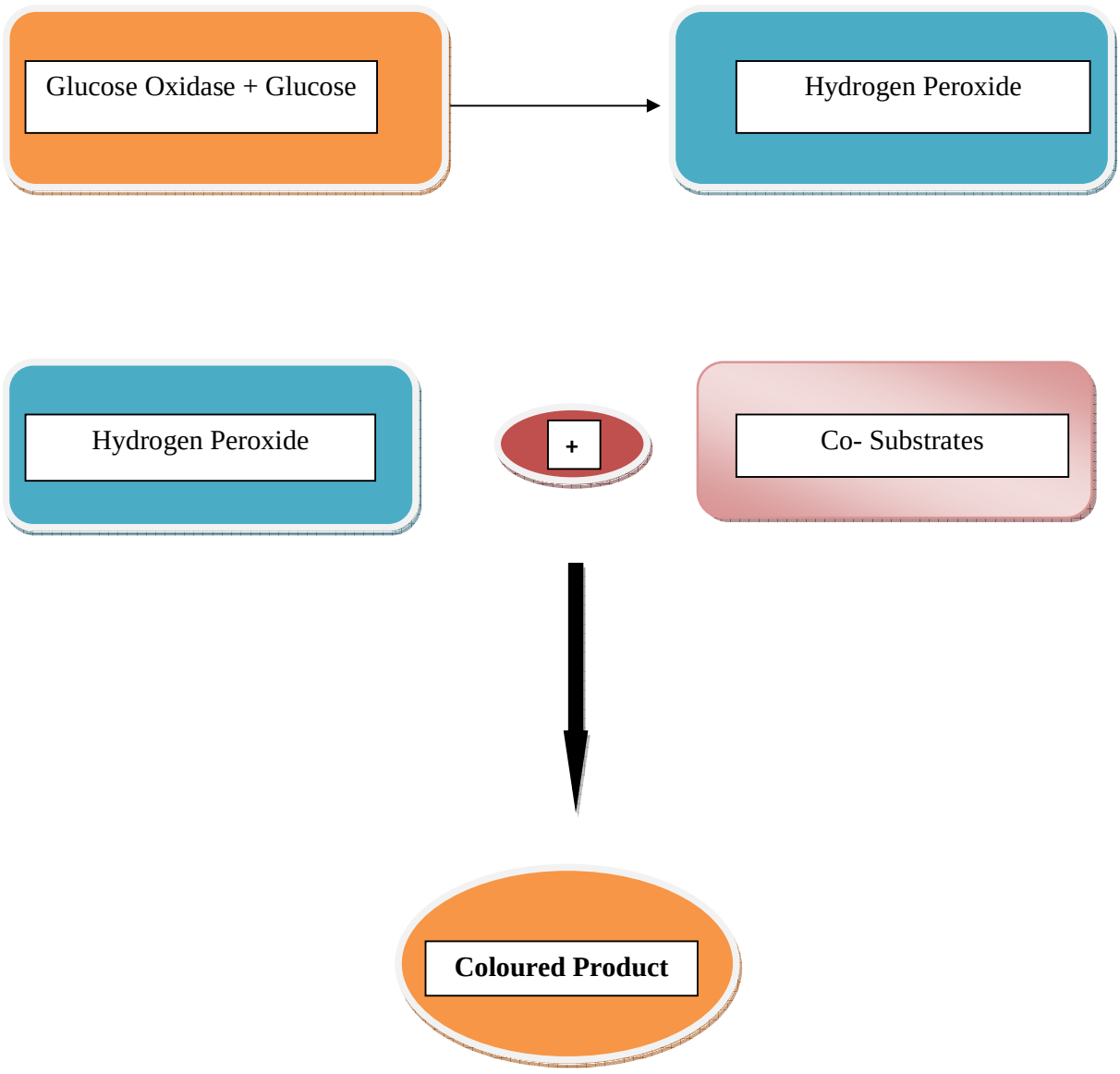
A number of methods are available for the assay of enzymes; most of the existing methods are having their own demerits some of them need costly instruments, highly toxic reagents, solubility problem, lack of stability and time consuming. To overcome these entire problems attempt was made to develop new simple spectrophotometric and spectrofluorimetric methods.

The methods developed in the present investigation require simple, inexpensive and readily available equipment and chromogens, including the possibility of extensive automation, which is the important requirement for the regular analysis of clinical and environmental samples. The major advantages with our proposed reagents are that they , are colorless, water soluble, colour develops instantaneously, results are reproducible, less carcinogenic, easy to handle and the coloured product is stable for longer time and the methods can be effectively employed for majority of plant and clinical samples. The investigator has made sincere attempts and has successfully developed a few new chromogens and fluorogens for the assay of biologically important enzymes like HRP, Catalase and GOD.

The novel reagents or chromogens used in the development of new analytical methods for the assay of some enzymes like Horseradish peroxidase, catalase and glucoseoxidase include:

- 3-Methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH) – for the assay of catalase
- Pyrocatechol (PC) - for the assay of catalase
- Para phenylenediamine dihydrochloride (PPDD) - for the assay of horseradish peroxidase (HRP)
- Iminodibenzyl (IDB) - For the assay of HRP
- 3-Aminophenol (3-AP) - For the assay of horseradish peroxidase
- *N*, *N*-Diethyl-*P*-phenylenediamine (DPD) - For the assay of HRP
- 5-Sulfosalicylic acid dehydrate (SSA) - for GOD and glucose assay
- Para acetyl aminophenol- For HRP assay.

SECTION 1.5: GRAPHICAL ABSTRACTS OF THE RESEARCH FINDINGS



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