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Graduate Project (Chem.774)

Voltammetric Determination of Hydrogen Peroxide with Enzyme Modified Carbon Paste Electrode

BY

Etsubneh Chekole

Department of chemistry

Approved by	**Signature**
Dr. Mesfin Redi Examiner	
Dr. Merid Tessema Advisor	
Dr. Shimelis Admassie Advisor	

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1. Introduction

1.1. Hydrogen Peroxide

At room temperature, pure hydrogen peroxide is colorless, syrup-like liquid that freezes at –0.41 ^{0}C and boils at 150.2 ^{0}C. The pure liquid can decompose violently and exothermically in to water and oxygen [1].

$$2\,H_2O_2(\ell) \rightarrow 2\,H_2O + O_2(g), \quad \Delta H^0 = -196\ KJ \qquad (1)$$

Hydrogen peroxide plays an important role in clinical and biological fields. The oxidation of many biological substances in the body fluids produces a certain amount of hydrogen peroxide [2]. Furthermore, small amounts of hydrogen peroxide can be found in our tissues or in our cells as a product of cellular metabolism. It can also be found in our saliva, which causes the foaming in our mouth in contact with toothpaste [3]. It is a very important intermediate in environmental and biological reactions and a universal oxidant in industrial processes [4]. Further, the presence of hydrogen peroxide in the environment is important because it is a key species in the reaction of the troposphere [2]. Photolysis of hydrogen peroxide produces hydroxyl radical that activates the oxidation of saturated hydrocarbons and abstract hydrogen atom from any organic molecule. The resulting organic radical combines with molecular oxygen to make organoperoxy radicals. These decompose photochemically. The ultimate products are carbondioxide and oxygen [3]. In addition, since H_2O_2 breaks down into water and O_2, it does not form any persistent or toxic residual compounds.

Hydrogen peroxide is a strong oxidizing agent and a weak acid in water solution. The formula is similar to that of water, with an extra atom of oxygen attached, H_2O_2. It is completely soluble in water [3].

A 3 % solution of H_2O_2 is widely used medically as an antiseptic to treat body sores, broken blisters, animal bite wounds (cleaning wounds), sore throats, removing dead tissue, and abrasives, and is readily available in pharmacies [5].

H_2O_2 can also be considered as bactericidal at low concentration (up to 35%). Its bactericidal and sporicidal properties have made it a useful tool in the food processing industry and packing system.

Hydrogen peroxide is well established as an environmentally friendly deodorizing and bleaching agent. Its use includes organic and inorganic chemical processing, textile and pulp bleaching, meat treating, cosmetic applications, catalysis of polymerization reaction, municipal odor control and industrial waste treatment (detoxification) [6].

Furthermore, it is used in restoring the original colors to paintings that have darkened through the conversion of the white lead used in the paintings to lead sulphate. The H_2O_2 oxidizes the black lead sulphide to white lead sulphate. It is also used as a source of oxygen in the fuel mixture for many rockets and torpedoes, and in very low concentration, typically around 6% to bleach human hair [6].

A solution of hydrogen peroxide is very slightly acidic through the reaction:

$$H_2O_2\,(aq) + H_2O\,(\ell) \rightarrow H_3O^+\,(aq) + HO_2^-\,(aq) \qquad (2)$$

In acidic media, hydrogen peroxide is an oxidizing agent.

$$2\,Fe^{+2}\,(aq) + H_2O_2\,(aq) + 2\,H^+\,(aq) \rightarrow 2\,Fe^{+3}\,(aq) + 2\,H_2O\,(\ell) \qquad (3)$$

However, in alkaline condition hydrogen peroxide is used as a reducing agent [7].

$$2\,Fe^{3+}(aq) + H_2O_2\,(aq) + 2\,OH^-\,(aq) \rightarrow 2\,Fe^{2+}(aq) + 2\,H_2O\,(\ell) + O_2(g) \qquad (4)$$

The decomposition of H_2O_2 is catalyzed by light and by numerous substances, including dust particles and metal ions such as Cu^{+2} and Fe^{+3}. Decomposition is inhibited by hydrogen peroxide solution in dark bottles with preservatives that bind and inactivate metal ions. Most commercial uses of hydrogen peroxide are based on its strong oxidizing abilities [1].

Hydrogen peroxide is used for the preparation of useful compounds like Caro's acid (used in mining industries) and peroxyacetic acid (used for disinfection, sterilization and as an epoxidizing agent). The equilibrium reaction for the production of Caro's acid and that of peroxyacetic acid are given in equation (5) and equation (6), respectively.

$$H_2O_2 + H_2SO_4 \longleftrightarrow H_2SO_5 + H_2O \quad (5)$$

$$H_2O_2 + CH_3COOH \longrightarrow CH_3COO\text{-}OH + H_2O \quad (6)$$

H_2O_2 is a poisonous by product of metabolism that can damage cells if it is not removed [8, 9]. Catalase is an enzyme that speeds up the breakdown of hydrogen peroxide in to water and oxygen.

$$2\,H_2O_2 \rightarrow 2\,H_2O(\ell) + O_2 \quad (7)$$

Hydrogen peroxide is an irritant of the eyes, mucous membranes, and skin. Inhalation of concentrations of the vapour or mist may cause extreme irritation of the nose and throat. The inhalation of 7 ppm causes lung irritation in humans. Severe systematic poisoning may cause headache, dizziness, vomiting, diarrhea, unconsciousness and shock. Exposure for a short period to the mist or spray may cause stinging and tearing of the eyes. Splashes of high concentration of hydrogen peroxide in the eyes may cause severe corneal damage. Skin contact with liquid hydrogen peroxide causes a temporary whitening or bleaching of the skin; if the skin is not washed promptly, redness and blisters may develop. Ingestion of hydrogen peroxide may cause irritation of the upper gastrointestinal tract and severe damage to the esophagus and stomach [3].

Reactive oxygen intermediates may be generated in the lungs during various pathological processes. These intermediates are toxic to cells in addition to hydrogen peroxide through their oxidizing effects on proteins, membranes and DNA. Here, detection of submicromolar concentrations of H_2O_2 is vital because these peroxide levels can damage mammalian cells [10]. This by-product is not only toxic but also considered to be an aging factor. Moreover, suitable hydrogen peroxide monitoring systems may prove to be use for environmental monitoring because hydrogen peroxide causes the acidification of rain by oxidizing sulfurdioxide in the atmosphere to SO_4^{2-}, resulting in the deforestation of vast areas as evidenced by research

indicating that acid rain has serious effects on the shape and internal structure of spruce and needles of green plants [11].

1.2. Objective

In this work the voltammetric behavior of H_2O_2 at carbon paste electrode modified with immobilized horseradish peroxidase (HRP) and the analytical application of the modified electrode for the determination of hydrogen peroxide were studied. In addition, the optimum experimental parameters for the determination of hydrogen peroxide were established.

2. Enzyme

Enzymes are proteins that catalyze chemical reactions in living systems. Such catalysts are not only efficient, but also extremely selective. These characters make them to use as sensors

2.1. Enzyme Based Sensors

The construction of a robust and reliable enzyme based sensor requires among other things, the choice of the enzyme with regard to its cost, ease of handling, stability, specificity, and suitability for enzyme immobilization. In addition to the immobilized enzyme, the sensor may require other reagents that contribute to the performance of the immobilized enzyme. These include a redox mediator to shuttle electrons between the enzyme and electrode to enhance the catalytic activity, and stabilizers to give long-term stability to the enzyme. Membranes preventing enzyme leaching from the electrode or contamination by species that adsorb on the surface are also common [12].

Amperometric biosensors based on the incorporation of biological entities within carbon paste are gaining considerable attention. Coupling plant tissues and pure enzyme as sensing agents to biosensors enables the detection of a wide range of chemicals from the agricultural, pharmaceutical and fermentation industries. Such a versatile strategy allows the co-immobilization of the enzyme, its mediator, or cofactor, and another enzyme or stabilizer, as needed for a reagentless biosensing device [13].

Enzyme electrodes are based on the coupling of a layer of an enzyme with an appropriate electrode. Such electrodes combine the specificity of the enzyme for its substrate with the analytical power of the electrochemical devices. Because of such coupling, enzyme electrodes have been shown to be extremely useful for monitoring a wide variety of substrates of analytical importance in clinical, environmental and food samples [14].

A biosensor consists of two components: a bioreceptor and a transducer. The bioreceptor is a biomolecule that recognizes the target analyte where as the transducer converts the recognition event into a measurable signal [15]. The uniqueness of a biosensor is that the two components are integrated into one signal sensor (Figure 1). This combination allows the measurement of target analyte without using reagents. For example, the glucose concentration in a blood sample can be measured directly by a biosensor by simply dipping the biosensor in the sample. This is in contrast to the conventional assay in which many steps are used and each step requires a reagent to treat the sample. Therefore, the simplicity and the speed of measurements are the main advantages of a biosensor [16].

Biorecogntion reactions often generate chemical species that can be measured by electrochemical methods. In amperometry, typically the reaction product is hydrogen peroxide or the reactant is hydrogen peroxide, which can be measured by a pair of electrodes. When suitable voltage is applied on one of the electrodes against the reference electrode, the target species is reduced or oxidized at the electrode, and this generates electric current.

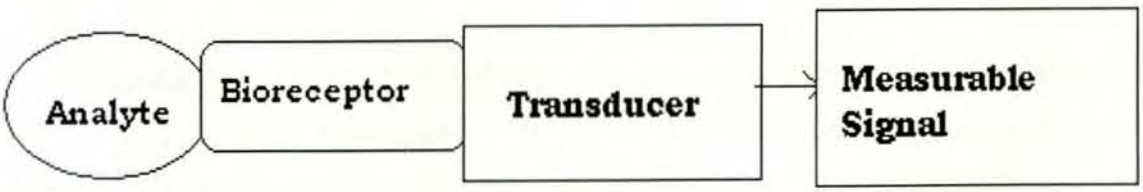


Figure1. Biosensor Configuration

Aqueous solution of enzymes lose their catalytic activity fairly rapidly and the enzyme can be neither recovered from such solutions nor its activity regained. These difficulties have been removed or minimized by the development of enzyme immobilization techniques. The enzyme is immobilized so that it retains its catalytic properties for a much longer time than the free enzyme and can be used continuously for many more analysis. In order to produce a reagentless electrochemical biosensor and to promote electron transfer between an enzyme and an electrode, the enzyme must be immobilized at the electrode surface [17].

Since the late eighties considerable interest has been shown to the development and characterization of Horseradish peroxidase (HRP) modified electrodes, such electrodes allow both direct and mediated electron transfer reactions for substrates, inhibitors, and activators of the enzyme. Thus, interest in HRP is mainly derived from the possibility of direct electron transfer between the enzyme and the electrode matrix allowing the construction of "reagentless" electrochemical biosensors [13].

HRP catalyzed oxidation/reduction of mediator, that are both electron and enzymatically active can be followed at low potential, thus diminishing the influence of electrochemically interfering substances [18].

It has been shown that immobilized HRP maintains its bioactivity and native structure, and displays a high affinity to hydrogen peroxide, making possible the construction of sensor for H_2O_2 [14, 19].

For the electrochemical detection of hydrogen peroxide by its oxidation/reduction at different electrode materials, the electrodes need to be operated at a relatively high potential; this makes the sensor for hydrogen peroxide detection susceptible to many interfering substances. Low applied potential, promoting selective and sensitive detection, can be obtained by using HRP as a biocatalyst for the electrochemical reduction of hydrogen peroxide [17].

The application of a potential between a reference and an indicator electrode enables current to be measured when an electroactive analyte is oxidized or reduced, depending on the voltage at

the indicator electrode. The current is related to the rate of the electrochemical reaction that occurs [17].

But a series problem with protein electrochemistry is the slow mass transport process and strong adsorption of protein molecules on the electrode surface. As result, direct electron transfer to or from the electrode surface is possible only for the first layer of protein on the electrode.

HRP has a very high specific activity for H_2O_2, up to about 600 units (mg protein^{-1}) (one unit catalyzes the reduction of 1 μmol H_2O_2 min^{-1} at 25 ^{0}C) [20]. Although the catalytic nature of enzymes was known in 19th century, the real effect on analysis was delayed until the mid 1960's. The reason for this was limitation in instability, purity and high cost. The present trend to lesson the cost of enzymes per assay is to use them in immobilized forms for applications in biosensors [12].

2.1.1. Catalytic Cycle and Physico-Chemical Properties of Peroxidase

The reaction of HRP with hydrogen peroxide produces HRP-I and HRP-II (Figure 2), which are intermediate compounds that are not enzyme–substrate complexes. HRP has been most thoroughly studied and frequently used to exemplify the peroxidase reaction cycle as shown in Figure 2.

$$\text{Native peroxidase } (Fe^{+3}) + H_2O_2 \rightarrow \text{Compound-I} + H_2O \quad (8)$$

$$\text{Compound-I} + AH_2 \rightarrow \text{Compound-II} + AH^{\bullet} \quad (9)$$

$$\text{Compound-II} + AH_2 \rightarrow \text{Native peroxidase } (Fe^{+3}) + AH^{\bullet} + H_2O \quad (10)$$

The first reaction (equation 8) involves a two-electron oxidation of the ferriheme prosthetic groups of the native peroxidase by H_2O_2. This reaction results in the formation of an intermediate, compound-I consisting of oxyferryl iron ($Fe^{+4} = O$) and a porphyrin π cation radical (Figure 2a).

In the second reaction (equation 9) compound-I lose one oxidizing equivalent upon one electron reduction by the first electron donor, AH_2 and forms compound-II (oxidation state +4) (Figure 2b). The latter in turn accepts an additional electron from the second donor molecule AH_2 in the third step (equation 10), where the enzyme is returned to its native resting state, ferric peroxidase (Figure 2c). [10]. The catalytic cycle of HRP of shown in Figure 2 is summarized into oxidized and reduced forms in Figure 3.

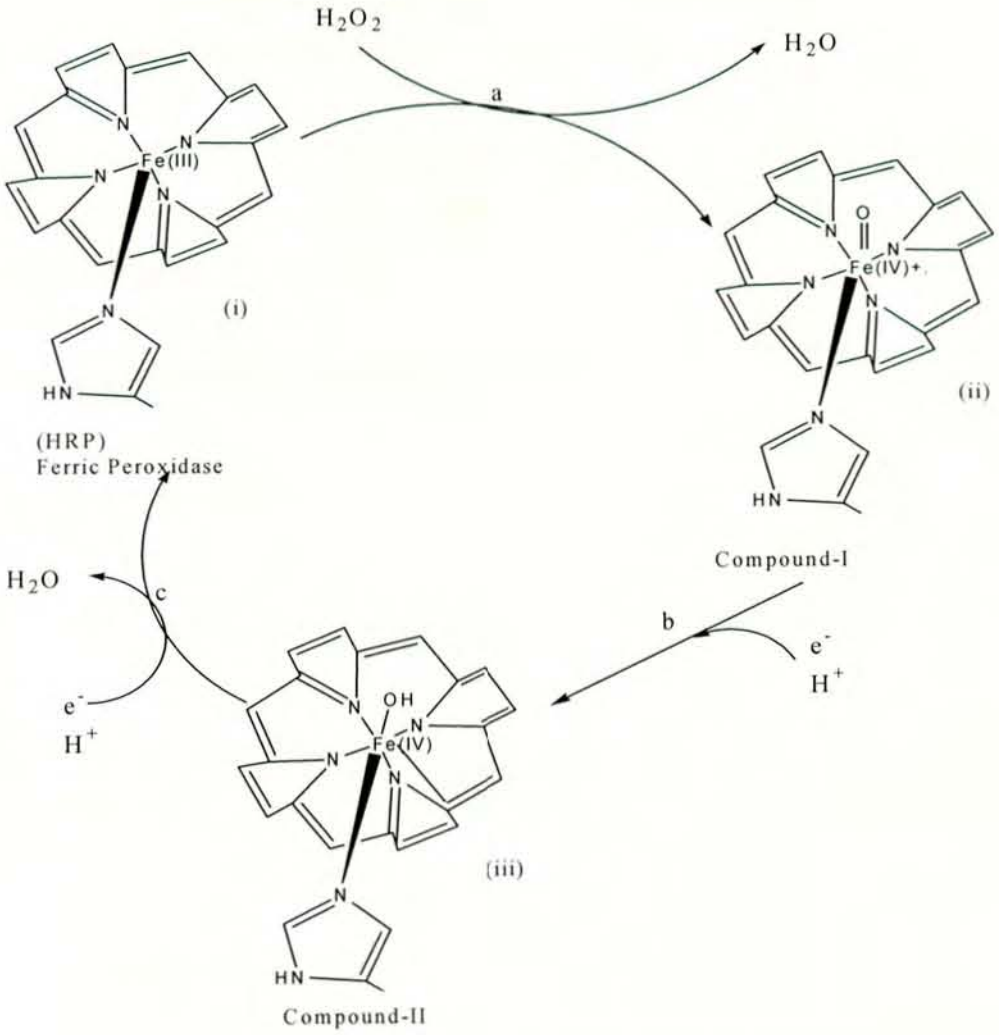


Figure 2. The reaction mechanism for the reaction between hydrogen peroxide and HRP [21].

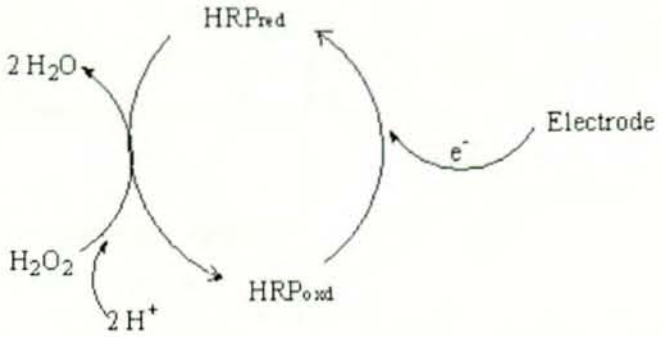


Figure 3. Schematic representation of direct bioelectrocatalytic reduction of hydrogen peroxide at HRP modified carbon paste electrode

In ferric peroxidase, iron exists in the Fe(III) state (Figure2i),where as in HRP-I and HRP-II, it is present in the Fe(IV) state. Compared to HRP-II, one π electron is removed from the porphyrin cycle in HRP-I (Figure 2 (ii, iii)) [22].

Figure 3 can be summarized by schematic equation as:

$$HRP_{oxd} + 2e^- \rightarrow HRP_{red} \qquad (11)$$

$$H_2O_2 + HRP_{red} + 2\,H^+ \rightarrow HRP_{oxd} + H_2O \qquad (12)$$

Virtually any reducing agent is capable of donating electrons to compound-I and compound-II. The necessary protons are donated either by the reducing agent or taken from the surrounding media [23].

2.2. **Characteristics of Enzyme**

The following can be considered as the main characteristics of enzymes. Enzymes form reversible complexes with substrates or are not irreversibly changed by the reaction, so they can catalyse repeated reaction. They commonly required in small amounts, show specificity for the reaction and influence reaction rate only.

Enzymes do not supply energy for the reaction, so they do not affect the stoichiometry, equilibrium state and thermodynamics of the reaction.

2.3. Factors Affecting Enzyme Activity

Being a protein, an enzyme loses its catalytic properties if subjected to agents like heat, strong acids or bases, organic solvents, or other conditions, which denature the proteins. Furthermore, substrate concentration and enzyme loading can also affect the rate at which an enzymatic reaction proceeds.

2.3.1. Effect of pH on Enzyme Activity

Since enzymes are proteins, pH changes will seriously affect the ionic character of the amino and carboxylic acid groups on the protein and will markedly affect the catalytic site and conformation of the enzyme. The –COOH and $-NH_2$ readily gain or lose H^+ ions. As the pH is lowered, an enzyme will tend to gain H^+ ions, and eventually enough side chains will be affected so the enzyme's shape is disrupted. Likewise as the pH is raised, the enzymes will lose H^+ ions and eventually lose its active shape. Therefore, low or high pH values can cause considerable denaturation and hence inactivation of the enzyme protein. The optimum pH at which most enzymes show maximum activity is around neutral pH and is represented as the peak of the bell shaped curve (Figure 4).

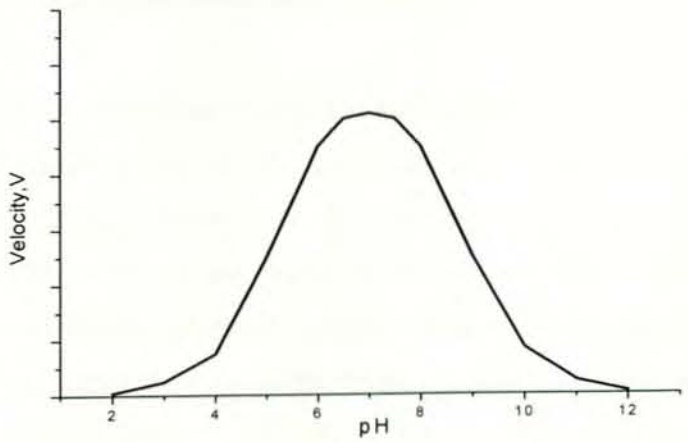


Figure 4. Effect of change in pH on velocity of an enzyme reaction.

2.3.2. Effect of Temperature on Enzyme Activity

Increase in temperature of the system results in increases in the kinetic energy of the system. This has several effects on the rates of reactions. Effect of temperature on rates of reaction can be explained by:

2.3.2.1. Energy of Collision

When molecules collide, the kinetic energy of the molecules can be converted into chemical potential energy of the molecules. Thus the greater KE of the molecules in a system, the greater is the resulting chemical potential energy. As temperature of the system is increased, it is possible that more molecules per unit time will reach at their activation energy. Thus, the rate of the reaction may increase [24].

2.3.2.2. The Number of Collisions per Unit Time

In order to convert substrate into product, enzymes must collide with and bind to the substrate at the active site. Increasing the temperature of a system will increase the number of collisions between enzyme and substrate per unit time. Thus, within limits, the rate of the reaction will increase [24].

2.3.2.3. The Heat of the Molecules in a System

As the temperature of the system is increased, the internal energy of the molecules in the system will increase. The internal energy of the molecules may include the translational energy, vibrational energy and rotational energy of the molecules, the energy involved in chemical bonding of the molecules as well as the energy involved in non bonding interactions. Some of this heat may be converted into chemical potential energy. If this chemical potential energy increase is great enough, some of the weak bonds that determine the three-dimensional structure of the active proteins may be destroyed. This could lead to a thermal denaturation of the protein and thus inactivation of the protein. Thus too much heat can cause the rate of an enzyme-

catalyzed reaction to decrease because the enzyme or substrate becomes denatured and inactive. Taking the above reasoning into consideration, each enzyme has a temperature range in which it achieves a maximum rate of reaction. This maximum is known as the temperature optimum of the enzyme. Outside this temperature range the enzyme is rendered to be totally inhibited. This occurs because as the temperature changes, it supplies enough energy to break some of the intramolecular attractions between polar groups (hydrogen boning, dipole-dipole attractions) as well as the hydrophobic forces between non-polar groups with in the protein structure. When these forces are disturbed and changed, they causes a change in the secondary and tertiary levels of protein structure, and the active site is altered in its conformation beyond its ability to accommodate the substrate molecules it was intended to catalyze. Thus, storage of enzymes at 4 ^{0}C or below is generally the most suitable [24].

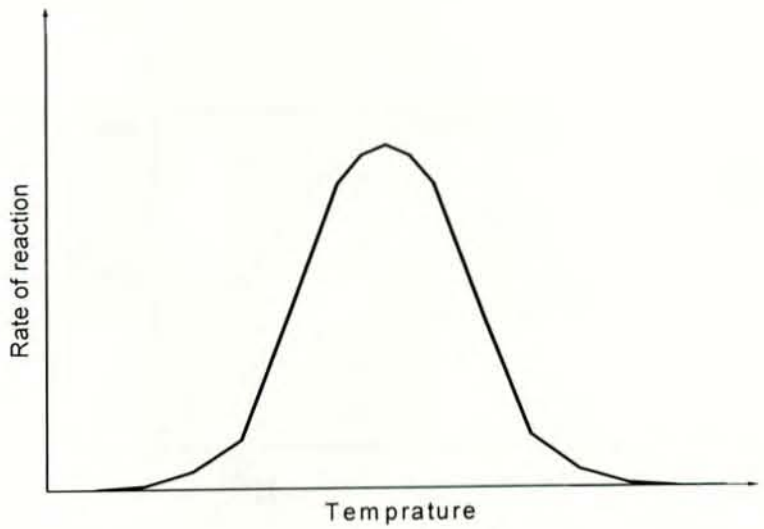


Figure 5. Effect of change in temperature on the rate of an enzymatic reaction.

2.3.3. Effect of Substrate Concentration on Enzyme Activity

As it is true for any catalyst, the rate of an enzyme-catalyzed reaction depends directly on the substrate concentration. The maximum velocity (V_{max}) of the reaction is reached when substrate molecules occupy all the active site.

With a fixed concentration of enzyme, an increase in substrate concentration will result at first in a very rapid rise in velocity or reaction rate. As substrate concentrations continue to increase, however, the increase in the rate of reaction begins to slow down until no further change in velocity is observed and V_{max} is obtained. This is attributed to the fact that all active sites of the enzymes are occupied with substrate molecules (what is called enzyme saturation).

To determine the maximum rate of an enzyme mediated reaction, the substrate concentration is increased until a constant rate of product formation is achieved. This is the maximum velocity (V_{max}) of the enzyme. In this state, enzyme active sites are saturated with substrate. It should be noted that at the maximum velocity, the factors that affect the rate of enzyme-mediated reactions (i.e., pH, temperature, etc) are at the optimum values [11].

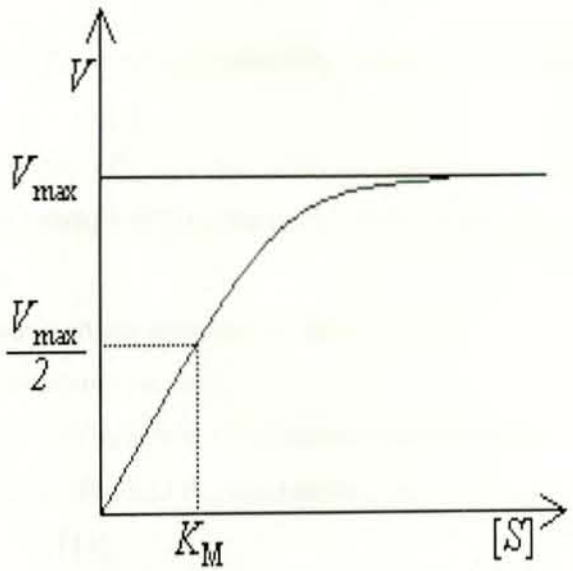


Figure 6. The effect of substrate concentration [S] on the velocity (V) of an enzymatic reaction

The speed (V) means the number of reactions per second that are catalyzed by an enzyme. With increasing substrate concentrations, the enzyme is asymptotically approaching its maximum speed, V_{max}, but never actually reaching it. Because of that no substrate concentration for V_{max} can be given. In stead, the characteristic value for the enzyme is defined by the substrate concentration at its half-maximum speed ($½V_{max}$) (Figure 6). This is considered as K_m value for the enzyme and is called Michaelis - Menten constant.

2.3.4. Kinetics of Enzyme Catalyzed Reaction

For a single substrate homogenous enzyme catalyzed reactions, a plot of reaction rate (V) as a function of substrate concentration [S], usually gives a hyperbolic relationship as shown in Figure 6.

The mechanism of reaction for the conversion of the substrate [S] to the product [P] is given in the reaction:

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \underset{K_4}{\overset{K_3}{\rightleftharpoons}} E + P \qquad (13)$$

Where, E is free enzyme, ES is enzyme-substrate complex, S is Substrate and P is Product

In a first step, the enzyme [E] and the substrate [S] form a complex [ES], which in turn decomposes to form the product [P] and the native form of the enzyme.

The mechanisms are based on the assumptions that:

i) the enzyme concentration is constant.

ii) the concentration of the substrate [S] is much more higher than that of the enzyme [E] and

iii) the formation of ES is fast and the rate limiting step of the overall reaction is the slow decomposition of ES [12].

At initial rate (t = 0, [P] = 0) the backward reaction can be neglected. Reaction (equation) 13 is therefore reduced to:

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_3} E + P \qquad (14)$$

At steady state, the rate of formation of ES equals the rate of its decomposition. With a series of mathematical manipulations, the following formulations can be obtained.

$$K_1 [E][S] + K_4 [E][P] = K_2 [ES] + K_3 [ES] \qquad (15)$$

Since it is assumed that almost none of the products reverts to the initial substrate ($K_4 \approx 0$), then

$$K_1 [E][S] = K_2 [ES] + K_3 [ES]$$

From which it follows; $$\frac{[E][S]}{[ES]} = \frac{K_2 + K_3}{K_1} \qquad (16)$$

Defining a new constant, K_m, the Michaelis- Menten constant, equal to $\frac{K_2 + K_3}{K_1}$

Equation (16) can be rewritten as, $\frac{[E][S]}{[ES]} = K_m$

or $$[ES] = \frac{[E][S]}{K_m} \qquad (17)$$

The rate (or velocity) of the reaction is:

$$\frac{d[P]}{dt} = K_3[ES] \qquad (18)$$

Where, $\frac{d[P]}{dt}$ is reaction rate, or reaction velocity (V_0) of formation of product and V_{max} is maximum rate or maximum velocity [12].

The total concentration of enzyme is:

$$[E_0] = [E] + [ES]$$

$$[E] = [E_0] - [ES] \qquad (19)$$

Substituting equation (19) into (17) gives

$$[ES] = \frac{([E_0] - [ES])[S]}{K_m}$$

After rearranging, $[ES] = \frac{E_0}{\frac{Km}{[S]} + 1}$ (20)

Substituting (20) into (18) gives:

$$\frac{d[P]}{dt} = K_3 E_0 \frac{[S]}{K_m + [S]} \quad (21)$$

i.e., $V_0 = V_{max} \frac{[S]}{K_m + [S]}$

The maximum velocity (V_{max}) is attained when the total enzyme [E_0], is completely complexed with substrate, S. Hence $V_{max} = K_3[E_0]$. Moreover the initial velocity, V_0, is proportional to [ES], i.e., $V_0 = K_3[ES]$

The constant K_m, is the Michaelis- Menten constant of the enzyme for the given substrate. K_m is the substrate concentration at which the reaction rate is half of the maximum. It is noted to be that if [S] is large compared to K_m, $\frac{[S]}{K_m + [S]}$ approaches to one. Therefore, the rate of product formation is equal to $K_3[E_0]$, (equation 21).

The relevance of K_m becomes evident when $[S] = K_m$, and then $\frac{[S]}{K_m + [S]} = \frac{1}{2}$ (equation 21).

In this case, the rate of product formation is half of the maximum rate (½ V_{max}) (Figure 6). By plotting V_0 against [S], one can easily determine V_{max} and K_m. The value of K_m characterizes the affinity between the substrate and the enzyme. At known K_m and V_{max}, V_0 can be calculated for each value of substrate concentration. A low K_m value reflects high affinity. At low substrate concentrations, $[S] << K_m$, the reaction rate is directly proportional to the substrate concentration. At high substrate concentration ($[S] >> K_m$), $V_0 = V_{max}$, the reaction rate is no longer dependent on the substrate concentration but only on the enzyme activity [24].

3. Electrochemical Measurements

3.1. Cyclic Voltammetry

Voltammetric techniques are based on controlling the electrode potential and measuring the resulting current. Cyclic voltammetry (CV) is perhaps the most versatile electroanalytical technique for the study of electroactive species. Its versatility combined with ease of measurements has resulted in extensive use of CV in the fields of electrochemistry, inorganic chemistry, organic chemistry and biochemistry. CV is often the first experiment performed in an electrochemical study of an inorganic or organic compound, a biological material, or an electrode surface. The effectiveness of CV results from its capability for rapidly observing redox behavior over a wide potential range. The resulting voltammogram is analogous to a conventional spectrum in that it conveys information as a function of an energy scan.

CV consists of cycling the potential of an electrode, which is immersed in an unstirred solution, and measuring the resulting current. The potential of this working electrode is controlled versus to a reference electrode, a SCE or a Ag/AgCl electrode.

A cyclic volammogram is obtained by measuring the current at the working electrode during the potential scan. The current can be considered as the response signal to the potential excitation signal. The voltammogram is a display of current (vertical axis) versus potential (horizontal axis). Because the potential varies linearly with time, the horizontal axis can also be thought of as a time axis. This is helpful in understanding the fundamentals of the techniques [25].

A cyclic voltammogram can quickly show the presence of all the species that undergo oxidation-reduction reactions at the electrode within the limits set by the solvent, electrolyte and electrode. The important parameters of a cyclic voltammogram are the magnitudes of the anodic peak current ($i_{p,a}$), cathodic peak current ($i_{p,c}$), anodic peak potential ($E_{p,a}$) and cathodic peak potential ($E_{p,c}$) [26, 27].

A redox couple in which both species rapidly exchange electrons with the working electrode is termed an electrochemically reversible couple. The formal reduction potential (E^0) for a reversible couple is centered between Ep,a and Ep,c:

$$E^0 = \frac{E\,p,a + Ep,c}{2} \qquad (22)$$

The number of electrons (n) transferred in the electrode reaction for the reversible couple can be determined from the separation between the peaks potential:

$$\Delta E = Ep,a - Ep,c = \frac{0.059}{n} V \qquad (23)$$

The peak current for a reversible system is described by the Randles – Sevcik equation for the forward sweep of the first cycle.

$$i_p = 2.69 \times 10^5\, n^{3/2} A C D^{1/2} v^{1/2} \qquad (24)$$

Where, i_p is peak current (A); n is electron stoichiometry (eq/mol); A is electrode area (cm^2)
D is diffusion coefficient (cm^2/s); C is concentration (mol/cm^3) and v is can rate (V/s)

Accordingly, i_p increases with $v^{1/2}$ and is directly proportional to concentration. Its relationship to concentration is particularly important in analytical applications and studies of electrode mechanism.

For an irreversible process, i.e., with sluggish electron transfer at the electrode surface, the peak current is given by:

$$i_p = 2.99 \times 10^5\, n(n_a\alpha)^{1/2} A C D^{1/2} v^{1/2} \qquad (25)$$

where, n_a is the number of electrons in the rate determining step and α is the transfer coefficient. All the other quantities have the same meaning as in equation (24).

3.2. Square Wave Voltammetry

Square wave voltammetry is a large amplitude differential technique in which a waveform composed of a symmetrical square wave, superimposed on a base staircase potential, is applied to the working electrode. The pulse techniques are all based on the difference in the rate of the decay of the charging and the faradaic currents following a potential step. This difference arises when a sequence of potential steps, each with duration of about 50 minutes, is applied on to the working electrode. After the potential is stepped, the charging current decays exponentially, while the faradaic (for a diffusion–controlled) current decays as a function of $(1/t)^{1/2}$; that is, the rate of decay of the charging current is considerably faster than the decay of the faradaic current. Thus, by sampling the current late in the pulse life, an effective discrimination against the charging current is achieved. Therefore, after pulse application the measured current consists solely of the faradaic current; that is, measuring the current at the end of a potential pulse allows discrimination between the faradaic and charging current [17, 26, 27,28].

The square wave signal is defined by its frequency f, the amplitude E_{sw}, and scan increment ΔE. The square wave amplitude is one half of the peak-to-peak amplitude and the potential increment ΔE is the step height of the staircase waveform as shown in Figure 7 [17, 27, and 29].

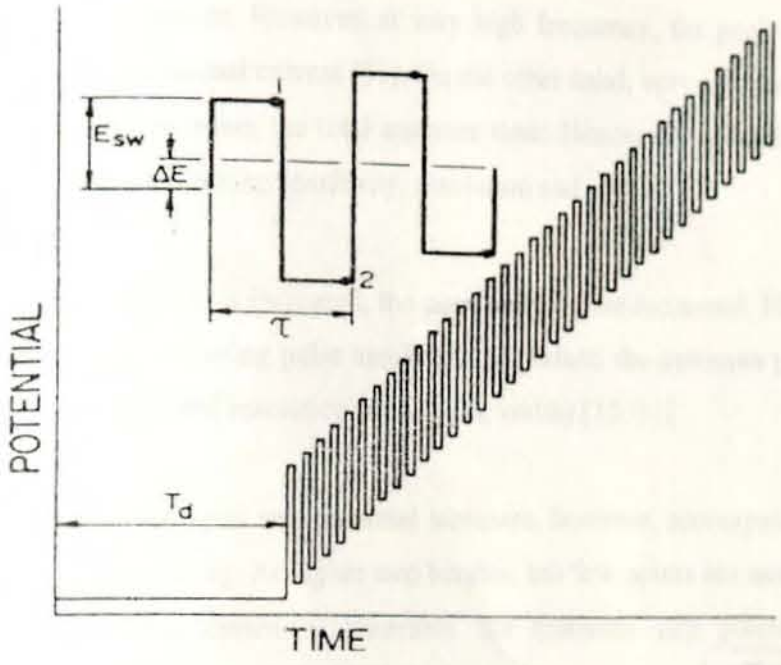


Figure 7. Excitation signal for square-wave voltammetry

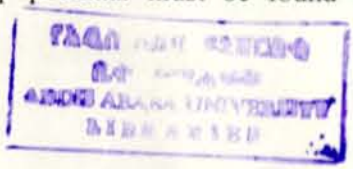
The current is measured at the end of each half cycle, and the current measured on the reverse half–cycle (i_r) is subtracted from the current measured on the forward half-cycle (i_f). This difference current (i_r–i_f) is displayed as a function of the applied potential. The resulting peak shaped voltammogram is symmetrical about the half wave potential, and the peak current is proportional to the concentration. Excellent sensitivity is achieved from the fact that the net current is larger than either the forward or the reverse components (since it is the difference between them); coupled with the effective discrimination against the charging back ground current, very low detection limits near $1x10^{-8}$ M can be attained [17, 27, 30, 31].

The effective scan rate is given by $f\Delta E$. Kinetic parameters can also benefit from the rapid scanning and the reversal nature of square wave voltammetry. The operating parameters in square-wave voltammetry are SW frequency, SW amplitude and step potential.

An increase in frequency of SWV results in an increase in the effective scan rate, which in turn increases the peak current. However, at very high frequency, the peak current is unstable and obscured by a large residual current [31]. On the other hand, very low frequency gives a low but narrow signal, and increases the total analyses time. Hence, the selection of frequency usually requires a compromise among sensitivity, resolution and speed.

When the SW amplitude is increased, the peak currents are increased. However, the peak width also increases with increasing pulse amplitude. Therefore, the optimum pulse amplitude must be found to maximize i_p, and resolution (small peak width) [30, 31].

The net current increases as step potential increases, however, accompanied by peak broadening and loss of peak symmetry. At higher step heights, too few points are sampled, thus affecting the reproducibility of the detection. Therefore, the optimum step potential must be found to maximize sensitivity and to obtain better peak symmetry.



3.3. Amperometry

Amperometry uses a carbon electrode to record changes in the chemical composition of the reduced components of analyte solution. Oxidation and reduction is accomplished by changing the voltage at the active surface of the electrode in a process known as "scanning" [32].

Amperometric measurement is recording the current flow in the cell at a single applied potential. On the other hand, a voltammetric measurement is made when the potential difference across an electrochemical cell is scanned from one preset value to another and the cell current is recorded as a function of the applied potential. In both cases, the essential operational feature of voltammetric or amperometric device is the transfer of electrons to or from the analyte. The basic instrumentation requires controlled-potential equipment and the electrochemical cell consisting of three electrodes (W.E, R.E, and A.E) immersed in a suitable electrolyte [32].

In chronoamperometric measurement, a stationary electrode and unstirred solution are used. The resulting current-time dependence is monitored. As mass transport under these conditions is

solely by diffusion, the current-time curve reflects the change in the concentration gradient in the vicinity of the surface of the electrode [25].

The analysis of chronoamperometric (CA) data is based on Cottrell's equation, which defines the current – time dependence for linear diffusion control:

$$i = nFACD^{1/2} \Pi^{-1/2} t^{-1/2} \qquad (26)$$

where, n is number of electrons transferred/molecule, F is Faraday's constant (96,500C/mol), A is electrode area (cm^2), D is diffusion coefficient ($cm^2 s^{-1}$), Π is 3.14 and C is concentration (mol/cm^3).

This indicates under this condition, there is a linear relationship between the current and the inverse of square root of time. A plot of i vs. $\frac{1}{\sqrt{t}}$ is referred to as the Cottrell's plot.

The analysis of chronocoulometry (CC) data is based on the Anson equation, which defines the charge- time dependence for linear diffusion control:

$$Q = 2\, nFCD^{1/2} \Pi^{-1/2} t^{1/2} \qquad (27)$$

Where, Q is the charge and all the other quantities have the same meaning as in equation (26)

3.4. Electrodes

3.4.1. Working Electrode

The reaction of interest occurs at the working electrode. Electrical current at the working electrode due to electron transfer is termed as faradaic current. The faradaic current at the working electrode is transduced to a potential out put at a selected sensitivity, expressed in amperes per volt, and recorded in a digital or analog form. The CV response is plotted as current

versus potential. The electrode should provide high signal-to-noise characteristics, as well as a reproducible response. Thus its selection depends primarily on two factors, the redox behavior of the target analyte and the background current in the potential region required for the measurement. The most popular working electrodes are carbon, mercury, or noble metals (particularly platinum or gold) [17].

3.4.2. Reference Electrode

It provides a stable potential (independent of the sample composition) against which the potential of the working electrode is compared. Such "buffering" against potential changes is achieved by a constant composition of both forms of its redox couples, e.g., Ag/AgCl or Hg/Hg_2Cl_2, as common with the silver-silver chloride and saturated calomel reference electrodes, respectively.

To minimize contamination of the sample solution, the reference electrode may be insulated from the sample through an intermediate bridge. To avoid large junction potentials, the reference electrode solvent should be as close in nature as possible to the cell solvent system [26].

The cell is filled with saturated solution (KCl, Hg_2Cl_2) and is separated from the solution in which the electrode is to be used by a fritted glass disc, asbestos fiber, or a salt bridge, which maintains electrolyte contact between the internal solution and the sample solution, but prevents the internal solution from rapidly leaking out [33]. A practical reference electrode should be easily and reproducibly prepared and maintained, relatively inexpensive, stable over time, and usable under a wide variety of conditions [34].

3.4.3. Auxiliary or Counter Electrode

The auxiliary electrode is typically a platinum wire that is often placed directly in to the solution. These inert conducting materials, platinum wire or graphite rod is driven by the potentiostatic circuit to balance the faradaic process at the working electrode with an electron transfer in opposite direction (e.g., if reduction takes place at the working electrode, oxidation takes place at the auxiliary electrode). The process at the auxiliary electrode is typically not of interest, and in

most experiments the small currents observed mean that the electrolytic products at the auxiliary electrodes have no influence on the processes at the working electrodes [17].

The auxiliary electrode provides the current required to sustain the electrolysis at the working electrode. This arrangement prevents the reference electrode from being subjected to large currents that could change its potential. The potentiostat applies the desired potential between a working electrode and a reference electrode as shown in Figure 8 [25].

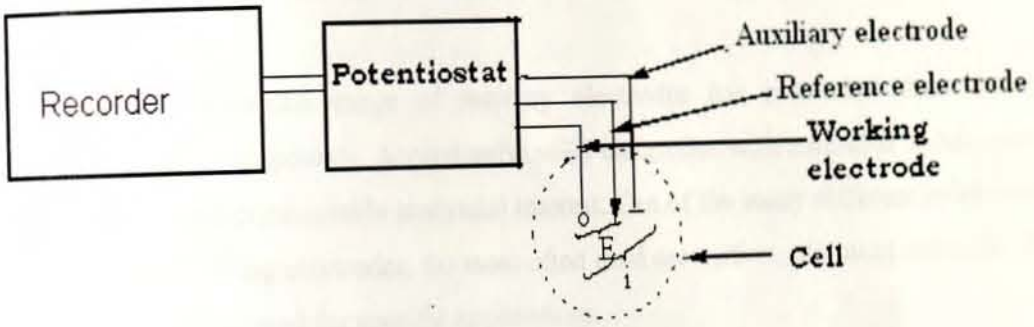


Figure 8. Instrumentation for voltammetry

3.5. Solvents and Supporting Electrolyte

Electrochemical measurements are commonly carried out in a medium, which consists of solvents containing a supporting electrolyte. The choice of solvent is dictated primarily by the solubility of the analyte and its redox activity, and by solvent properties, such as the electrical conductivity, electrochemical activity and chemical reactivity. The solvent should not react with the analyte (or products) and should not undergo electrochemical reactions over a wide potential range [17].

Often, to minimize evaporation of the cell solvent and consequent changes in concentration, the purge gas (Ar) is passed through the same solvent used in the electrochemical cell. This step is particularly necessary for solvents with low vapor pressure.

Supporting electrolytes are required in controlled potential experiments to decrease the resistance of the solutions, to eliminate electron migration effects, and to maintain a constant ionic strength. Buffer systems (such as acetate, phosphate, or citrate) are used when pH control is essential. The electrolyte, added to enhance conductivity, to minimize double layer and migration current effects, is chosen on the basis of solubility in a given solvent as well as inertness toward the electroactive substances and its electrolysis products [33]. The background current, which flows under given experimental conditions in the absence of electroactive material, must be determined before the intended experiments can be made. [35].

3.6. Composite Electrodes

The limited anodic potential range of mercury electrodes has precluded their utility for monitoring oxidizable compounds. Accordingly, solid electrodes with extended anodic potential windows have attracted considerable analytical interest. Out of the many different solid materials that can be used as working electrodes, the most often used are carbon, platinum and gold. Silver, nickel and copper can be used for specific applications.

An important factor in using solid electrodes is the dependence of the surface of the electrode in which reaction takes place. Accordingly, the use of such electrodes requires precise electrode pretreatment and polishing to obtain reproducible results. Unlike mercury electrodes, solid electrodes present a heterogeneous surface with respect to the electrochemical activity, and their true surface is very rough (in the microscopic scale). Such surface heterogeneity leads to deviations from the behavior expected for homogeneous surfaces. Solid electrodes based on carbon are currently in wide spread use in electroanalysis, primarily because of their broad potential, low background current, low cost, chemical inertness, and suitability for various sensing and detection applications.

In contrast, electron transfer rates observed at carbon surfaces are often slower than those observed at metal electrodes. The electron transfer activity is strongly affected by the carbon surface structure. A variety of electrode pretreatment procedures have been proposed to increase the electron transfer rates. The types of carbon, as well as the pretreatment method, thus have a profound effect up on the analytical performance. The most popular carbon electrode materials

are those involving carbon paste, glassy carbon, carbon fiber, or carbon films. The interest of this graduate project is in carbon paste electrodes [17].

3.6.1. Unmodified Carbon Paste Electrode

Carbon paste electrode, which use graphite powder mixed with various water immiscible organic binders (pasting liquids) offer easily renewable and modified surface, low cost, and very low background current contributions.

In addition, since it is simply hand mixing, the analysts can themselves choose the individual components as well as their ratio between the graphite powder and the pasting liquid.

A wide choice of pasting liquids is possible, but practical considerations of low volatility, purity, and economy narrow the choice to a few liquids. These include Nujol (mineral oil), paraffin oil, silicone grease, and bromonaphthalene. The first appears to perform the best. The paste composition strongly affects the electrode activity; in which increase in the pasting liquid content decreases the electron transfer rates, as well as the background current contributions. In the absence of pasting liquids, the dry graphite electrode yields very rapid electron transfer rates (approaching those of metallic surface). It is possible that some of the electrochemistry observed at this electrode involves permeation of the pasting-liquid layer by the electroactive species. Carbon paste represents a convenient matrix for the incorporation of appropriate modifying moieties [36].

3.6.2. Modified Carbon Paste Electrode

Chemically modified electrode is an electrode coated with a selected monomolecular, multimolecular or ionic chemical modifier by means of faradic (charge transfer) reactions or interfacial potential difference (no net charge transfer) exhibits chemical, electrochemical, and or optical properties of the film [37].

The main component of modified carbon paste electrodes is usually a mixture of graphite powder and non-electrolyte binder. Another constituent in the mixture is then a modifier itself (mediator,

enzyme, etc). Modifying agents are usually one substance, but the paste can also be modified with two or even more components. The amount of enzyme in the paste usually varies between 10 to 30% (w/w) depending on the character of modifying agents and its capability of forming enough active site in modified paste

3.6.3. Reasons for Modification

The main reason behind modifying an electrode surface is to improve its analytical performance either by increasing sensitivity and selectivity or by protecting the surface from unwanted reaction. Carbon paste electrodes are advantageous as compared to simple carbon rod since they are bulk modified so that all the necessary components needed for the bioelectrodes such as enzymes, cofactors/ coenzymes, mediators, or activators can be mixed in to the paste.

4. Experimental Section

4.1. Materials

4.1.1. Reagents

Horseradish peroxidase (HRP) (SIGMA, EC, 1.11.1.7 typeVI, 290 Purpurogallin units/mg, 250,000 units, one unit will form 1.0 mg pupurogallin from pyrogallol at pH 6 at 20^0C). Graphite powder (Fluka); KH_2PO_4 (Fluka); Na_2HPO_4 (NICE); paraffin oil (Fluka, Cat.no.76235); and hydrogen peroxide (BDH, U.N.no.2014) were used as received. Deionized water was used in all the experiments.

4.1.2. Apparatus

For amperommetric measurements, the three electrode system with CPE as working electrode; Ag/AgCl as reference electrode; and Platinum wire as the auxiliary electrode was used. All the cyclic voltammetric experiments were carried out using electrochemical analyzer (BAS CV-50W, USA). In addition to this, magnetic stirrer (Model 04803-02, USA)); suction filtration apparatus; plastic syringe for the preparation of working electrode; pH meter (430, JENWAY, UK); balance (SCIENTECH, Model No SA120, Rev–B, USA); freezer (Model: WRD 233, Whirlpool, USA) were used. The experimental set up is shown in Figure 9.

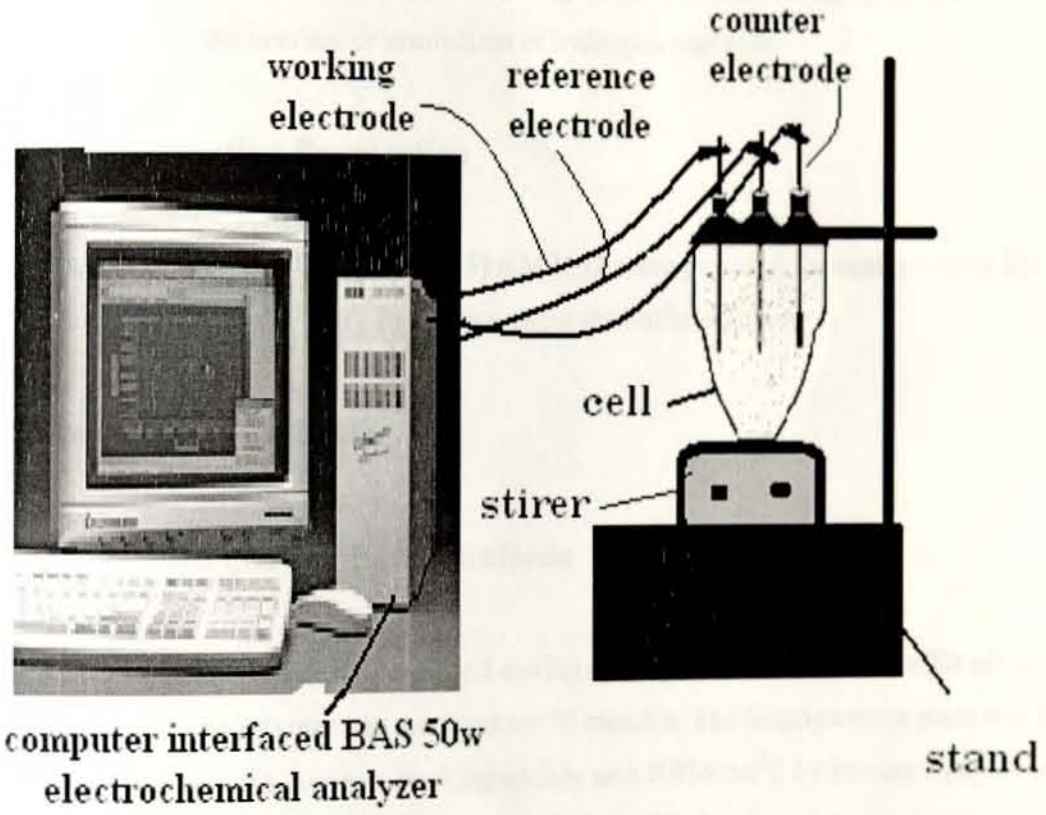


Figure 9. Experimental set up for the electrochemical studies.

4.2. Solution Preparation

4.2.1. Buffer Preparation

The buffer (pH 7) used for dissolving the analyte was prepared by the following procedures: 0.02 M KH_2PO_4 was prepared by measuring 2.72 g of KH_2PO_4 and dissolving in one liter of deionized water. 0.01 M of Na_2HPO_4 was prepared by measuring 1.42 g of Na_2HPO_4 and dissolving in one liter of dionized water. Then the two solutions were mixed and the pH was adjusted to 7 using a pH meter. 1.0 M phosphoric acid (H_3PO_4) and 0.1 M sodium hydroxide (NaOH) were used for adjusting the pH of the phosphate buffer. This solution was used in the preparation of H_2O_2 solutions for the electrochemical determination of hydrogen peroxide.

4.2.2. Sample Solution Preparation

Concentrations of (0.1, 0.3, 0.5, 1, 2, 3, 4, 5) mM H_2O_2 were prepared for analysis from H_2O_2 stock solution (30% w/w, 9.71 M), by dilution using the buffer solution.

4.3. Preparation of Electrode

4.3.1. Unmodified Carbon Paste Electrode

Carbon paste electrode was prepared by hand mixing of graphite powder and paraffin oil in a 75:25 weight ratio. The mixture was mortared for 30 minutes. The homogeneous paste was filled into a plastic syringe (1 mL Syringe, working surface area 0.038 cm^2); by leaving a gap of about 3 - 4 mm at the tip to be filled subsequently with HRP modified carbon paste as shown in Figure 10.

4.3.2. Modified Carbon Paste Electrode (HRP – CPE)

The HRP-CPE was prepared according to the following procedure: 100 mg of graphite powder and 4 mg of horseradish peroxidase (HRP) were taken and dissolved in 200 µL buffer (pH 7.0). The solution was mixed and stirred for 2 hours with a stirrer. The mixture was dried using suction filtration set up for 5 hours. The dry composite was mixed with 40 µL paraffin oil and mortared for 30 minutes to get a homogeneous paste, and it was filled into the tip of the plastic syringe initially filled with unmodified carbon paste to obtain the final electrode. Inserting a copper wire into the plain carbon paste from the backside of the syringe to have electrical contact between the source and the sample in the cell. The electrodes, which were prepared, contained 3%, 6%, 9%, 12%, 15%, 21% HRP w/w. After the tip of the syringe was filled with the modified paste, the end was gently rubbed on tissue paper to produce a flat electrode surface. The HRP – CPEs were stored in a dry state at 4 ^{0}C until and after use.

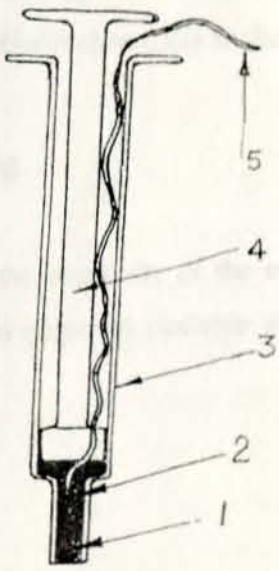


Figure 10. Horseradish peroxidase modified carbon paste electrode: 1 – HRP modified carbon Paste; 2 - unmodified carbon paste; 3 - Syringe body; 4 – piston; 5 - copper wire

5. Results and Discussion

5.1. Voltammetric Determination of Hydrogen Peroxide with HRP Modified Carbon Paste Electrode

5.1.1. Cyclic Voltammetric Determination

Electrochemical analyzer (BAS CV-50 W) was used to run all electrochemical experiments. All potentials were described (reported) versus to Ag/AgCl reference electrode. Before each experiment was carried, the solutions were purged with a stream of argon and blanketed with argon throughout the experiments.

The electrochemical potential window of the supporting electrolyte (0.02 M KH_2PO_4 and 0.01M Na_2HPO_4) solution was examined before the electrochemical studies and was carried out using cyclic voltammetry. There were no interfering electrochemical reactions with in the potential range where the hydrogen peroxide was electrochemically studied.

5.1.1.1. Effect of Enzyme Loading

Enzyme loading was found to affect the sensitivity of the enzyme electrode. Increasing the amount of the immobilized HRP caused improved electrode sensitivity which levelled off to a maximum at 15% HRP (w/w).

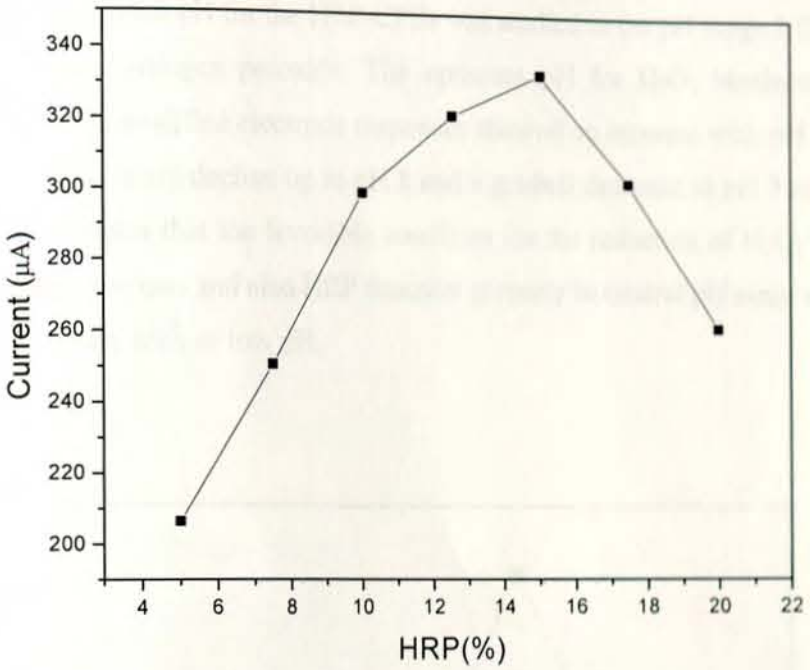


Figure 11. Effect of enzyme loading on the current responses of the modified carbon paste electrode for 5 mM H_2O_2

All measurements were carried out in phosphate buffer at pH 7.0. The amount of HRP in the carbon paste has a significant influence on the voltammetric signal. The maximum current was obtained with the ratio of 15% HRP (mass ratio of HRP in the paste). Higher ratio of HRP (21% w/w) decreased the current significantly, the reason for which may be more HRP at the electrode surface reduces the amount of conductive areas (carbon particles); so that the electrochemical reduction of H_2O_2 at higher HRP ratios is reduced and the chemical reduction of H_2O_2 becomes more probable. Lower ratios of HRP (3% w/w) also decreased the current due to inability to transfer electron effectively from the electrode to the analyte since less contact would occur between the analyte and the modifier. Thus higher or lower HRP ratio reduced the extent of the electrochemical reaction. Hence, an electrode modified with 15% HRP was employed through out these experiments (Figure 11).

5.1.1.2. Effect of pH

The optimum operational pH for the HRP-CPEs was studied in the pH range 3.0 - 9.0 using 5 mM solution of hydrogen peroxide. The optimum pH for H_2O_2 bioelectro reduction was observed at 7.0. The modified electrode responses showed an increase with pH to a maximum at pH 7, followed by a sharp decline up to pH 8 and a gradual decrease to pH 9 as shown in Figure 12. The result indicates that the favorable condition for the reduction of H_2O_2 is around neutral conditions. Many enzymes and also HRP function properly in neutral pH range and are denatured at either an extremely high or low pH.

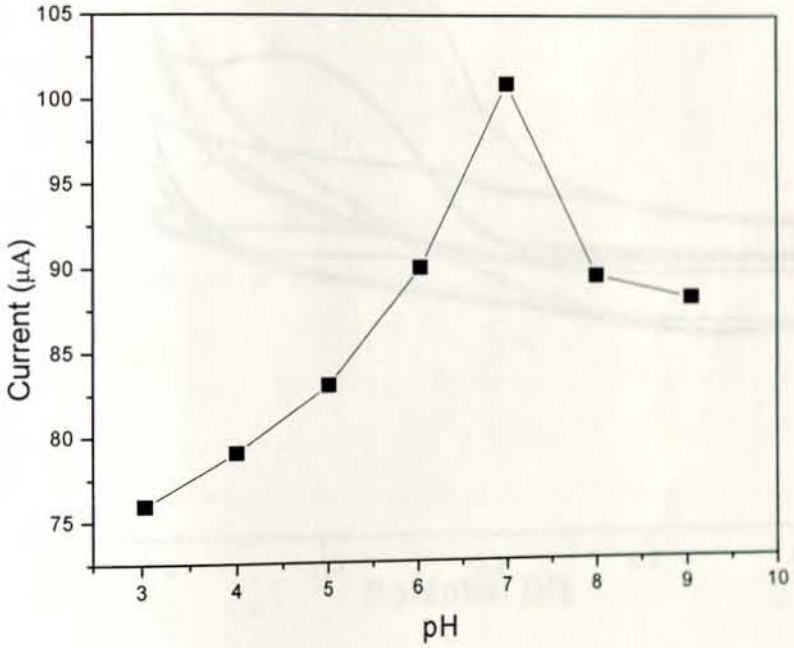


Figure 12. The influence of pH on the catalytic activity for 5 mM H_2O_2 in phosphate buffer on HRP modified CPE, scan rate 100 mVs^{-1}

5.1.1.3. Comparison of Current Responses and Potential of H_2O_2 Reduction Using Modified and Unmodified Carbon Paste Electrodes

Once the appropriate potential window was known, the electrochemical reduction of hydrogen peroxide was studied. The voltammograms of modified and unmodified CPE for the buffer and a solution containing 5 mM H_2O_2 were recorded as depicted below.

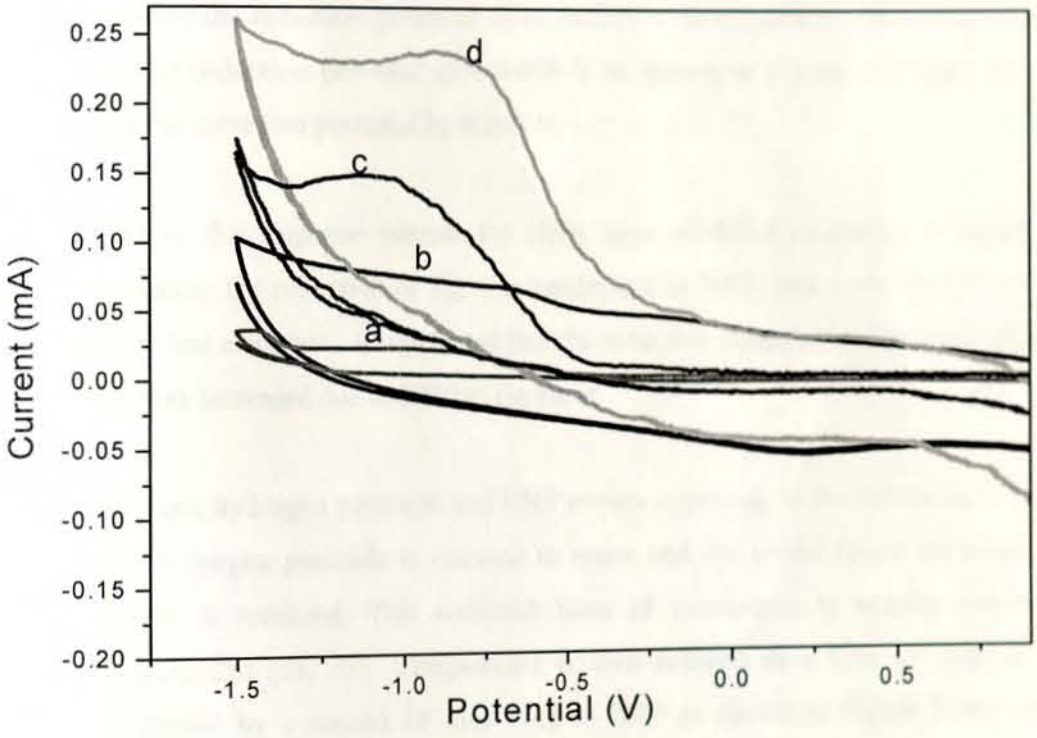


Figure 13. Cyclic voltalmmogram for (a) buffer with unmodified (b) buffer with HRP modified (c) 5 mM H_2O_2 with unmodified (d) 5 mM H_2O_2 with HRP modified carbon paste electrodes, pH 7, and scan rate 100 mVs^{-1}.

The experiment showed that the response of the sensor for hydrogen peroxide is dependent on the activation of the modified electrode at the negative potential. Hence, voltammetric experiments were carried out by cycling the potential in the range of 1.0 V to -1.5 V.

At the unmodified electrode, reduction of H_2O_2 appears at a negative potential of – 1.229 V as shown in Figure (13) curve (c). This potential of unmodified is higher comparing with the potential response of modified CPE.

From analytical point of view, one interesting feature of HRP modified electrodes is the low operating potential which offers low background currents and minimizes the risk of surface fouling and interference by electractive species. It was observed that, a CPE modified with HRP shows no cathodic/ anodic peak in the absence of hydrogen peroxide (buffer).

The modifier shifted the reduction potential from –1.229 V to – 0.886 V. The voltammogram displayed a distinct reduction potential at – 0.886 V as shown in Figure (13) curve (d). The modifier reduces the reduction potential by 0.343 V.

On the other hand, the response current for H_2O_2 with modified electrode is higher than unmodified, indicating the reduction of H_2O_2 is catalayzed by HRP, and more H_2O_2 is reduced than at the unmodified electrode. It was found that the reduction potential was reduced while the response current was increased due to HRP in the paste.

The reaction between hydrogen peroxide and HRP occurs according to the following: In a first $2e^-$ transfer step hydrogen peroxide is reduced to water and the bound factor (in most cases ferroprotoporphyrin is oxidized. This oxidized form of peroxidase is usually denoted as compound–I (Figure 2ii) [38, 39]. Compound-I is then reduced in a first $1e^-$ step to form compound-II, followed by a second $1e^-$ step back to HRP as shown in Figure 2 and also in equation (8, 9 and 10).

5.1.1.4. Effect of Concentration

The basic feature of a voltammogram (i.e., a plot of current versus potential in cyclic voltammetry) is the appearance of a current peak at a potential characteristic of the electrode reaction taking place. The voltammetric response of HRP modified CPE for solutions containing various concentrations of hydrogen peroxide are shown in Figure 14 and 15.

Figure 14(a) has no observable peak since it was a buffer solution. After H_2O_2 was added, an obviously catalytic characteristic appeared resulting in an increase of the reduction current with increase in H_2O_2 concentration. Figure 14 (i) shows the voltammogram for 5 mM H_2O_2 while 16 (b) shows for 0.1 mM, in which the reduction current appears at -0.886 V. The increase in current is ascribed to the electron transfer between the electrode and HRP. These results indicated the HRP could effectively induce electron transfer to the active center of the electrode. Therefore, as the concentration increased from 0.1 mM to 5 mM, the reduction peak current also increased significantly (Figure 14).

It is shown that, the magnitude of the peak current in the cyclic voltammogram provides information regarding the concentration of the analyte according to the Randles–Sevcik equation (equation 24), [26]. Accordingly, the peak current increases with the square root of the scan rate, and is directly proportional to concentration of hydrogen peroxide

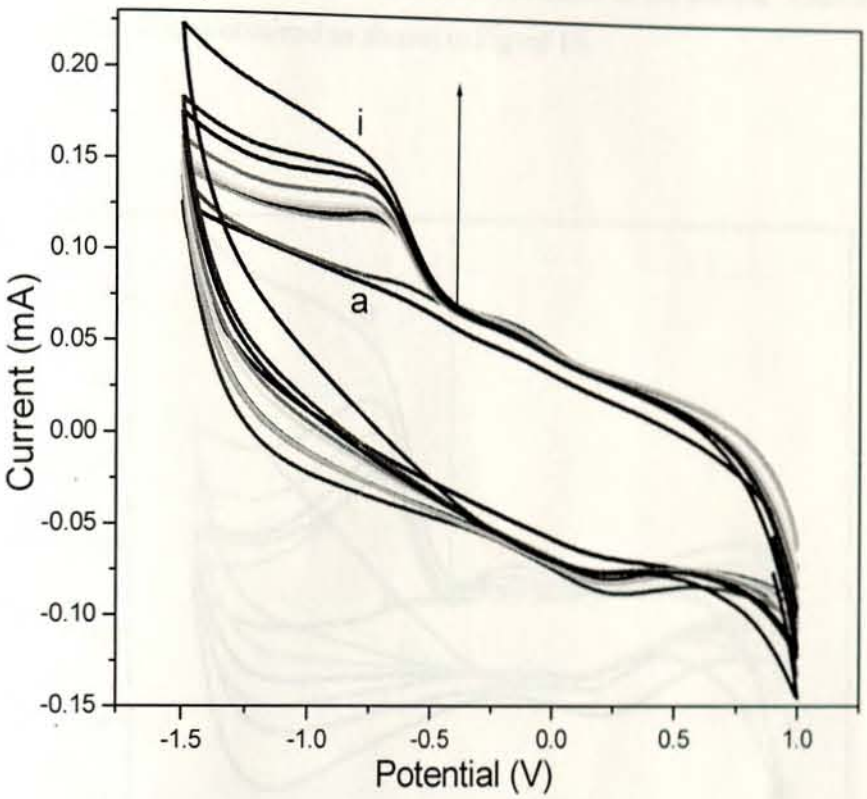


Figure 14. Cyclic voltammograms for a solution containing (a) buffer; (b) 0.1; (c) 0.3; (d) 0.5; (e) 1; (f) 2; (g) 3; (h) 4; (i) 5 mM H_2O_2 solutions (pH 7) using HRP modified CPEs, scan rate 100 mVs^{-1}

Cyclic voltammogram shown in Figure 14 also contains cyclic voltammograms for solutions containing different concentration of H_2O_2 in which the background current was not subtracted.

The background current that flows in the absence of the electroactive species of interest is composed of contributions due to the double layer charging process and redox reactions of solvent, electrolyte or electrode. The background current (the current due to double layer charging or redox reaction of solvent or electrolyte) was subtracted, and the pure faradaic current of analyte was obtained. The reduction peak is clearly seen in the voltammograms of Figure 15

and can be compared with those in Figure 14. This indicates that the contribution of the background current for the net current is high and should be taken into consideration in the explanation of the peak current for different concentrations of the analyte. Then the pure faradaic current of the analyte was obtained as shown in Figure 15.

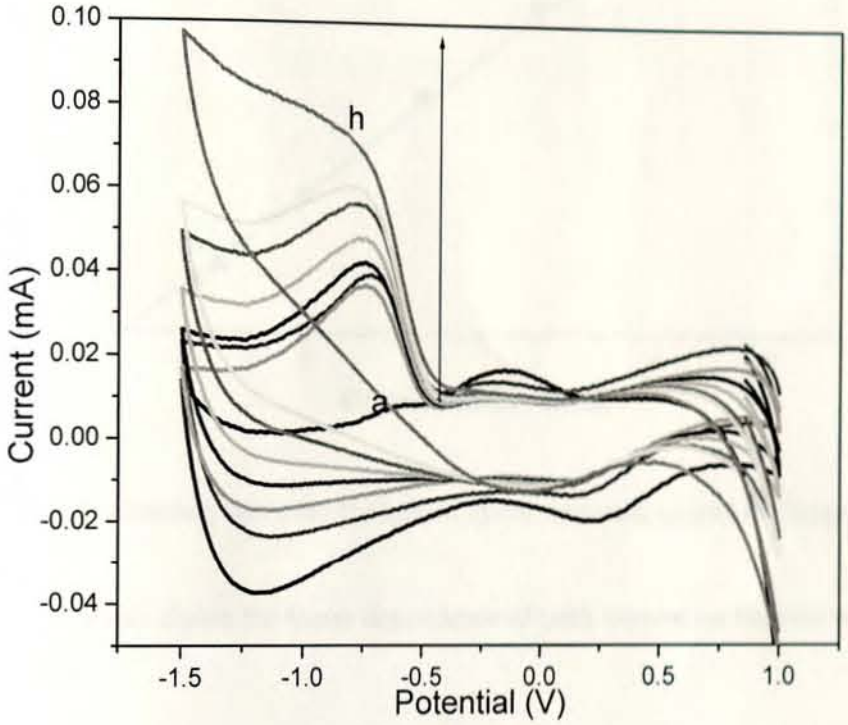


Figure 15. Cyclic voltammograms for a solution containing (0.1, 0.3, 0.5, 1, 2, 3, 4, 5) mM H_2O_2 represented by a to h, respectively (pH 7) using HRP modified CPEs, scan rate 100 mVs^{-1}.

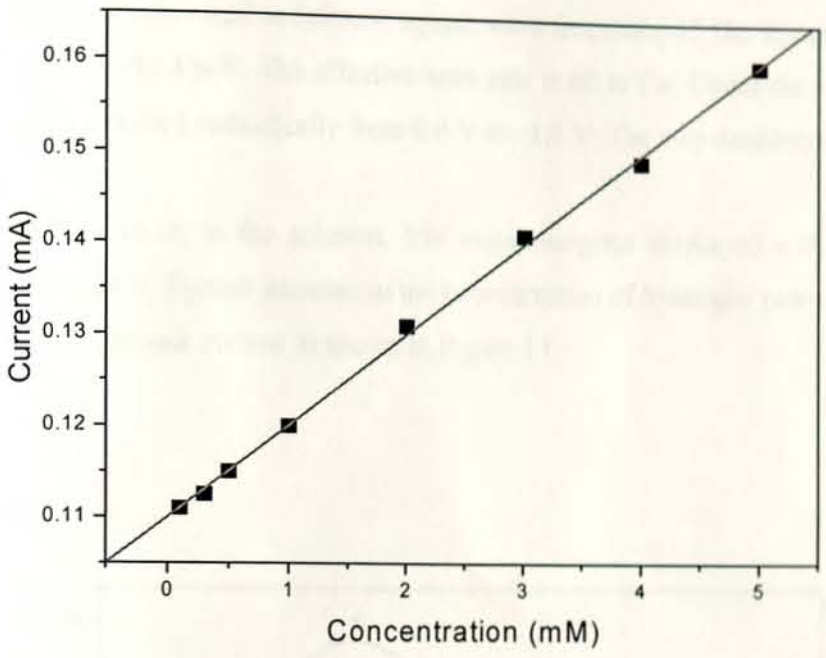


Figure.16. Calibration curve for H_2O_2 from cyclic voltammograms in Figure 15

The calibration graph shows the linear dependence of peak current on the concentration of H_2O_2 from 0.1 – 5 mM.

5.1.2. Square Wave Determination of Hydrogen Peroxide

The peak current obtained in Osteryoung Square Wave Voltammetry (OSWV) is dependent on various instrumental parameters such as square-wave amplitude, square wave frequency, and step height. These parameters are interrelated and have a combined effect on the response, but here only the general trends will be examined. When the square wave amplitude was varied between 10 and 50 mV, the peak current increased with increasing amplitude. However, the peak width was also increasing at the same time, in particular when the amplitude was greater than 25 mV. Hence, 25 mV was chosen as the square wave amplitude. The step height together with the frequency defines the effective scan rate. Hence, an increase in either the frequency or the step height results in an increase in the effective scan rate. However, very high frequency has an

influence on stability of peak current and increasing step potential has an effect of peak broadening. By considering these interrelated (combined) effects, the overall optimization parameters can be summarized as follows: square wave frequency 15 Hz, square wave amplitude 25 mV and step height 4 mV. The effective scan rate is 60 mV/s. Under the optimal condition, the potential was scanned cathodically from 0.0 V to –1.5 V. The step duration was 2 seconds.

In the presence of H_2O_2 in the solution, SW voltammogram displayed a distinct increase in reduction peak current. Further increase in the concentration of hydrogen peroxide resulted in an increase of reduction peak current as shown in Figure 17.

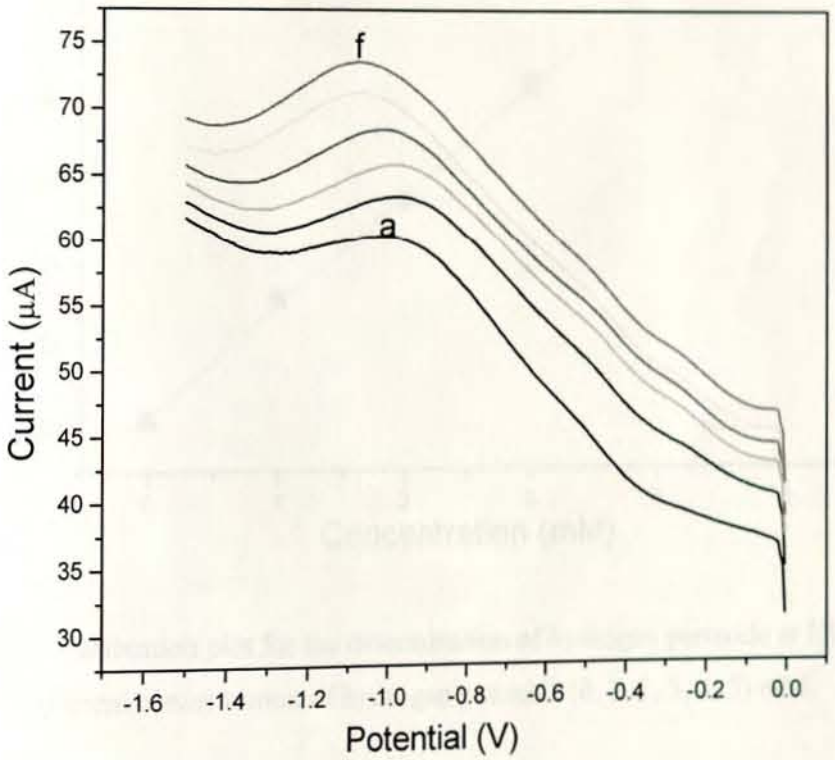


Figure 17. Square wave voltammograms for the determination of H_2O_2 at HRP modified CPE for different concentration of H_2O_2 ((a) 0; (b) 1; (c) 2; (d) 3; (e) 4; (f) 5)mM.

The relationship between the SW voltammetric peak current and H_2O_2 concentration was examined. Using the results in Figure 17, a linear calibration plot was constructed for the concentration of H_2O_2 between 0 mM and 5 mM in buffer solution (pH 7) with slope 2.661x10 (μA/mM), and (R = 0.99946) as shown in Figure 18. On the basis of these results obtained by SW voltammetry, the procedure can be applied to determine the concentration of hydrogen peroxide.

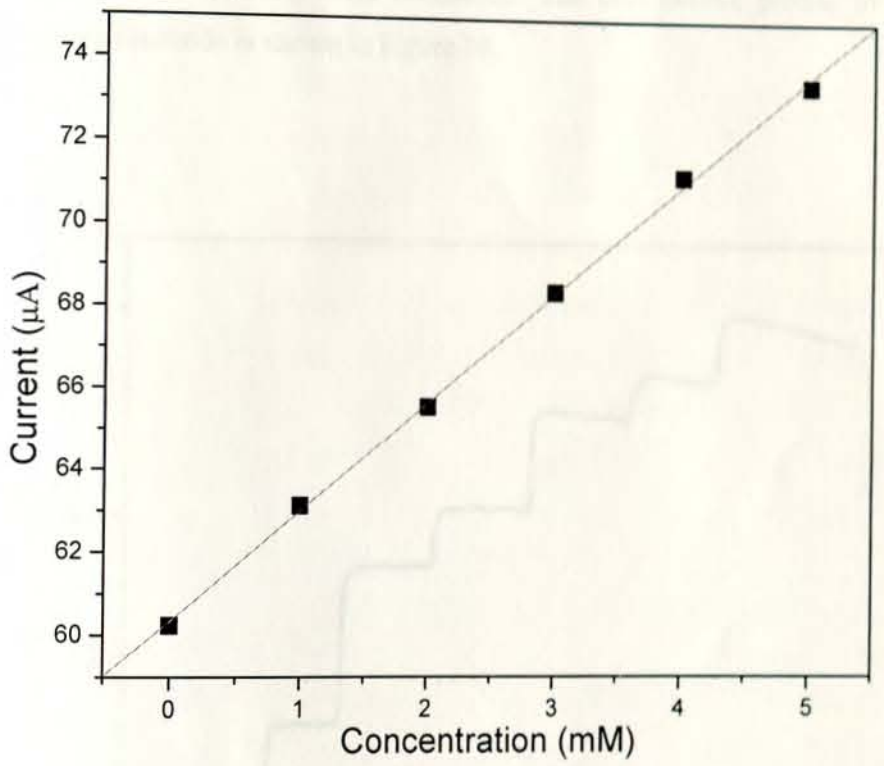


Figure 18. Calibration plot for the determination of hydrogen peroxide at HRP modified CPE for different concentration of hydrogen peroxide (0, 1, 2, 3, 4, 5) mM.

5.1.3. Amperometric Determination of Hydrogen Peroxide

In amperometric measurments, a constant potential is applied between the sensing electrode and a reference electrode. The current generated by the redox reaction of the analyte at the sensing electrode is directly proportional to the analyte concentration. The amperometric response of HRP modified CPE to hydrogen peroxide were measured by stepwise injection of 1 mL of 10 mM hydrogen peroxide with micro liter syringe into 50 mL of phosphate buffer (pH 7) with stirring for 5 seconds to homogenize the injected solution after which the resulting reduction currents were monitored. The current-time profile of HRP modified carbon paste electrode is shown in Figure 19.

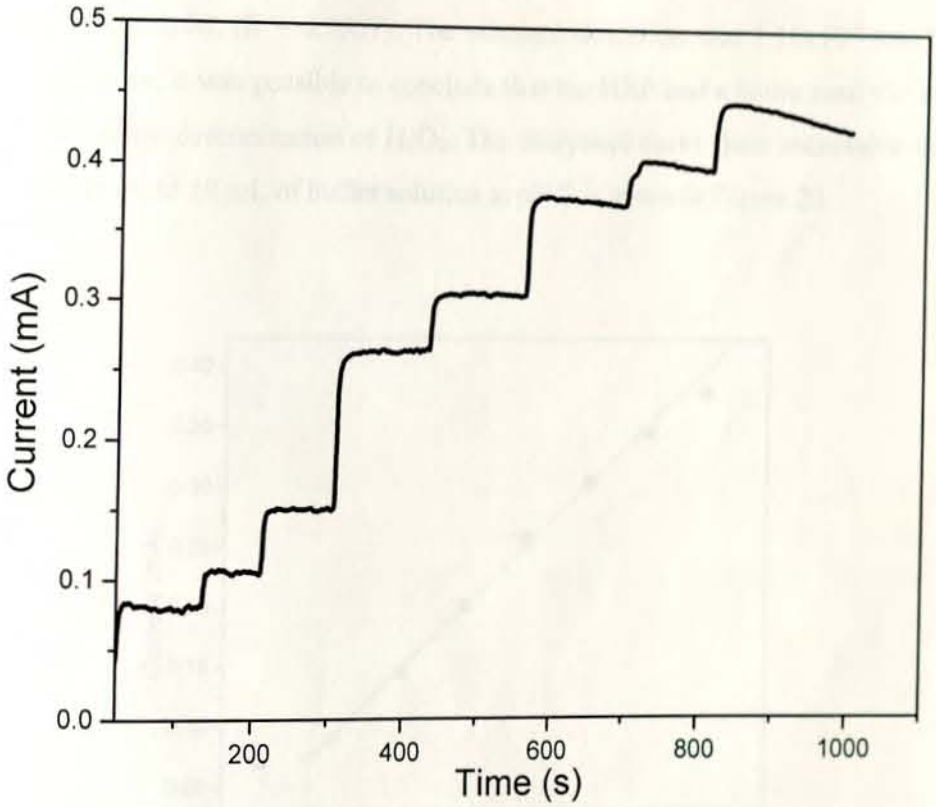


Figure 19. Amperograms of HRP modified CPE to successive additions of 1 mL of 10 mM H_2O_2 to 50 mL buffer solution (pH 7) at applied potential of – 0.8 V versus Ag/AgCl.

It was found that the HRP modified CPE displayed a more significant current response after addition of hydrogen peroxide. A well-defined and fast amperometric response was observed at –0.8 V with successive injection of 1 mL of 10 mM H_2O_2. For successive addition of 1 mM H_2O_2, the concentration increased from 0 to 1.228 mM. The measurements were made over a long time as possible to ensure reliable results. For concentrations of hydrogen peroxide higher than 1.071 mM, a decline of response was observed, showing a characteristic of the Michaelis-Menten kinetics mechanism. In addition, since mass transport is solely by diffusion, the current-time curve reflects the change in the concentration gradient in the vicinity of the electrode surface. This involves a gradual expansion of the diffusion layer associated with the depletion of the reactant and hence decreased slope of the concentration profile as time progress. Accordingly, the current decays with time, as given by the Cottrell's equation (equation 26). The calibration curve of the sensor response is given in Figure 20. The linear range of H_2O_2 responses was

0.196 mM – 1.071 mM, (R = 0.9957). The standard deviation was 1.16×10^{-5} for 7 successive additions. Therefore, it was possible to conclude that the HRP had a better catalytic performance for the amperometric determination of H_2O_2. The analytical curve from successive addition of 1 mL of 10 mM H_2O_2 to 50 mL of buffer solution at pH 7 is given in Figure 20.

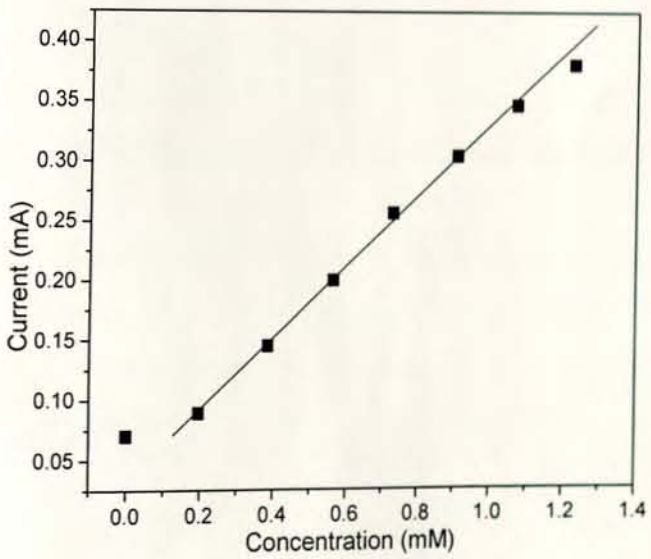


Figure 20. Calibration curve for amperometric hydrogen peroxide determination at an applied potential of –0.8 V versus Ag/AgCl

6. Summary

High catalytic activity of the peroxidasees with a broad range of substrates and their application, in a variety of fields, is the attractive property of these enzymes for the development of amperometric peroxidase modified electrode.

Amperometric determination of hydrogen peroxide using HRP modified CPE is characterized by high sensitivity and speed with low sensing potential. The redox process can involve the HRP-I/HRP two electron transfer or the two-step HRP-I/HRP-II and HRP-II/HRP mechanism. The electrochemical reduction (oxidation) calls for the expenditure of one proton per electron as in the reduction of HRP-I and HRP-II by a reducing agent in the bulk solution. The phosphate buffer (pH 7) has no interference in the selected potential window.

The electrocatalytic reduction of hydrogen peroxide was obtained at around -880 mV versus Ag/AgCl giving high current responses. To maintain high enzyme activity, the temperature and the pH of the environment were maintained at appropriate working conditions.

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