STUDIES ON IMMOBILIZED ENZYMES FOR BIOSENSOR APPLICATIONS

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By

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CERTIFICATE

This is to certify that the thesis entitled ***"Studies on immobilized enzymes for biosensor applications"*** is the result of the work carried out by **Mr. M. Devaraja Gouda** during the period 1996-2000 under my guidance at the Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore 570013.

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This work incorporated in the thesis *"Studies on immobilized enzymes for biosensor **applications*** " was carried out by me at the Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore, 570013, under the guidance of **Dr. N.G. Karanth,** deputy director and head Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore during the period 1996-2000.

I further declare that the work embodied in this thesis has not been submitted for the award of degree, diploma and any other similar title.

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Place Mysore

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M. Devaraja Gouda

Dedicated to my beloved
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Introduction

INTRODUCTION

Quantification of sugars like glucose and sucrose in various food and fermentation industries is important for efficient monitoring and quality control of various operations. For example, in sugar manufacturing, beverages and confectionery industries, the concentration of sugars needs to be estimated rapidly and accurately. Various methods for the estimation of sugars range from the conventional Shaffer and Hartman and dinitrosalicylic acid (DNSA) methods to modern techniques like HPLC and GC. Most of these techniques are often non-specific or expensive. One of the modern techniques which overcomes many of the disadvantages of the conventional methods is the use of biosensors.

The basic principle behind the biosensor utilizes the specificity of the reaction involving the biological component, the selection of which is based on the nature of the analyte to be detected. Based on the biological component, biosensors are classified into catalytic, affinity and tissue type biosensors. Catalytic biosensors in particular are attractive as they offer a specificity for the analyte and the depletion of cosubstrate or the formation of the coproducts can be easily monitored by using a variety of transducers. Although the application of enzyme based biosensors for various analytical purposes have been explored, actual field application has been hampered due to the relatively poor stability of immobilized enzymes. A literature review indicates that the stability of immobilized enzymes can be improved further for biosensor applications. Though a number of methods exist for immobilization of enzymes, all of them are not suitable for

biosensor applications. One of the popular enzyme immobilization methods for biosensor application is the glutaraldehyde cross linking method. Glutaraldehyde being a strong bifunctional reagent, modifies the enzyme drastically and leads to inactivation of the enzymes. Methods have been reported to minimize this drastic modification of enzymes by the addition of inert proteins like gelatin, bovine serum albumin (BSA) etc. during the process of immobilization (Broun. 1976). While it is known that potentially useful enzymes/proteins that provide a better stability for soluble enzymes are available, the use of such protein based stabilizing agents (PBSAs) during immobilization is restricted to gelatin and BSA. Our investigations indicate that the stability of desired enzymes can be improved by selecting a suitable PBSA, with an inherent higher stability and renaturation ability. In the present work we have directed our efforts to construct an immobilized enzyme and amperometry based biosensor, study its operational characteristics and the stability of enzymes immobilized with various PBS As against temperature, pH, denaturants, and operational stability. The stability studies of immobilized enzymes is difficult by using Circular Dichroism (CD), Differential Scanning Calorimetry (DSC) and Fluorescence Spectroscopy (FS). We however have used a simple modified dissolved oxygen electrode that can be efficiently used for stability studies and also for the denaturation-renaturation studies.

The literature survey starts with a brief description of biosensor, followed by a classification of biosensors based on the biological component as well as transducer. This is followed by a detailed review of various methods of immobilization and the stability of the biological component with reference to the biosensor applications.

1.1 Conventional analytical methods for the estimation of sugars.

1.1.1 Chemical methods.

Most of the chemical methods which are mentioned in literature are based on the estimation of total reducing sugars either by titration using copper sulphate (Shaffer and Hartman.1921) and phenol and sulphuric acid (Dubois et al., 1956) or by spectrophotometric method using dinitrosalicylic acid (Miller. 1959). These methods are simple, but a major disadvantage of these methods is the non-specific estimation of the desired sugar which is not adequate in most of the biotechnological processes. Furthermore, these methods are laborious, time consuming, encounter interference of colour from the sample itself and difficulties in the automation limit the use of these methods.

1.1.2 Chromatographic methods.

In order to quantify the desired sugars in a specific manner, advanced methods like Fligh Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) have been employed. HPLC using refractive index detector and acetonitrile : water in the ratio of 80:20 as mobile phase has been reported (McGinnis and Fang. 1980). GC method for sugars reported (Sawaradekar et al., 1965) utilizes the flame ionization detector at 250°C and nitrogen as carrier gas. However, these conventional methods have several disadvantages. HPLC is expensive and needs laborious sample pretreatment to get accurate results. GC is also expensive and sample preparation by derivatization is generally elaborate. Additionally, these instruments require column clean up also.

1.1.3 Enzymatic methods.

Sugar estimation based on enzymatic methods (Boehringer Mannheim) involve wastage of enzyme which gets consumed for each analysis and most of the enzymatic estimation methods involve indicator enzymes which increase the cost of analysis. Due to increased current importance of various biotechnological processes for the production of value-added products, development of rapid and reliable analytical methods for sugars plays an important role in quality control and maximizing the process efficiency. Further, conventional analytical methods are not suitable where on-line monitoring is desirable. One of the modern techniques which overcomes the disadvantages of conventional methods is the use of biosensors.

1.2 Biosensors.

Biosensors are a subgroup of chemical sensors in which a biologically based mechanism is used for selective detection of desired analytes. The first enzyme based biosensor for glucose using dissolved oxygen electrode was reported in 1962 (Clark and Lyons. 1962). Updike and Hicks developed the first operational glucose biosensor in 1967 based on amperometric measurement of oxygen depletion in an immobilized glucose oxidase (GOD) gel (Updike and Hicks. 1967). Since then, much research work has been carried out and a number of papers, reviews and books have been published on these biosensors. A schematic diagram of the general biosensor is shown in figure 1.2. The selectivity and specificity of biosensors originates from the biological component connected to the sensor. In any biosensor, the property of specific molecular recognition based on affinity between complementary structures (enzyme-substrate; antibody-antigen; receptor-hormone), is used to obtain concentration dependent signals.

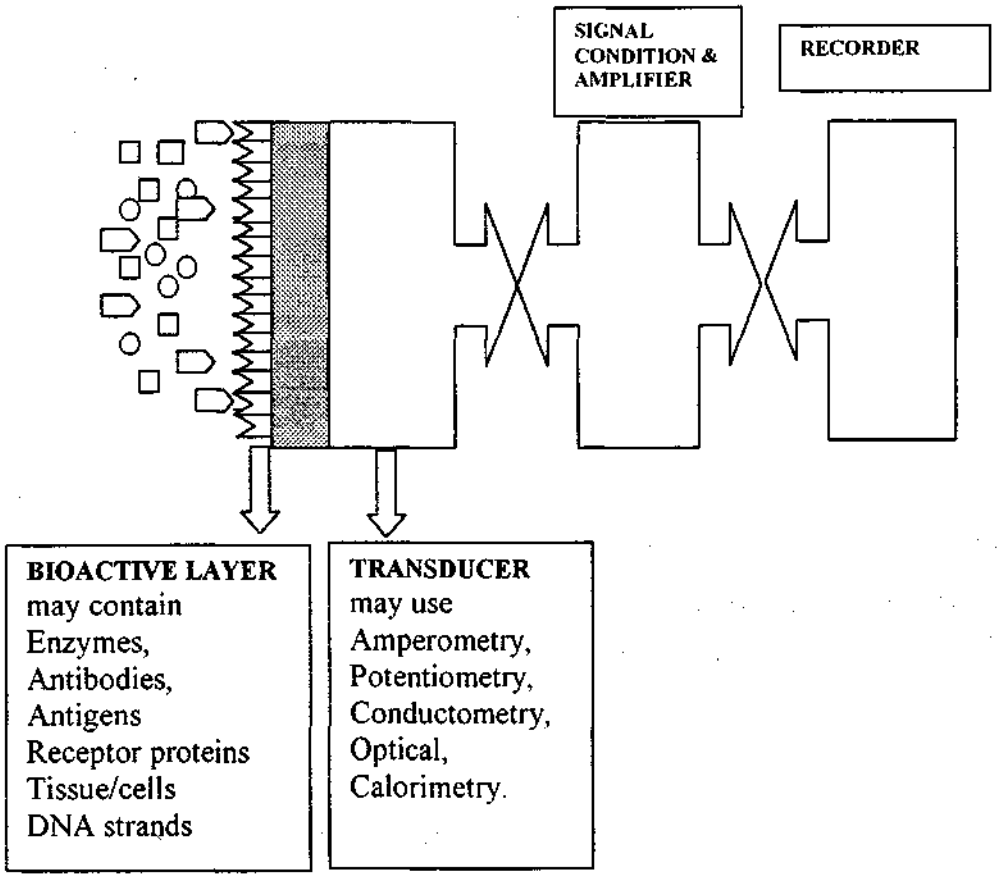


Figure 1.2 A generalized scheme of biosensor configuration.

(Schmidt et al., 1992)

Advantages of biosensors over conventional methods:
(Mulchandani and Bassi. 1995)

1. Specific in response to analyte
2. Speed of response
3. Simple to operate
4. Require no pretreatment of sample-A major advantage; Can operate in turbid / coloured solutions and without addition of reagents
5. Ease of miniaturization
6. Economical
7. Do not require consumption or withdrawal of body fluids during patient monitoring
8. Amenability for continuous operation
9. Can be easily interfaced with microprocessor or computer for data processing and control

Definition of a biosensor.

Many definitions have been proposed for biosensor, the following one being accepted in general

*"A biosensor is an analytical device that combines the specificity of **a** biological sensing element for the analyte of interest with a transducer to produce **a** signal proportional to target analyte concentration"* (Turner. 1987).

1.3 Classification of biosensors.

In general, biosensors can be classified either on the basis of the biological component or on the basis of the transducer.

1.3.1 Biological component.

Specificity of any biosensor primarily relies on the biological component. Primary event of signal generation in the biosensor occurs when the biological component interacts with the analyte. A variety of biological components such as enzymes, tissues/ cells and affinity ligands are utilized for biosensor construction, based on which biosensors are divided into three following classes.

1.3.1.1 Enzyme based biosensors.

Because of their specificity and catalytic properties, enzymes have found wide-spread applications in biosensor construction. Majority of biosensors that are constructed and commercialized for various applications are based on enzymes and amperometry. In enzyme based biosensors either an enzyme will be fixed on to the electrode surface, as in a batch type biosensor or an enzyme reactor will be connected to the transducer through flow line as in the continuous Flow Injection Analysis (FLA) system. A variety of enzymes belonging to the classes of oxido-reductases, hydrolases and lyases have been integrated with different transducers to construct biosensors for applications in clinical, veterinary, medical and pharmaceutical areas, food and fermentation processes, environmental monitoring and defense applications (Guilbault. 1984). Since not all enzyme catalysed reactions involve transducer-active compounds such as hydrogen ions, oxygen, or hydrogen peroxide, only a limited number of analytes can be determined by using mono-enzyme sensors. However, in such cases coupled enzyme reactions for analyte conversion provide a favorable alternative. The primary product of the conversion of the analyte is further converted enzymatically with the formation of measurable secondary product (Xu et al., 1989; Scheller and Renneberg. 1983). However, the optimal operating conditions for each component are different, so that a compromise becomes necessary and may lead to poor stability. As shown in table 1.3.1.1, enzyme based biosensors can be used for measurement of a wide range of substrates. Although, a primary aim of the biosensor research is the preparation of operationally stable biosensor for long term use in monitoring systems (Appleton et al., 1997), the development of highly satisfactory enzyme based biosensor is hampered because of their poor stability (Mulchandani. 1998). In order

to improve the stability of enzymes, many different techniques have been employed; such as protein engineering (Nosoh and Sekiguchi.. 1993), the use of enzymes from naturally thermostable microorganisms (McNail et al., 1989; Rain et al., 1995), immobilized enzymes (Scouten et al., 1995; Kullary et al., 1992) and by addition of stabilizing agents to the enzymes (Gibson et al., 1992; Gibson et al., 1993). Another limitation involves the removal of interferences while analysing the real samples particularly with multienzyme based biosensors, for example, in sucrose estimation, a major potential limitation being the interference of glucose. However, in such biosensors, interference of glucose has been avoided by using a two electrode system. One enzyme electrode for the estimation of glucose and other for the estimation of both glucose and sucrose (Xu et al., 1989; Scheller and Renneberg.1983). The difference in the signal responses of the two electrode is utilized for the net sucrose concentration calculation in the given sample. Though the problem of using two electrode system has been indicated elsewhere (Riche et al., 1995), no reports are available on a systematic study on a elimination of the effect of glucose on the sucrose biosensor. A detailed discussion on the stability of enzymes and removal of glucose interference in sucrose biosensor which forms a part of this work has been discussed in chapter 4 in this thesis.

1.3.1.2 Tissue/cell based biosensors.

Tissue/cell based biosensors are also based on enzymes, wherein, instead of using purified enzymes, whole tissue/cell which is a rich source of desired enzyme is used as biological component, in intimate contact with a suitable transducer. Various methods of immobilization have been used. A variety of tissues/microorganisms have been utilized in combination with different transducers to determine simple analytes like glucose (Riedel

et al., 1988), sucrose (Riedel et al., 1990), benzoate (Riedel et al., 1991) etc, and complex analytes like biological oxygen demand (BOD) (Riedel et al., 1988) and chlorinated phenols (Riedel et al., 1993). **Table 1.3.1.1. Enzyme based biosensors for various analytes.**

Analyte	Enzymes	Transducer	Reference
Glucose	GOD	O_2	Clark and Lyons. (1962)
Sucrose	Invertase, Mutarotase & GOD	O_2 H_2O_2	Xu et al., (1989) Scheller and Renneberg. (1983)
Amino acids	Amino acid oxidase	O_2	Váradit, et al., (1999)
Organophosph-orous pesticides	Butyryl cholinesterase	H^+	Imato and Ishibashi. (1995)
Morphine	Morphine dehydrogenase	Electron mediator	Holt et al., (1995)
Urea	Urease	Optical	Konchi et al., (1995)
Metal ions	Urease	Optical	Tescione and Belfort., (1993)

Tissue/microorganism based biosensors have some advantages over enzyme based biosensors (Kurabe and Nakanishi. 1994; Reidel. 1998) such as, elimination of enzyme purification step and thus improved process economy, cofactor not being required, higher tolerance to suboptimal pH and temperatures etc. The whole cell may perform multistep transformations that could be difficult to achieve with purified enzymes, e.g., for **BOD.** However, a major disadvantage of the tissue based biosensors is the lack of selectivity, due to the multi-receptor behavior of the intact living system. Other disadvantages like long response and washing time, limit the use of microbial biosensors.

1.3.1.3 Affinity Sensors.

Affinity sensors are based on monitoring the change in mass or optical properties of biological component and analyte interactions. In affinity biosensors, biological component may be antigen, antibody, receptors and DNAs.

Affinity sensors based on antigen or antibody are called as immunosensors, defined as analytical devices that detect the binding of an antigen to its specific antibody by coupling the immunochemical reaction to the surface of the device known as a transducer (Vizeli and Cristopher. 1996). They have been a subject of increasing interest during the past decade mainly because of their potential applications as an alternative immunoassay technique in areas such as clinical diagnostics (Mattiasson and Nilson. 1977), environmental monitoring (Brecht et al., 1995; Bart et al., 1997; Ozgoev et al., 1999) and defence applications (Bart et al., 1997).

The specific binding property of certain proteins like lectins, with their ligands is utilized for the construction of affinity based biosensors. Plant proteins like concanavalin A (Con A), capable of binding to certain carbohydrate groups has been used for the construction of affinity sensor with an optical transducer for the detection of mannan (Janata. 1975). Affinity biosensor for β-lymphocytes has also been constructed by monitoring the decrease in catalase activity caused by binding of β-lymphocyte (Aizawa. 1983). A major disadvantage of these sensors is the non-specific binding of interfering compounds. Furthermore, since these biosensors are based on equilibrium, time required to reach steady state is very high.

Affinity sensors based on biological receptors are called as receptrodes. They are based on the chemoreceptors (in purified form or in intact membranes) integrated with a transducer that can quantify the physical changes during their complex formation. Isolated nicotinic acetyl choline as well as plant receptors have been employed for the determination of nicotine and auxins respectively (Rechnitz. 1987; Thompson et al., 1986). The major disadvantage of these biosensors are the inherent instability of the receptors within the sensor which are stable for less than a few hours (Frederick. 1998).

In recent years, DNA biosensors have gained increased importance. The development of DNA hybridization based biosensors, holds great promise for obtaining sequence-specific information for clinical, environmental and forensic investigations. DNA biosensors typically rely on the immobilization of a single-stranded DNA sequence (the probe) and its hybridization with the complementary (target) strand to give a suitable electrical or optical signal (Gópel. 1998). In spite of extensive research and development, most DNA biosensors are not yet capable of discriminating selectively against single-base mismatches, for example, in the detection of disease related point mutations (Gópel. 1998).

1.4 Transducers.

The physicochemical change of the biologically active material resulting from the interaction with analyte must be converted into an electrical output signal by an appropriate transducer. In order to construct an efficient biosensor, selection of suitable transducer is crucial and has been reviewed in literature (Scheller and Schubert. 1992; Mulchandani and Rogers. 1998). On the one hand, unspecific, but broadly applicable

transducer may be used, which indicates general parameters such as reaction enthalpy (thermistor), mass change (Piezoelectric crystal) or layer thickness (reflectometry). On the other hand, specific indication may be achieved with amperometric or potentiometric electrode for species such as H^+, OH-, $C0_2$, NH_3, H_20_2, 0_2 or with optical methods such as photometry or fluorimetry. The advantages and disadvantages of various transducers are given in table 1.4

Table 1.4 Advantages and disadvantages of various transducers commonly used for biosensor constructions (Luong et al., 1991)

Transducer	Advantages	Disadvantages
O_2 electrode	Simple, high selectivity	Low sensitivity
H_2O_2 electrode	Simple, high sensitivity	Low selectivity
Ion selective electrode	Simple and reliable	Slow response, requires stable reference electrode and susceptible to electronic noise
Piezoelectric	Fast response, simple, no special sample handling	Low sensitivity in liquid conditions and interference resulting from non-specific binding
Optical	Remote sensing, simple, no reference electrode is required and can be free from electrical interferences	Interference from ambient light, requires high energy sources, applicable to only narrow range of analytes.
Calorimetric	Versatility, free from optical interferences	Expensive, cumbersome, drift in baseline due to change in sample temperature

The operating principle of commonly used transducers are described below.

1.4.1 Electrochemical Transducers.

There are three types of electrochemical transducers that are used in biosensor construction: Amperometric, Potentiometric and Conductometric.

1.4.1.1 Amperometry.

The basic concept of the biosensor as pointed out previously, was first proposed in 1962 (Clark and Lyons. 1962). The first operational glucose biosensor based on amperometric measurement of oxygen depletion in an immobilized GOD gel was reported by Updike and Hicks (1967). Amperometric enzyme biosensors form the majority of commercial biosensor devices available in the market today. Amperometry is based on the oxidation or reduction of an electro-active compound at an electrode while constant potential is applied to the working electrode with respect to reference electrode. The resulting current is measured by using either these two electrodes (working and reference electrodes) or a three electrode system (working, reference and counter electrodes) to compensate for the potential drop caused by passage of current through the solution. The current, I is a direct measurement of the electrochemical reaction rate (oxidation or reduction rate of the analyte at the electrode) as described by Faraday's law.

$$\mathbf{I = ZF\,(dn/dt)}$$

where dn/dt is the oxidation or reduction rate (in mol s^{-1}), Z is the number of electrons transferred , F is the Faraday constant. The rate of electron transfer can be accelerated by increasing the potential difference between the electrodes. Most of the amperometric biosensors are based on redox enzymes; thus, their appeal is caused by the availability of a

large number of oxido-reductase enzymes that can act on fatty acids, sugars, amino acids and phenols (Mulchandani. 1998). Table 1.4.1.1. shows a selected list of enzymes that are used with amperometry for the construction of biosensors. The redox enzymes use molecular oxygen as electron acceptor and produce hydrogen peroxide as co-product in their enzymatic reaction. The consumption of molecular oxygen can be monitored by using cathodic amperometry involving application of -650mV to the working electrode. Alternatively, production of hydrogen peroxide can be monitored by using anodic amperometry by applying +700mV to the working electrode with respect to reference electrode. The electrode potential is a decisive factor for the selectivity of the sensor. For example, biosensors based on hydrogen peroxide measurement may suffer from the non-specific electrochemical oxidation of compounds such as ascorbate, uric acid, catecholamine, cystine FADH2 and NADH at the applied potential +600 to +700mV versus Ag/AgCl electrode (Scheller and Schubert. 1992). Though, oxygen based biosensors, are influenced by fluctuations in dissolved oxygen concentration, these fluctuations can be minimized by continuously saturating the solution with oxygen concentration (Cleland. 1984). This method can be applied for the determination of even high analyte (substrate) concentration at which the enzyme reaction is otherwise limited by oxygen. Such biosensors are useful for monitoring in food and fermentation industries where the concentration of the analytes are relatively higher (1-10%). Further details of Clark type dissolved oxygen electrode will be discussed in chapter 2. Furthermore, amperometric based biosensors have also been constructed for non redox enzymes (Palleschi et al., 1992). In order to measure the acetyl cholineesterase (AChE) activity amperometrically, acetylethiocholine iodide was used as substrate. In the presence of

AChE, actylethiocholine iodide is hydrolyzed to thiocholine and acetate. The electrooxidation of thiocholine was monitored amperometrically by applying +410mV to the working electrode with respect to the reference electrode. Organophosphorus (OP) pesticides thus were monitored based on their inhibition of AChE activity (Skladal. 1991).

Table 1.4.1.1 Typical examples of the enzyme based biosensors.

Enzyme	Analyte	Electroactive sps.	Reference
GOD	Glucose	O_2	Clark and Lyons.(1962)
Invertase, mutarotase & GOD	Sucrose	O_2	Mohammad et al., (1987)
Lactate oxidase	Lactate	H_2O_2	Mascini et al., (1985)
Ascorbate oxidase	Ascorbate	O_2	Matsumoto et al., (1981)
Laccase	Catecholamines	O_2	Wollenberg et al., (1997)
AChE	OP pesticides	Thiocholine	Skládal. (1991)

In order to overcome the disadvantages of interference while analysing the real samples in anodic amperometry and baseline fluctuations during oxygen measurement in cathodic amperometry, electron mediators and conducting polymers have been used. Also attempts have been made to create direct electrical communication between redox enzyme and electrode. These are discussed below.

1.4.1.1a Electron mediators.

Electron mediators are low molecular weight compounds, which act as artificial cosubstrates by shuttling the electrons between the redox center of the enzyme and working electrode at low applied potential (+50 to 380 mV). Ideal electron mediator for an electrochemical biosensor should react rapidly with enzyme, exhibit reversible oxidation at the low applied potential and should be stable with respect to pH, temperature and

oxygen (Pandey. 1998). Commonly used electron mediators for biosensor applications are benzoquinone, ferricyanide, ferrocene and its derivatives. Though many electron mediators have been reported there is no single mediator working efficiently with all the redox enzymes. A major breakthrough was reported by Cass et al (1984) using ferrocene and its derivatives as electron mediators. However, disadvantages like solubility of one of the redox couple of ferrocene in the aqueous solutions, sensitivity to ascorbic acid and other redox centers of the enzyme and a large increase in background noise (Pandey. 1998), limit the use of electron mediators for biosensor applications. These problems have been tackled by immobilizing redox enzymes and the mediator in carbon paste electrodes. Although carbon paste electrodes without mediator immobilization has been reported, electrochemical interference caused by applying high potential (+0.9 to 1.1 V) to the working electrode (Wang and Teha. 1991; Amine et al., 1991; Matazzewski and Trojanowicz. 1988) necessitates the use of electron mediators immobilized along with enzymes (Wang and Chen. 1993).

1.4.2 Potentiometry.

Montalvo and Guilbault (1969) developed the first operational potentiometric enzyme electrode. Their first electrode was based on a combination of urease trapped in polyacryl amide and held in contact with univalent cation electrode which is sensitive to ammonium ions. Potentiometric measurement involves the determination of the potential between two electrodes when there is no current flowing between them. The most commonly used potentiometric electrodes are pH electrodes and other ion selective electrodes (Booker and Haslam. 1974; Cullen et al., 1975; Mulchandani et al., 1999). Accuracy of potentiometric measuring systems depends on the reproducible fabrication of

the reference electrode and the junction between the electrolytes (Camman. 1979). Therefore the requirement of a stable reference electrode may be a limitation of these sensors (Luong. 1991). Furthermore, one of the major difficulties of the potentiometric electrodes has been poor selectivity of the base sensor (Carr and Bowers. 1980; Guilbault. 1984). Attempts have been reportedly made to improve the selectivity of base sensor by using solid state or silicon based ion-selective field effect transistors (ISFETs). An example of enzyme based FETs (ENFETs) design can be a combination of a pH-sensitive ISFET with an immobilized hydrolase enzyme layer (Brand et al, 1991).

1.4.3 Conductometry based biosensors.

Biosensors based on conductometric measurement exploit the fact that the changes in substrate and product concentrations resulting from catalytic action of some enzymes may be accompanied by a net change in the electrical conductivity of the solution. As reported by Lawrence (1971), a number of mechanisms may be responsible: the generation of ionic groups, separation of unlike charges, proton generation and buffering, changes in the size of the charging groups and changes in the degree of association of ionic species etc. More detailed discussions of the theory and practice of conductivity measurement are given by Brad and Faulkner (1980). Since, in conductivity based biosensors, the measuring signal reflects the migration of all ions in the solution, interference is a major problem (Scheller and Schubert. 1992).

1.5 Stability of enzymes and enhancement of stability.

Stability of enzymes is one of the important considerations for biotechnological applications. Stabilization of enzymes can be in terms of a decrease of inactivation rate constants leading to an increase in half life of enzymes or resistance against denaturation by agents such as urea and guanidine hydrochloride (GdmCl) or by exposure to varying pH and temperature conditions.

The different types of interactions within proteins which affect the stability include disulphide bonds, hydrophobic forces, hydrogen bonds, ionic interactions and dispersion forces. Influencing these parameters through external agents can contribute to increasing the enzyme stability (Braxton. 1996). In order to increase the stability of enzymes, attempts have been made by using cosolvents like sugars, polyhydric alcohols, salts, free amino acids and proteins and by immobilization. These cosolvents, free amino acids and proteins tend to increase the stability of the native structure of the enzymes by different mechanisms- e.g. sugars such as sucrose mainly by increasing the surface tension of the medium (Lee and Timasheff. 1981), polyethylene glycols by both stearic and charge repulsion mechanisms (Lee and Lee. 1979), salts by binding to hydrophobic sites (Arakawa and Timasheff. 1981) and free amino acids like arginine, lysine and histidine at lower concentrations by increasing the hydrogen bonding (Arakawa and Timasheff. 1983) and proteins both by hydrophobic and hydrophilic interactions (Chang and Mohany. 1995).

By analysing mechanisms of enzyme inactivation, it has been reported that the main cause is the unfolding of protein (Martinek et al., 1977). Therefore rigidly fixing the enzyme molecule on to a solid support thus rendering the protein more difficult to unfold,

may lead to stabilization of enzymes. It has been reported (Martinek et al., 1977) that along with the multipoint attachment through crosslinkages, complementarity surface between the enzyme and support provide better stability of the immobilized enzymes. Most of the reports on the stabilization of enzymes by means of immobilization have tried to minimize the unfolding step during thermal inactivation of enzymes.

1.5.1 **Techniques for studies on stability of enzymes.**

In order to quantify the stability of enzymes as a function of temperature, denaturants like GdmCl and urea and pH, a variety of techniques have been employed where change in the structural or functional properties of enzymes can be monitored. Most of the reported stability studies on soluble enzymes are carried out using circular dichroism (CD), fluorescence spectroscopy (FS) and differential scanning calorimetry (DSC).

CD technique has been efficiently employed to monitor the structural changes like secondary structure (α-helix, β-sheet and β-turn) and tertiary structure of protein as a function of denaturant effect (Fasman. 1996). The protective effect of stabilizing agent on proteins can also be quantified (Devi and Rao. 1998).

In proteins, fluorescence is generally due to tryptophan side chains. Change in both the intensity and wavelength of emission are sensitive measures of the polarity of the tryptophane environment in the presence of denaturant and hence provide useful information about the tertiary structure of proteins (Sudarshan and Rao. 1999). This principle has been made use of in FS method.

By using DSC, change in the thermodynamic parameters like heat capacity, melting temperature (Tm) and calorimetric enthalpy (ΔHc_at) and Van't Hoff enthalpies

(ΔH_{VH}) and free energy (ΔG), entropy (ΔS) of proteins can be monitored as a function of denaturant concentration and pH values. Similarly stabilizing effect of additives on the structural properties of proteins can be calculated (Pace. 1986).

Change in the functional properties as a function of temperature, denaturant concentration and pH values can be quantified by measuring the residual activity of enzymes. This method can be used for the enzymes even if they exhibit irreversible thermal inactivation. Using this method, activation parameters like activation enthalpy (ΔH^*), free energy of activation (ΔG^*), activation entropy (ΔS^*) can be calculated (Devi and Rao. 1998).

Although information on stability studies of soluble enzymes is fairly well documented, with respect to immobilized enzymes, the above mentioned methods viz., CD, FS and DSC have several constraints. In most of the reported immobilized enzyme stability studies, the residual activity of enzymes has been quantified as a function of temperature, denaturant concentration and pH for examining the operational and storage stability. Since in these studies, an indicator enzyme is used along with the enzyme to be studied, stability measurement may not be possible as a function of chemical denaturants like GdmCl, urea and pH due to denaturation of the indicator enzyme. Furthermore, adsorption of colour on the surface of the immobilized enzyme due to the indicator enzyme activity may lead to erroneous results. These constraints have hampered immobilized enzyme stability studies using indicator enzyme. In this thesis attempts have been made to overcome these difficulties by using the simple Clark type dissolved oxygen electrode and the results of which are discussed in chapter 5 and 7.

1.6 Immobilization of enzymes for stability.

As pointed out earlier, long term stability and reliability of the biosensor are entirely dependent on the biological component fixed closely to the surface of the transducer. In particular, when using enzymes as biological component in biosensors, two important considerations are storage stability and operational stability. Attempts have been reported in literature, to improve the stability of enzymes by various methods of immobilization involving the fixing the biological component to the solid support by means of physical or chemical methods. Though the principles of stabilization of enzymes by means of immobilization for biotechnological applications is well documented (Farahdiba et al., 1993; Gupta. 1991; Klibanov. 1979), information with respect to biosensor applications is scanty. For example, lipase immobilized on ion exchange resins shows better stability in organic solvents (Turner et al., 1995), but the same is not true in aqueous media which is important for biosensor applications. On the other hand, methods which are widely used for biosensor applications like immobilization by using carbon paste and direct immobilization by activating electrode surface may not be suitable for other biotechnological applications. The stability of immobilized enzymes should be considered with respect to their applications. Operational and storage stability have been considered as critical for biosensor applications. An understanding of the stability of immobilized enzymes involves a study of the effect of temperature, pH and denaturants.

Methods of immobilization of enzymes.

Existing enzyme immobilization methods, may be broadly classified into physical and chemical methods. The advantages and disadvantages of the various methods of immobilization used for biosensor construction are listed in table 1.6.

Table 1.6 Common immobilization methods for biosensor construction.

Method	Advantages	Disadvantages	Example	Reference
Adsorption (physical)	No modification of biocatalyst, matrix can be regenerated, low cost	Binding forces are susceptible to change in pH, temperature and ionic strength, poor stability	Adsorption of GOD on graphite powder for glucose detection	Ikeda et al., (1984)
Entrapment (physical)	Only physical confinement of the biocatalyst near the transducer, low cost	High diffusion barrier, low stability	Entrapment of L-lysine oxidase in gelatin, support for lysine detection	Romette et al., (1983)
Covalent bonding (chemical)	Low diffusional resistance, stable under adverse conditions	Harsh treatment by toxic chemicals, matrix not regeneratable	GOD bound via acyl azide derivatives of polyacrylamide for glucose detection	Guilbault. (1988)
Cross-linking (chemical)	Loss of biocatalyst is minimum, moderate cost, can be prepared in desired shapes	Harsh treatment of biocatalyst by toxic chemicals	Glutaraldehyde crosslinking of GOD with BSA for glucose detection	Wingard et al., (1984)

1.6.1 Physical methods.

In the physical the methods of immobilization, there is no chemical modification of the enzyme. Therefore chances of inactivation of enzyme is very minimum. Physical methods of immobilization may be again divided into two classes.

1.6.1.1 Adsorption

The adsoφtion of an enzyme onto a water insoluble material is the simplest method for obtaining enzyme-support conjugates. Basically it consists of placing an aqueous enzyme solution in contact with a solid support for a fixed time after which the excess enzyme is washed off from the support. Numerous surface active materials have been used in the adsorption technique. Commonly used supports are cation and anion exchange resins, activated charcoal, silica gel, alumina, controlled pore glass (CPG) and ceramics (Table 1.6.1.1). Though the method is simple, a major disadvantage is that

changes in pH, temperature, ionic strength and solvents have a significant adverse effect on the efficiency of immobilization and stability (Carr and Bowers. 1980 and table 1.6.1.1)

Table: 1.6.1.1 Typical examples of the enzyme biosensor that are based on adsorption.

Analyte	Enzyme	Carrier	Transducer	Stability	Reference
Pencillins	Pencillinase	Glass frit	pH- electrode	6 weeks	Papariello et al., (1973)
Acetyl choline	Choline esterase	Cation exchange resins	pH- electrode	10 runs	Guilbault and Iwase. (1976)
Uric acid	Uric acid oxidase	Hydroxy ethyl cellulose	CO_2 gas sensor	10 days	Kawashima and Rechnitz (1976)
Glucose	GOD	Diatomaceous earth	H_2O_2 sensor	50 runs	Silver et al., (1976)
Lactic acid	Lactate dehydroge-nase (LDH)	Co-adsorbed with collagen on platinum electrode	NADH oxidation on platinum electrode	~8 runs	Suzuki et al., (1975)
H_2O_2	Catalase	Collagen-enzyme electroche-mically adsorbed membrane	O_2 electrode	~9-10 runs	Aizawa et al., (1974)

1.6.1.2.Entrapment.

The preparation of physically entrapped cholinesterase for analytical purposes has been described by Bauman et al (1965). Important matrices that are used for entrapment are calcium alginate, collagen, cellulose triacetate, poly acrylamide, gelatin, BSA, agar, silicon rubber, polyvinyl alcohol and polyurethanes (Table 1.7.1.2). In physical entrapment techniques, enzymes are introduced during the solidification/crosslinking stage of the matrix. Disadvantages of this method are the irregular pore size of the gel, lack of mechanical strength and diffusional limitations encountered by substrate and products. Diffusional resistance causes sensors using this method of enzyme immobilization to have

low sensitivity and poor lower detection limit (Mulchandani. 1998). Also poor stability of the biosensor is another disadvantage (Table 1.6.1.2).

Tablel.6.1.2. Typical examples of enzyme biosensors based on entrapment method.

Analyte	Enzyme	Carrier	Transducer	Stability	Reference
Urea	Urease	Polyacryla-mide	Cation electrode	14 days	Guilbault and Montalvo (1970)
Lactic acid	LDH +catalase	Gelatin	$Fe(CN)_6^{3-}$ redox electrode	20days	Shinbo et al., (1979)
Galactose	Gal-OD	Gelatin	H_2O_2	10 days	Schumacher et al., (1994)
Glucose	GOD	Polyacryla-mide	O_2	N.R	Updike and Hicks (1967)
Phenol	Tyrosinase	Polyacryla-mide	O_2	24	Schiller et al., (1978)
Glutamine	Glutaminase	Hydoxyethyl methacrylate ethylene glycol	pH	21 weeks	Moser et al., (1995)
Glutamine	Glutamate oxidase		H_2O_2	21 weeks	
Malate	Salicylate hydroxylase and malate dehydrogenase	Gelatin	O_2	N.R	Scheller et al., (1998)

Gal-OD = Galactose oxidase; N.R.= Not reported

1.6.2 Chemical Methods.

In these methods of immobilization, enzymes are chemically modified and coupled to carrier with the help of bi- or multifunctional reagents. As reported by Guilbault (1984), there are many amino acid side chains that are amenable to chemical modifications of the enzyme. Depending on the nature of chemical modification, chemical methods are divided into two classes: Covalent coupling and Crosslinking.

1.6.2.1 Covalent coupling.

In covalent coupling, usually, first the water-insoluble support is activated, to which enzyme is then coupled. As reported by Guilbault (1984) any of the reactive sites on the protein surface can be used for the coupling of the enzyme to the solid support.

Most commonly used matrix for the biosensor applications is CPG and direct immobilization on the electrode surface. Unlike the organic matrices, in the majority of the cases the surface of the inorganic support must be derivatized before activation. Organosilanes like amino propyl triethoxysilane, marcapto propyl trimethoxysilane or Glycidoxy propyl trimethoxysilane is used to bridge the inorganic to organic interface (Shriver-Lack. 1998). A variety of enzymes have been immobilized by the covalent method for flow injection analysis (FIA) applications (Table 1.6.2.1). There are advantages and disadvantages of the use of covalent immobilization of enzymes. Advantages are that these immobilized enzyme preparations can be fabricated in sheets, beads, or membranes, which makes this method very useful for industrial and analytical applications (Carr and Bowers 1980). However, among several disadvantages of the technique, the most significant is the relatively low recovery of the enzyme activity.

In recent years, direct immobilization of the enzymes on the electrode surface has gained considerable interest, due to its unique advantages like quick response due to direct electrical communications between the redox enzymes. Chemical immobilization of enzymes on the electrode surface involves derivatization of gold surface with organosulfur compounds (Liedberg and Jonathan. 1998). The gold surface was derivatized with organosulfur compounds like glutathione and cystamine. Carboxyl group of the organosulphur was then activated by carbodiimide and finally the enzyme was coupled to the electrode surface. However these methods of immobilization are at the early stage of development. Furthermore, the amount of enzyme loaded on the electrode surface is small and may require a highly sensitive base sensor. These factors limit the application of immobilization by covalent coupling.

Table 1.6.2.1 Features of some reported biosensors based on covalent immobilization using CPG

Analyte	Enzyme	Transducer	Stability	References
Lactate/glucose	LOD/GOD	O_2	500/1000 assays	Kyröläinen et al., (1995)
Cholesterol esters	Cholesterol esterase/ oxidase	H_2O_2	N.R	Carpenter and Purdy. (1990)
Ascorbic acid	AAO	Thermistors	N.R	Mattiasson and Danielsson. (1992)
Sucrose	Invertase	Thermistors	6 months	Mandenius et al., (1981)
Lactate	LOD	H_2O_2	10 days	Nelson et al., (1990)

AAO = Ascorbic acid oxidase; N.R = Nor reported

1.6.2.2 Covalent crosslinking method.

Though in this method chemical modification of the enzyme remains same as in covalent coupling, the major difference between them is that in the latter case the enzyme is insolubilized by coupling with preactivated matrix, whereas in the former case, the enzyme is insolubilized directly by adding the crosslinking agent which may be either bifunctional or polyfunctional. Commonly used agents for this purpose include glutaraldehyde, bisisocyanate derivatives, bisbenzidine and succinyldisalicylate. When acting on a homogeneous solution of proteins, homobifunctional reagents react with identical reactive sites of the same molecule, resulting in a crosslinked aggregate of the molecule. This method of immobilization is also called as chemical aggregation method. Since glutaraldehyde can act under ambient conditions and in a wide range of pH, immobilization of enzyme using glutaraldehyde has been extensively studied (Wold. 1967). However, glutaraldehyde being a strong bifunctional reagent, modifies the enzyme drastically, leading to conformational changes and loss of activity (Broun. 1976). This

deleterious effect can be minimized by using inert proteins like BSA, gelatin and collagen (table 1.6.2.2).

Table 1.6.2.2 Features of some reported biosensors based on covalent crosslinking immobilization.

Analyte	Enzyme	Matrix	Transducer	Stability	Reference
Sucrose	Inv, Mut, & GOD	BSA + Glutaraldehyde	O_2	200 days	Xu et al., (1989)
Maltose	Amyloglucosi-dase + GOD	Pig small intestine + Glutaraldehyde	H_2O_2	200 assays	Varadit et al., (1993)
Sucrose	Inv, Mut, GOD	Gelatin + Glutaraldehyde	O_2	30 days	Filipiak et al ., (1996)
Amino acids	Amino acid oxidase	BSA+ Glutaraldehyde	O_2	100 days	Guilbault and Lurano. (1978)
Phosphate	ACP+GOD	BSA+ Glutaraldehyde	O_2	90 days	Guilbault and Nanjo. (1975)
Carboxylic acids	Alcohol oxidase	BSA+ Glutaraldehyde	O_2	120 days	Nanjo and Guilbault. (1975)
Penicillin	Penicillinase	BSA+ Glutaraldehyde	H^+	1000 assays	Zhong and Li. (1993)
Glucose	GOD	Gelatin + Glutaraldehyde	Optic fiber	170 days	Schaffer and Wolfbeis. (1990)

Inv = invertase; mut = mutarotase; ACP = Acid phosphatase

These proteins and amino acids avoid excessive intermolecular crosslinkages within the enzyme and enhance the intermolecular linkages between the enzyme and inert proteins. The advantages of crosslinking are the simplicity of the procedure and strong chemical binding of biomolecules. An other advantage is that the microenvironment of the enzyme can be defined by using suitable stabilizing agent, enabling better stability through surface complementarity with the desired enzyme may provide better stability (Chang and Mohany. 1995). The main drawback is the possibility of activity losses due to chemical alterations of the catalytically essential sites of the protein.

1.7 Desirable Characteristics of Immobilized Enzyme Biosensors Based on Amperometry

The performance of the biosensor is assessed by certain technical and functional characteristics which are listed below (Alexander. 1990).

- Selectivity
- Sensitivity
- Calibration Requirement
- Stability
- Response Time
- Recovery Time

1.7.1 Selectivity.

Selectivity reflects the capability of the device to avoid the response caused by interfering substances. Interfering substances can be classified into two categories: (l)Those which interfere with the electrode sensor (2) Those which interfere with the enzyme itself (Guilbault. 1976). Interferences may be positive or negative depending on their respective effects of increasing or decreasing the electrode output. Positive interference occurs when certain substances structurally and chemically similar to the substrate are converted by the immobilized biocatalyst into products, for which the electrode gives a response. Negative interferences correspond to those substances that act as inhibitors of the enzyme used. Such interfering substances reduce the electrode response. Desirably the biosensor should not react with substances other than the desired analyte, present in the sample (Guilbault. 1976). As a general practice, biosensors use semipermeable membranes like teflon membrane to prevent electrochemically interfering substances like ascorbic acid, citric acid, etc, from reaching the electrode surface (Xu et al., 1989; Scheller and Renneberg. 1983).

1.7.2 Calibration requirement.

Response for the substrate in terms of the electric signal will decrease when the immobilized enzyme electrode is used repeatedly for a large number of analyses. Complicated changes in electrode response are also not uncommon (Carr and Bowers. 1980). This change in electrode response necessitates calibration of the enzyme electrode at regular intervals. Generally when there is a linearity of signal response, a two point calibration is used and the instrument is calibrated every day before starting the sample analysis. The electrode response in terms of current/voltage is plotted against the concentration of analyte to obtain a straight line graph. At low substrate concentrations, the response is not useful for measurement, as the electrode is not sensitive to very low analyte concentrations. At very high substrate concentrations, the enzyme concentration becomes the reaction rate limiting factor and the reaction becomes independent of the substrate concentration, thereby resulting in a levelling off of the graph (Guilbault. 1976). The linearity region of calibration is used for measurements. One advantage of an amperometric transducer is the linear relationship existing between the output current and the analyte concentration, in contrast to potentiometric detection, which leads to a logarithmic relation for calibration (Blum and Coulet. 1991).

1.7.3 Sensitivity and detection limit.

The slope of the calibration graph of the electrode current versus analyte concentration represents the sensitivity of the sensor (Blum and Coulet. 1991). The greater the slope, the greater is the sensitivity of biosensor.

The sensitivity of the biosensor is distinguished from the detection limit, which is defined as the lowest analyte concentration that can be detected with an

acceptable signal to noise ratio (For example analyte concentration that gives response twice that of the noise level) (Sumathi. 1995). In case of biosensors making use of inhibition, some authors have considered the concentration of inhibitor that causes 10 % inhibition as the detection limit (Marty et al., 1995). It is possible to have biosensors with high sensitivity, but not a low detection limit, if the noise level is high (Sumathi. 1995).

1.7.4 Stability and operating life.

Another characteristic of biosensors important from the viewpoint of practical utilization is the stability of the electrode response which determines the useful life. An operating life in the range 10-60 days has been reported for both enzyme (Verduyn et al., 1983) and microbial (Arnold and Rechnitz. 1981) sensors. During prolonged usage of the enzyme electrode the activity of the enzyme decreases, thereby resulting in a reduced slope of the calibration graph. However, as long as the linearity is maintained and the signal is measurable with accuracy, the electrode is still useful, but it requires day to day calibration (Guilbault. 1976). Frequency of usage of the biosensor is an important factor in determining its life. More frequent the use, shorter will be the overall life time (Guilbault. 1976). Storage stability is defined as the ability of the preparation to retain its activity under some specified storage conditions (Carr and Bowers. 1980). Operating conditions, mainly pH and temperature also influence the biosensor life. Another factor adversely affecting the stability of some enzyme electrodes is the leaching out of a loosely bound cofactor from the active site, which is essential for its activity (Guilbault and Hrabankova. 1971).

1.7.5 Response time.

Response time is usually defined as the time needed for the electrode response to reach the steady state value. In the case of potentiometric electrodes with slow response, the absolute steady state value is never achieved as there is a gradual increase in response over a long period after coming close to steady state. Therefore, in such cases, the response time is defined as the time needed for the electrode response to reach 90, 95 or 99% of its equilibrium (steady state) value, the latest recommendation being 95 % (Uemasu and Umezawa. 1982). From a practical point of view, it is difficult to apply these definitions when the final electrode potential is not readily determined. Uemasu and Umezawa (1982) defined a quantity called differential quotient, $t(\Delta t, \Delta E)$ as a measure of practical response time which can be applied to simulations where the response is so slow that the final equilibrium potential is not determined readily. This definition has the advantage that it does not require a knowledge of the equilibrium potential value. Generally biosensors based on inhibition of the biocatalyst require considerably longer analysis time compared those for substrate determination. Multienzyme systems also show a longer response time because of the sequential enzyme reaction. Numerous other factors can influence the response time, including the nature and thickness of the membrane, the amount of immobilized enzyme, the substrate concentration, the geometry of the electrode tip and that of the sample cell, agitation rate and temperature. The type and efficiency of the electrode is another factor which determines the response time (Blum and Coulet. 1991). Faster the sensing of gives the potential or current proportional to the analyte, lower will be the response time (Guilbault. 1976).

1.7.6 Recovery time.

The minimum time needed between two consecutive analyses is known as the recovery time. Generally a fast response also means a smaller recovery time and high substrate concentrations are associated with longer recovery time. During use, the product and the unreacted substrate buildup in the enzyme membrane which necessitate adequate washing of the electrode sensing element after each analysis in order to reach the baseline potential. Recovery time also depends on the thickness of the enzyme membrane, nature of enzyme and the basic biosensor hardware such as the electrode used. Most of the time, the electrode requires only simple washing by keeping the electrode in water/buffer or under running distilled water. Membranes containing physically entrapped enzymes must be washed very gently under static water/buffer to prevent the weakly bound enzyme getting washed out of the membrane, thus taking a relatively longer time to return to base line potential. On the other hand, chemically bound enzymes are held strongly and can be washed under running water, which results in a quick return to the base line potential. Cathodic amperometry based electrodes show quick return to base line by simply placing in fresh buffer solution, whereas anodic amperometry based electrodes have to be pretreated before use by applying a potential of +0.6 V (against saturated calomel electrode) until the anodic current decays to a base line value (Guilbault. 1976).

1. 8 Scope and objectives of the present investigations.

Analysis of sugars like glucose and sucrose in various food and fermentation industries is important for efficient monitoring and quality control. Most of the conventional techniques for analysis are either non specific or expensive. One of the

modern techniques which overcome the disadvantages of the conventional methods is the use of biosensor which has great potential for industrial applications for a variety of analytes. Though literature on the biosensor technology is well documented, there is a gap between the growing knowledge of biosensor technology and limitations in the commercial viability of the biosensor. Most commercially available instruments utilize amperometric enzyme electrodes mainly with oxidases. This may be due to the requirements for highly reliable instruments and the difficulties encountered in the preparation of stable bioactive sensing element and interferences while analysing the real samples. Problems which have to be taken into account for designing devices usable in real conditions include the stability of the sensing layer and the removal of interferences. Though there are several reports in literature on the stability studies of immobilized enzymes, with respect to biosensor application, several unresolved questions arise such as

1). While knowing that the complementarity between the enzyme and support surfaces plays a crucial role in stabilization, information in this regard is scanty.

2). Although it is known that stability of the carrier plays an important role in stabilization of enzymes, in glutaraldehyde crosslinking method, use of inert proteins has been limited to BSA and gelatin. Is there a scope for improved stabilization effect by incorporation of other protein based stabilizing agents?.

3). There is a dearth of systematic stability studies of immobilized enzymes as a function of temperature, chemical denaturants and also operational stability of biosensors.

Use of indicator enzymes while quantifying the stability of immobilized enzymes may lead to erroneous results due to adsorption of colour on the immobilized enzyme.

Another limitation is the effect of interfering substances in the sample in accurate estimation of the desired analyte. Information on the accurate estimation of the desired analyte, particularly by using multienzyme based biosensor, is not available. Furthermore, though the potential application of biosensor for the various fundamental investigations on immobilized enzymes has been anticipated earlier, its application has been limited only to the quantification of various analytes.

Considering the above points, the objectives of the present investigations include construction of an immobilized enzyme based amperometric biosensor for glucose and sucrose in food and fermentation analysis, a study of the effect of various parameters on the performance of the biosensor and also a study of basic biochemical aspects of the immobilized single and multienzyme systems with respect to temperature, pH, denaturants as well as operational stability.

Chapter 2
Materials and methods

MATERIALS AND METHODS

2.1 Materials.

Enzymes/Proteins	**Source**
Mutarotase (Porcine Kidney)	Biozyme, UK
Glucose oxidase (*A.niger*)	Sigma, USA
Invertase (Baker's yeast)	Sigma
Ribonuclease	Sigma
Lysozyme (Hen's egg)	Sigma
Gelatin	Sigma
BSA	Sigma
Peroxidase (Horse radish)	Sigma
Chemicals (all analytical reagent grade)	
Glutaraldehyde	Sigma
Guanidine Hydrochloride	SRL, India
Buffer salts	SRL
Acrylamide	SRL
Bis-acrylamide	SRL
Glucose	SRL
Sucrose	SRL
Membranes	
Cellophane membrane MWCO (8-10,000)	SpectraPor, USA
Teflon membrane	WTW, Germany

Dissolved oxygen meter used in these studies was procured from M/S EDT, UK. The dissolved oxygen electrodes in the biosensor construction were from M/S Century Instruments, Chandigarh, India

2.2 Protein estimation.

The concentrations of protein were estimated by Lowry method (Lowry et al., 1951). To the 1 ml of appropriate diluted protein, 3 ml of alkaline copper tartarate reagent was added and the mixture was incubated at 25±1°C for 10 minutes. Then 0.5 ml of 1 N folin ciocalteu's reagent was added and incubated at 25±1°C for 30 minutes. The developed blue colour was measured at 660 nm by spectrophotometer.

2.3 Enzyme estimation.

Glucose oxidase.

GOD activity was measured by glucose oxidase and peroxidase method (Bergmeyer. 1974). To the 1.5 ml of the O-dianosidine containing buffer pH 5.0, one ml of the appropriately diluted GOD enzyme solution was added. To this, 0.5 ml of the glucose solution was added and shaken for proper mixing. The colour developed was measured at 436 nm in the spectrophotometer.

Invertase.

Invertase activity was estimated according to the method of Mc Ghee et al., (1978) using sucrose as substrate. The reaction mixture consisting of 0.5 ml of 30 mM substrate, 0.5 ml of suitably diluted enzyme solution in a test tube was incubated for 30 min at 40°C. The test tube was then placed in a boiling water bath for 5 minutes to inactivate the enzyme. Glucose liberated was estimated by DNSA method (Miller. 1959). One unit of enzyme activity was defined as the amount of enzyme which liberates one micromole of glucose per minute under the conditions of assay.

Mutarotase.

Mutarotase activity was measured by using dissolved oxygen meter as reported by Miva and Okudu (Miva and Okudu. 1974). Enzymatic activity of mutarotase can be measured by using α-D-glucose as substrate and GOD as indicator enzyme. Since the GOD is specific for β-D-glucose, the difference in the activity of GOD for α-D-glucose in presence and absence of mutarotase is considered as mutarotase activity. In order to measure the mutarotase activity, first known concentration of GOD in the absence of mutarotase was added to the sample cell of the oxygen electrode immersed in 5 ml of 50 mM phosphate buffer pH 7.0. Rate of oxygen consumption by GOD for α-D-glucose was measured by injecting freshly prepared 50mM α-D glucose solution. Then appropriately diluted mutarotase along with GOD was added to the sample cell of the oxygen electrode immersed in 5 ml of 50 mM phosphate buffer pH 7.0. Rate of α-D to β-D glucose conversion was measured by injecting freshly prepared 50 mM α-D glucose solution. The difference in the rate of oxygen consumption in presence and absence of mutarotase by GOD was considered as mutarotase activity. One unit of enzyme activity was defined as the amount of enzyme which depletes one μmole of oxygen per minute under the assay conditions. 2.4 Immobilized enzyme preparation.

Enzymes were immobilized by glutaraldehyde crosslinking method reported by Cass (Cass. 1990). In the case of single enzyme, GOD, 1 mg of GOD was dissolved in 180 μl of 50 mM phosphate buffer pH 6.0. 200 mg of the stabilizing agent (BSA, gelatin and lysozyme) were dissolved in 1 mL of 50 mM sodium phosphate buffer. 2.5 % (w/v) of glutaraldehyde solution was prepared by appropriate dilution of 70 % (w/v)

glutaraldehyde. On the 2 cm x 2 cm cellophane membrane, 10 µl (10 IU) of GOD and 30 µl (6 mg) of the stabilizing agent were placed and mixed thoroughly using a glass rod. 50 µl of 2.5 % glutaraldehyde was then added and mixed thoroughly so that enzyme and the stabilizing agent got distributed uniformly throughout the enzyme membrane. The mixture of the enzymes and stabilizing agent was allowed to remain for one hour and then the enzyme membrane washed repeatedly with 50 mM phosphate buffer pH 6.0 to remove the excess glutaraldehyde. For the immobilization of multienzymes (invertase, mutarotase and glucose oxidase), instead of using single enzyme all the three enzymes were used.

Immobilized enzyme activity estimation.

In order to determine the immobilized enzyme activity various methods have been reported in literature based on the colour formation in presence of indicator enzyme and dye (Mort et al., 1973; Lilly et al., 1966). However, these methods may not be suitable due to the adsorption of colour on the surface of the immobilized enzymes, which was observed in our studies on GOD immobilized with various PBSAs in presence of peroxidase and O-dianosidine dye. This may lead to a calculation of virtual rather than real activity yield of the immobilized GOD. In our case, immobilized GOD activity was estimated by the method using oxygen meter (Weibel and Bright. 1971). Oxygen electrode was dipped in a glass sample cell containing 25 ml of 50 mM phosphate buffer pH 6.0. The solution was kept agitated by means of a magnetic stirrer. Immobilized enzyme membrane was placed in the beaker and the depletion of oxygen was measured as a function of time by the addition of 1 ml of 50 mM glucose solution. Activity of the

soluble enzyme was conducted in the same way as the immobilized enzyme on equivalent enzyme basis.

Immobilized multienzyme activity was measured by Mosbach and Mattiasson method (1976) using the dissolved oxygen meter, which is a modified method of Weibel and Bright (1971). Overall activity of the immobilized multienzyme system (invertase, mutarotase and GOD) was measured by injecting the 0.1 M sucrose solution to the oxygen electrode immersed in 25 ml of the 50 mM phosphate buffer pH 7.0. In order to separately quantify the enzyme activities, individually immobilized enzymes (invertase, mutarotase or GOD) were prepared first, followed by the addition of the other two enzyme solutions (same concentration used for immobilization) in the sample cell using same concentration of substrate (sucrose). Overall activity of the soluble multienzyme system was estimated by adding same concentrations of all the three enzymes to the sample cell using sucrose as substrate.

Activity yield of immobilized enzymes.

Activity yield or the effectiveness of immobilization represents the activity of enzyme retained after immobilization. Activity yield of the immobilized single and multienzyme system was determined by the following expression.

$$\text{percent activity yield} = \frac{\text{Activity of the immobilized enzyme}}{\text{Activity of the soluble enzyme}} \times 100$$

The activity yield values for GOD immobilized with gelatin, BSA and lysozyme at the optimized concentrations of PBSA (6 mg), glutaraldehyde (25 µl of 5 %) and enzyme (10 IU) were to be found to be 22,27 and 24 % respectively.

Activity yield values of the multienzyme system immobilized with various PBSAs were also determined. The respective values with gelatin, BSA and lysozyme were found to be 50, 58 and 52 %. These values along with the activity yields of the three individual immobilized enzymes are shown in table 2.4.1.

Table 2.4.1 Activity yield of the single and multienzymes immobilized with various PBSAs at pH 7.0

PBSA	Overall activity yield of multienzyme system, %	% Activity yield of the individual enzymes		
		GOD	Invertase	Mutarotase
Gelatin	50	20	18	26
BSA	58	22	20	24
Lysozyme	52	21	22	28

Higher overall activity yield values of the immobilized multienzyme system when compared to single enzyme system, can be attributed to the accumulation of the first enzyme reaction product, in the microenvironment of the enzyme membrane, in turn leading to higher substrate concentration for the second enzyme. Mosbach and Mattiasson. (1976) reported that the overall activity yield of multienzyme system is always higher than the activity yield of individual immobilized enzyme. This phenomenon, they have called as proximity effect. 2.5 High Performance Liquid Chromatography.

In order to verify the biosensor results, glucose and sucrose were estimated by high performance liquid chromatography (HPLC) (McGinnis and Frang. 1988) using a aminophenoxy column with a mobile phase of acetonitrile : water (80:20) at a flow rate

1.0 ml/min and a refractive index detector (RID). The retention time for glucose and sucrose were 6.0 and 8.2 minutes respectively.

2.6 Circular Dichroism (CD) spectroscopy.

Circular dichroism measurements were made with a Jasco-J20C automatic recording spectropolarimeter fitted with a xenon lamp and calibrated with + d-10-camphor sulphonic acid. Dry nitrogen was purged continuously into the instrument before and during the experiment. Slits were programmed to yield 10 A band width at each wave length. The measurements were made at 27°C. The light path length of the cell used was 1 mm in the far-UV region and 10 mm in the near-UV region. The protein concentrations were 0.2-1.0 mg/ml. The samples were prepared in 20 mM sodium phosphate buffer pH 6.0.

The secondary structure of the GOD was analysed by a computer program, CD PROT (Menendez-Arias et al., 1988). In this program, the secondary structure of the protein was calculated as a linear combination of reference spectra based on known tertiary structure as interpreted by Chang et al (1978), Bolotina et al (1980) and Yang and Kubota(1985)

Purification of glucose oxidase.

In order to remove the traces of catalase from GOD for the CD studies, GOD was purified by using sephadex G-250. Sephadex G-250 was washed several times with distilled water and then the gels were allowed to swell overnight in 20 mM sodium phosphate buffer pH 6.0. The swelled gels were loaded to the column (1.5 x 50 cm) and equilibrated with 20 mM sodium phosphate buffer. The GOD sample was loaded on the column and 2 ml fractions were collected at a flow rate of 10 ml/hour. The protein

concentration and the activity of the fractions were measured. The GOD containing fractions were pooled and then lyophilized.

2.7 Electrophoresis.

Native-PAGE.

Polyacrylamide gel electrophoresis (PAGE) was performed on 8 % vertical slab gel in the absence of sodium dodecyl sulphate (SDS) according to the method of Laemmli (1970). The working buffer pH was 8.6 and a constant current of 30 mA was employed. Electrophoresis was carried out for 3 hours at room temperature. The gels were then fixed by using water: methanol: Trichloroacetic acid (TCA) (5:4:1 by volume) and stained with 0.1 % w/v coomassie brilliant blue in water: methanol: acetic acid (5:4:1 by volume). The gels were then destained in water: methanol: acetic acid (5:4:1 by volume) until the background was clear.

SDS-PAGE.

SDS-PAGE was performed on an 8 % vertical slab gel in presence of 0.1 % SDS. The enzyme samples were prepared in 2 % SDS and 5 % β-mercaptoethanol followed by boiling at 100°C for 5 minutes. The pH of the buffer, fixing of the gel, staining and destaining of the samples were as employed for native gel.

2.8 Biosensor construction.

The biosensor comprises the biological sensing element, the transducer, amplification and detector systems.

The biological sensing element.

In order to construct the biosensor for glucose and sucrose, immobilized enzyme membrane sandwiched between the teflon and a cellophane membrane was fixed on to

the oxygen electrode surface by using 'O' ring as shown in figure 2.8.1. The schematic diagram of the complete biosensor setup is shown in figure 2.8.2. Gas permeable teflon membrane is used as internal membrane in the sensor element. Since it is permeable only to the gases, electrode poisoning due to electrochemically interfering compounds like metal ions and ascorbic acid and metal chelating agents like citric acid, is avoided during real sample analysis. Though a higher pore size (MWCO 10,000-12,000) gives faster response, in order to avoid leaching of the enzymes, SpectraPorl membrane with lower pore size (MWCO 6000-8000) was used as the external membrane. A 25 ml glass container with 5 ml phosphate buffer was used as the sample cell. Air was continuously bubbled through a simple aquarium air pump to keep the content mixed as well as oxygen supplied continuously. Initially for the new electrode, the electrode kept in the sample cell containing buffer was polarized for 12 hours.

Construction of the transducer-amplifier-detector system.

In order to apply the potential to the electrode and to process the signal which is obtained from the oxygen electrode, a signal conditioning circuit constructed at our laboratory was used. The construction of signal conditioning circuit for cathodic amperometry based biosensor has been described elsewhere (Gouda et al., 1997; Thakur et al., 1999). The reduction of oxygen at the cathode gives an output voltage/current, which is proportional to the oxygen concentration in solution. The current generated due to the electrochemical reaction is in the range of few hundreds of nano amperes. This was amplified in the detector system into measurable voltage levels for data acquisition and further processing. This has been achieved by cascading two stage high gain operational amplifiers and signal conditioning circuit.

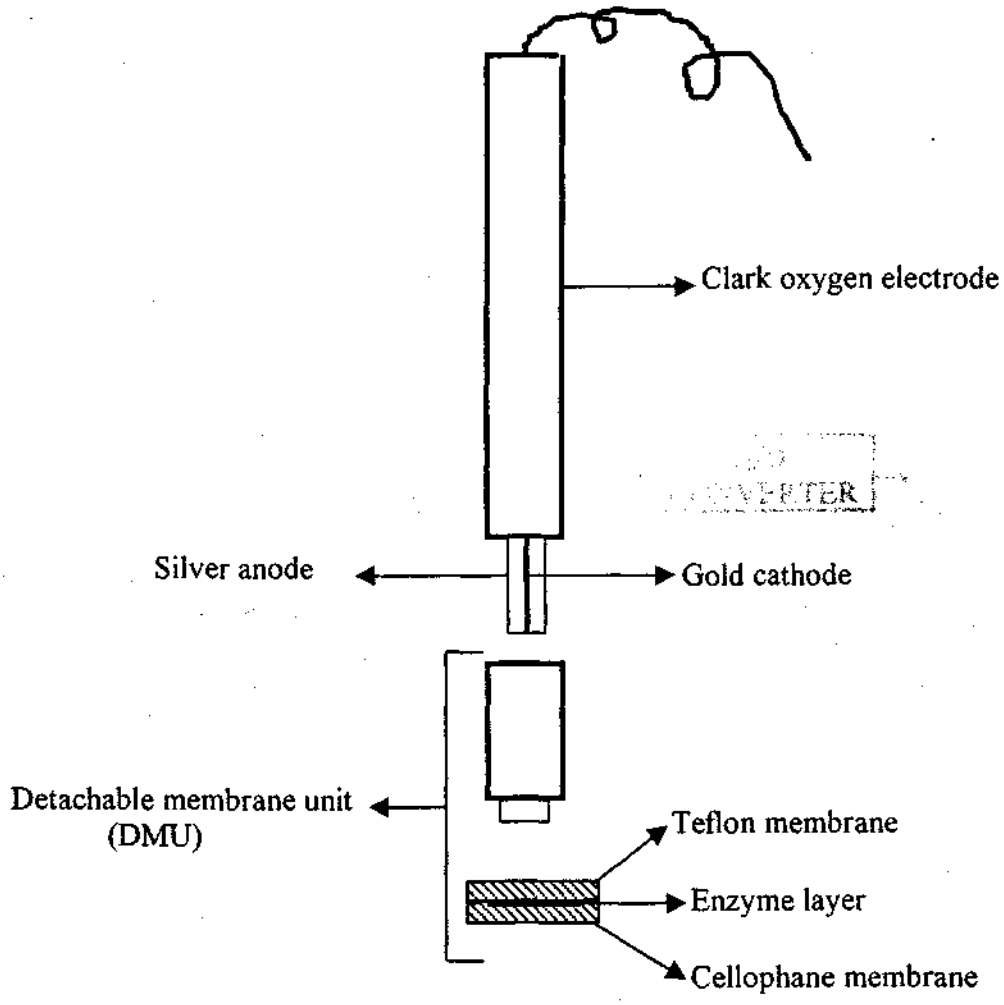


Figure 2.8.1 Schematic diagram of the enzyme electrode used for the stability studies

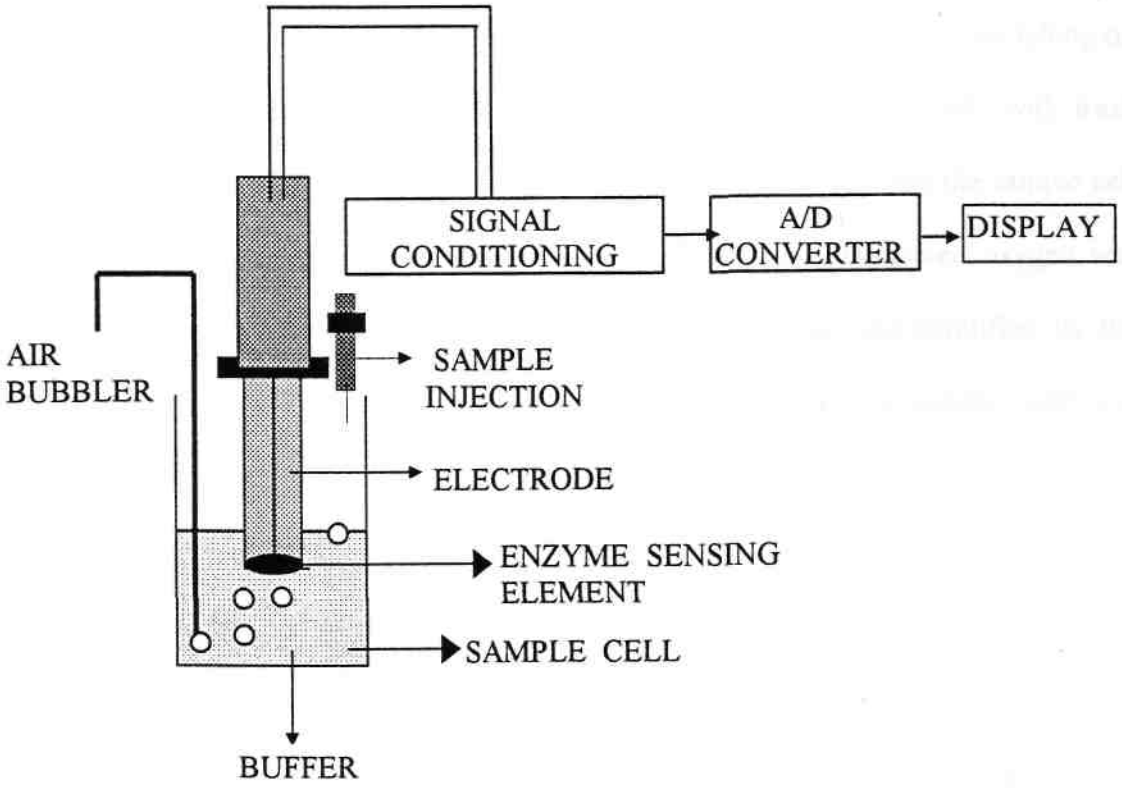


Figure 2.8.2 Schematic diagram of the biosensor device for the estimation of glucose and sucrose

Measurement method.

The measurement is based on monitoring the oxygen consumption through cathodic amperometry. Every day before starting the analysis, the enzyme electrode kept in the sample cell containing 5 ml buffer was polarized for 30 minutes by switching on the system. On polarization, the buffer was replaced in the sample cell with fresh buffer. In order to supply the oxygen, air was continuously bubbled into the sample cell containing buffer solution by using a simple aquarium pump. Dissolved oxygen was measured in terms of the current signal converted to voltage and amplified by the detector system. The reading prior to injecting the analyte to the sample cell was considered as the base line. Known concentrations of the analyte (glucose or sucrose) were then injected and the response of the biosensor was observed as a change in the output voltage for 3-4 minutes. After each analysis, the buffer in the sample cell was changed with fresh buffer solution.

2.9 pH activity relationship of the immobilized and soluble enzymes.

Effect of pH on the soluble and immobilized enzyme was studied in the pH range 4-8. The dissolved oxygen electrode containing the immobilized single/multienzyme membrane was immersed in 50 mM phosphate buffer pH 7.0. Rate of oxygen consumption by single/multienzyme membrane was measured by injecting 50 µL of 10 % glucose/sucrose solution respectively. The enzyme electrode was then immersed in a **solution** of desired pH value for 30 minutes. After this, the single or multienzyme activity was measured in the same buffer solution again by injecting 50 µl of 10 % glucose or sucrose solution. Rate of oxygen consumption by soluble single or

multienzyme was conducted in the same way as the immobilized enzyme on equivalent enzyme basis.

2.10 Enzyme stability studies.

An amperometric biosensor using a simple Clark type oxygen electrode was used for the investigations on stability of immobilized single and multienzyme system with various PBSAs as a function of temperature, GdmCl concentrations and pH as variables and also for the denaturation-renaturation studies. The dissolved oxygen electrode along with the enzyme membrane is shown schematically in figure 2.8.1

2.10.1 Operational stability.

The operational stability studies of the single and multienzyme system immobilized with different PBSAs was carried out at 27 + 1° C. The enzyme activity was measured by immersing the enzyme sensing element in a 25 ml glass sample cell having 5ml buffer, kept agitated continuously with air bubbled through an aquarium pump. After bubbling of air for 2 minutes (for saturation) the dissolved oxygen meter was set by fixing the dissolved oxygen at 100 %. 40 µl of 10 % glucose or sucrose solution was now injected and decrease in the %-dissolved oxygen at the end of three minutes (time taken to reach steady state) was monitored, which represents the activity of the immobilized enzyme for that concentration of glucose or sucrose. If the first analysis response in % dissolved oxygen is 'a' and response at any time on the n analysis is 'b', then % activity retained is calculated as **[b/a] x100.** In order to quantify the operational stability of PBSAs incorporated into the several immobilized enzyme preparations simultaneously, a detachable membrane unit (DMU) was constructed for each PBS A separately. The DMU containing the immobilized enzyme membrane system (shown in

figure 2.8.1) was then fixed to the electrode and the change in the response in % dissolved oxygen was measured by injecting 30 µl of 10 % glucose and 50 µl of 10 % sucrose respectively for the single and multienzyme membrane systems. After analysing about 5-6 samples of the same concentration of the sugar, the DMU was now kept in 10 ml of buffer at 27 ±1°C. An other DMU containing a different PBSA now replaced the earlier one and the activity was measured. The same procedure was repeated for all the DMUs, every day until the activity falls down to less than 40 % of the initial value.

2.10.2 Thermal stability studies.

For thermal stability studies on immobilized GOD, freshly immobilized GOD membranes with different PBSAs were kept at 27 ± 1° C overnight in 50 mM buffer solutions of respective pH values. Initial activity 'a' of the enzyme membrane for 10 % glucose solution was measured at 27 ± 1° C. The DMU was now removed from the dissolved oxygen electrode and immersed for 15 minutes in 10ml of the 50 mM buffer kept at desired temperature using a constant temperature bath. The enzyme membrane was immediately cooled to room temperature in ice bath for 2 minutes. Then the DMU was fixed to the electrode and set for 100 % dissolved oxygen. The residual activity 'b' of immobilized enzyme was observed at room temperature by injecting 10 % of glucose solution. The % residual activity is calculated as **[b/a]xl00.** The immobilized enzyme membrane was now replaced with a fresh enzyme membrane and the activity change was measured at the new desired temperature. Thus the residual activity was calculated at the different temperatures in the range of 40-80° C in steps of 2° C. Transition temperature (Tm), was calculated from a plot of % activity versus temperature at 50 % activity. Inactivation rate constant K was calculated from the semilog plot of % residual activity

versus temperature according to the equation, $A=Aoe^{-kT}$. In the similar manner, thermal stability of the multienzyme membrane was carried out by using the multienzyme membrane.

2.10.3 pH stability studies.

Buffer solutions used for the stability studies have been described earlier in this section. After measuring the initial activity at pH 6.0, the enzyme membrane was incubated at various pH values for 30 minutes and the residual activity was measured at pH 6.0 and expressed as a percentage of initial activity. In case of soluble enzyme the pH stability was investigated by adding known concentration of GOD to various pH solutions. After 30 minutes, aliquots were drawn and enzyme activity measured at pH 6.0.

2.11 Denaturation/renaturation studies on free and immobilized enzymes.

Studies on denaturation by GdmCl on GOD immobilized with various stabilizing agents and polyethyleneimine (PEI) were carried out at different GdmCl concentrations. Freshly immobilized GOD membrane was fixed to the electrode surface by using an 'O' ring. The initial activity 'a' of the enzyme membrane was measured by injecting 40 µl of 10 % glucose at 27±l°C. The enzyme electrode was then immersed in known concentration of GdmCl solution in phosphate buffer (50 mM) pH 6.0 for one hour with constant stirring. The residual activity 'b' of the immobilized enzyme membrane was observed by again injecting 40 µl of 10 % glucose solution. Percentage residual activity was calculated as **[b/a]xl00.** In order to measure the soluble enzyme activity, known concentration of enzyme (10 IU, in our studies) was injected first. Then 40 µl of 10 %

glucose solution was injected and activity 'a' was measured. Denaturation of the soluble enzyme was carried out as follows. The oxygen electrode sensor alone (without the enzyme membrane) was immersed in the desired GdmCl solution in phosphate buffer pH 6.0. 10 IU of the enzyme was now injected and incubated for one hour, after which the residual activity 'b' of the soluble GOD was measured by injecting 40 µl of 10 % glucose solution. Denaturation studies for the immobilized multienzyme system was conducted in the same way as the immobilized single enzyme system in 50 mM phosphate buffer pH 7.0 containing various concentrations of GdmCl.

Renaturation studies.

Renaturation of the immobilized GOD denatured in 8M GdmCl was carried out in 50 mM phosphate buffer pH 6.0 at 27±l°C for one hour. The dissolved oxygen electrode containing the denatured enzyme membrane was repeatedly washed by immersing the enzyme electrode in 50 mM phosphate buffer pH 6.0. At desired time intervals the regained enzyme activity 'c' was measured by injecting 40 µl of 10 % glucose solution. Then the % renaturation was calculated as [c/a]xl00, where 'a' is the initial activity of immobilized GOD. In the case of soluble enzyme renaturation was carried out by diluting the mixture of enzyme and denaturant with 40 volumes of phosphate buffer pH 6.0 and then activity was measured by using the oxygen electrode. Renaturation studies for the multienzyme system was carried out in the similar way as for immobilized GOD.

2.12 Calibration of the biosensor for glucose and sucrose.

In order to see the response for various concentrations of glucose, 25 µl of the various standard glucose solutions (1-10 %) were injected into the sample cell and depletion of oxygen was monitored by the biosensor with single (GOD) enzyme

electrode till steady state value was attained. After each measurement, the biosensor was washed with buffer. Response of the biosensor in volts was measured as a function of time by taking readings at 10 second intervals and plotted for different concentrations of glucose solution in the range 1-10 % (figure 2.12.1). It can be observed that steady state values are reached in all cases in 3 minutes. The difference between the initial and steady state value (3 minutes), was plotted against concentration of sugar solutions to get a calibration graph (figure 2.12.2). An excellent linearity is obtained with a high correlation coefficient of 0.9995. For the estimation of sucrose, the multienzyme (invertase, mutarotase and GOD) electrode was used. Various concentrations of standard sucrose solutions were injected into the sample cell of the biosensor and voltage response was measured for 4 minutes (time required to reach steady state) and a calibration graph constructed for various concentration of standard sucrose solutions in the range 1-10 % (figure 2.12.3.& 2.12.4). In this case also an excellent linearity is obtained with a high correlation coefficient of 0.997.

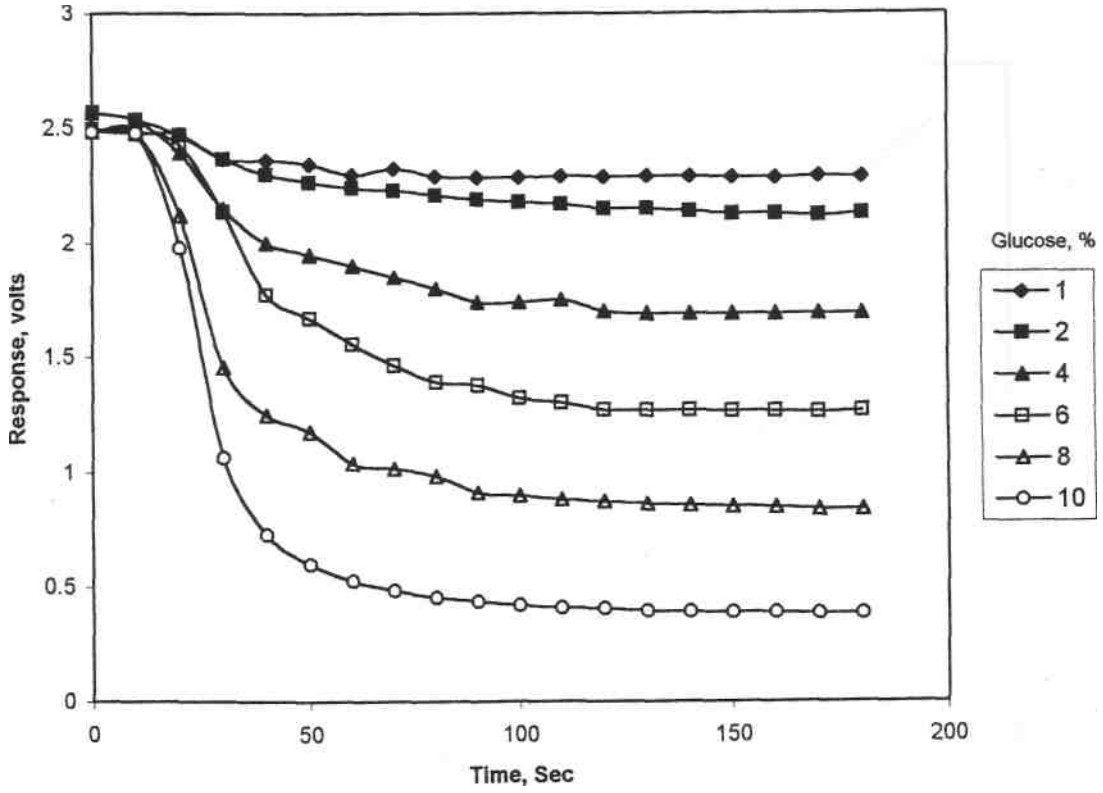


Figure 2.12.1 Response curve for various concentrations of glucose with glucose biosensor

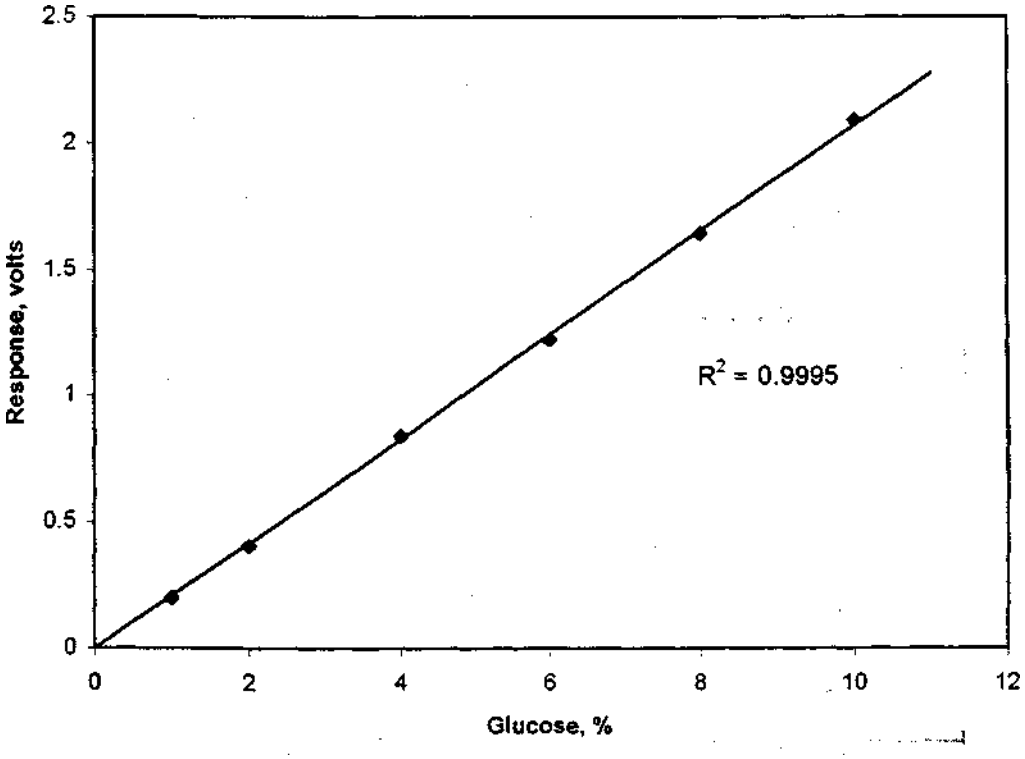


Figure 2.12.2 Calibration graph for the various concentrations of glucose with glucose biosensor

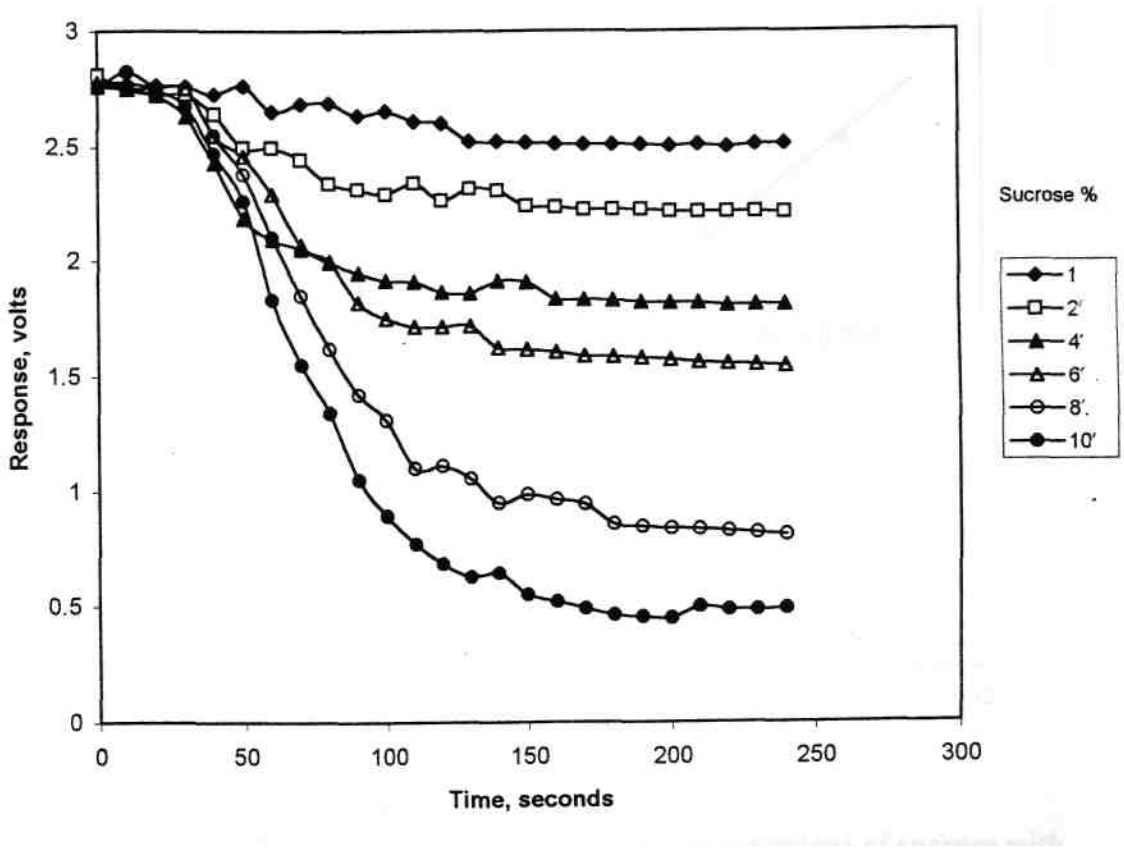


Figure 2.12.3 Response curve for various concentrations of sucrose with sucrose biosensor

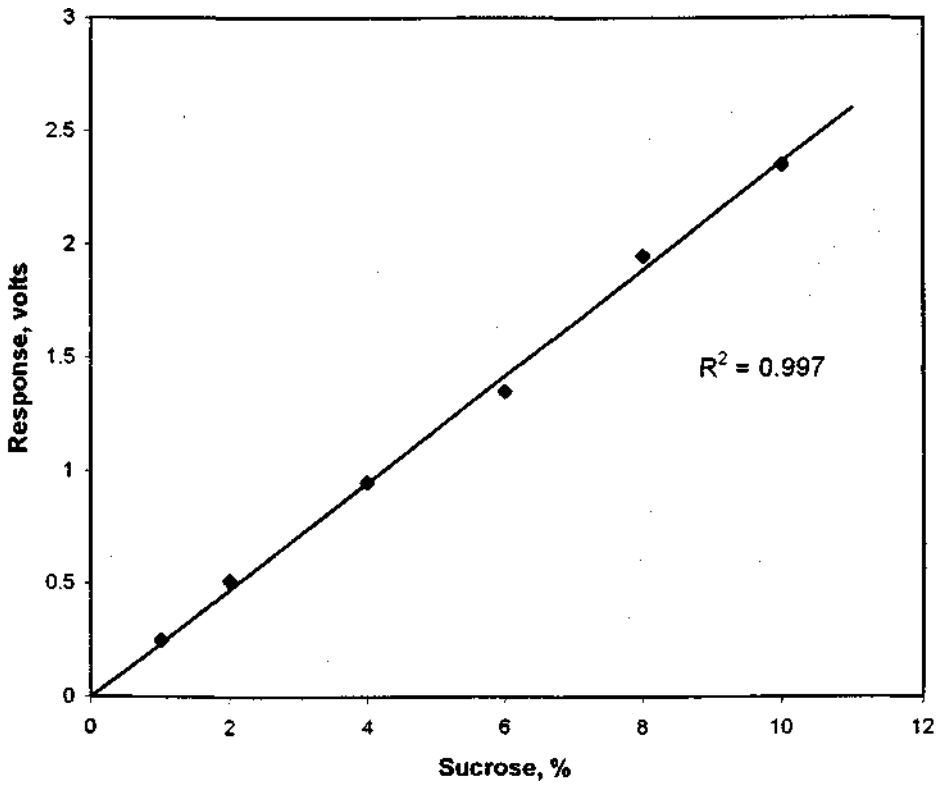


Figure 2.12.4 Calibration graph for the various concentrations of sucrose with sucrose biosensor

Results and Discussion

Chapter 3
Optimization of biosensor parameters

OPTIMIZATION OF BIOSENSOR PARAMETERS

Optimization of the key biosensor parameters, which minimizes the cost of the device and its operation is important for its commercial success. Towards this objective, optimization of biosensor parameters like operating pH, concentrations of glutaraldehyde, PBSA, and enzyme loading was carried out.

3.1 Optimum pH for the glucose biosensor.

Figure **3.1.1** demonstrates the pH dependent activity of the GOD immobilized with various PBSAs at 27±1°C in the pH range of 3.5-8.0. The immobilized GOD was exposed to the desired pH solution for 30 minutes followed by activity measurement. The soluble GOD shows maximum activity at pH 6.0, whereas GOD immobilized with gelatin, lysozyme and BSA respectively shows maximum activity at pH 6.0, 5.5 and 6.5. This shift in the optimum pH for GOD immobilized with lysozyme and BSA can be attributed to the physicochemical properties of the PBSA as explained in the following section on the optimum pH for the multienzyme system.

3.2 Optimum pH for the sucrose biosensor.

Figure 3.2.1 demonstrates the pH activity behaviour for the soluble and immobilized multienzyme system with various PBSAs at 27±l°C in the pH range of 5.0-8.4. The enzyme preparation was exposed to the desired pH solution for 30 minutes followed by activity measurement. The soluble multienzyme system shows maximum activity at pH 6.8, whereas the multienzyme system immobilized with BSA, gelatin and lysozyme shows maximum activity at pH 7.4, 7.0 and 6.2 respectively. Shift in the optimum pH for the single and multienzyme system immobilized with BSA and lysozyme towards alkaline and acidic pH values respectively can be attributed to the

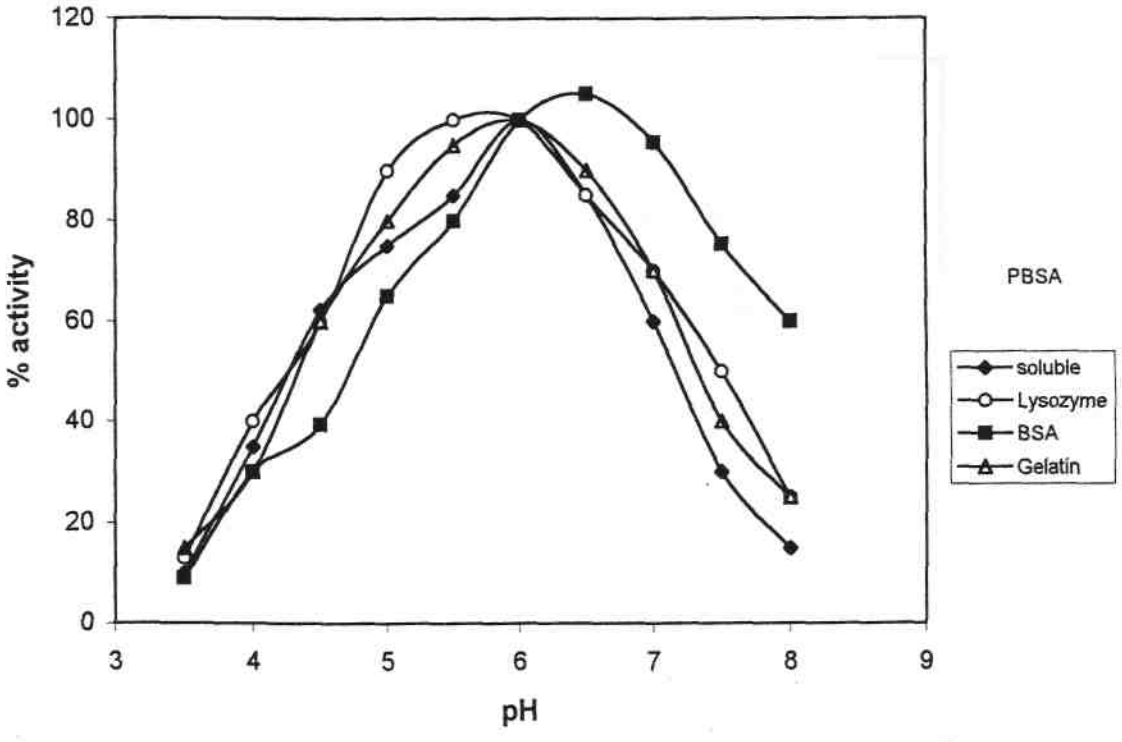
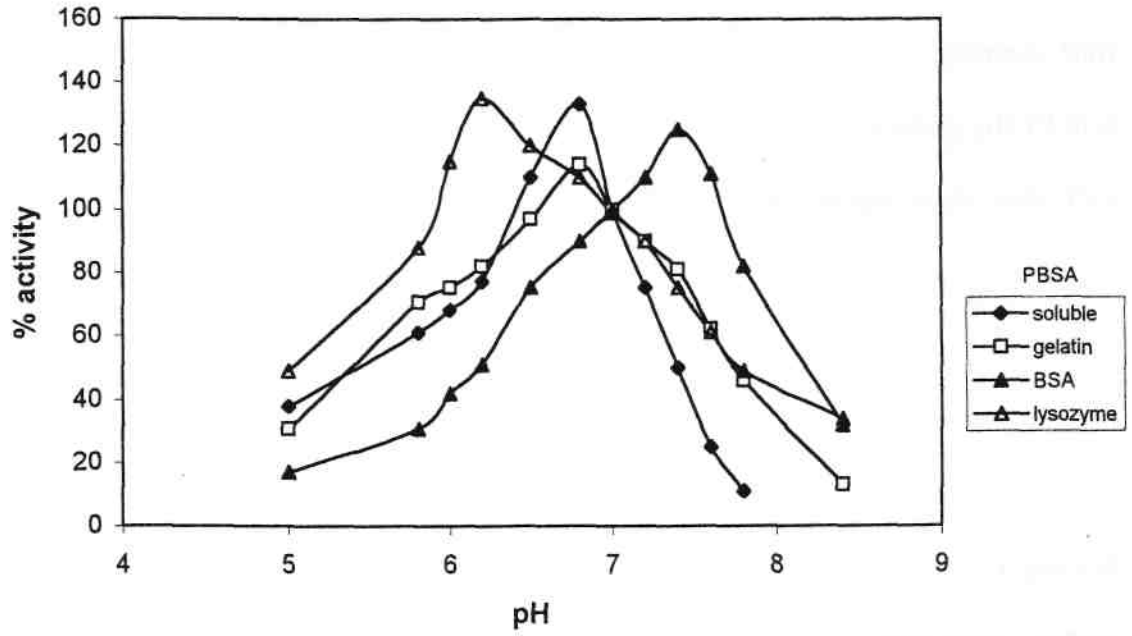


Figure 3.1.1 pH activity relationship of the soluble and immobilized GOD with various PBSAs at 27±1°C

Figure 3.2.1 pH activity relationship of the soluble and immobilized multienzyme system at 27+l°C



influence of physicochemical properties of the PBSA on the multienzyme system. Goldstein et al (1964) reported the influence of carrier on the pH - activity behaviour of the enzyme immobilized with various polyelectrolytes. If the carrier is negatively charged, then the optimum pH of the immobilized enzyme shifts towards alkaline pH and vice versa. This is due to the lower hydrogen ion concentration in the microenvironment of the negatively charged gel compared to the bulk solution. From our results (figure 3.1.1 and 3.2.1), it can be seen that with lysozyme as the PBSA, the pH optimum shifted towards acidic side, whereas with BSA as the PBSA the optimum shift was towards alkaline side. Since lysozyme is a basic protein, at the working pH (7.0) it carries net positive charge leading to a shift in the pH optimum towards acidic side. This occurs due to the higher hydrogen ion concentration in the microenvironment of the single and multienzyme system immobilized with lysozyme compared to that of bulk solution. Similarly BSA being acidic protein at the working pH (7.0) it carries a net negative charge, leading to a shift in optimum pH values towards alkaline side. This is due to the lower hydrogen ion concentration in the microenvironment of the single and multienzyme system immobilized with BSA. Corresponding multienzyme system immobilized with gelatin as PBSA has not shown any shift in the optimum pH since gelatin is a neutral protein, and at the working pH (7.0) it carries no net charge. Another significant observation from figure 3.2.1, is that although optimum pH for invertase and GOD in a free state in the range 5-6, up to a pH of 5.8, increase in overall activity for the soluble as well as immobilized multienzyme system was negligible. This may be due to the fact that optimum pH of the soluble mutarotase is considerably higher at 7.4 giving a relative low activity in the pH range of 4-6.0. Overall activity being dependent on all

the three enzymes, the low activity of mutarotase in the pH range of 4 to 5.8 probably results in a negligible increase in the overall activity of the multienzyme system in this pH range.

3.3 Effect of glutaraldehyde concentration.

The glutaraldehyde concentration during immobilization procedure plays an important role on the activity and stability of the immobilized enzyme, which are crucial for the biosensor performance. In order to optimize the glutaraldehyde concentration for the immobilization of GOD, various concentrations of glutaraldehyde were used at the fixed concentration of enzyme (10 IU) and PBSAs (6 mg). After preparing the enzyme membrane, activity was measured as biosensor response for 0.lmM standard glucose solution and the results are shown in figure 3.3.1. As the concentration increased from 5 to 25 µl of 5 % glutaraldehyde, relative activity of the GOD immobilized with BSA, gelatin and lysozyme increased. Further increase in concentration of crosslinking agent from 25 to 50 µl, resulted in a sharp decrease in the relative activity of immobilized GOD. Initial increase in the relative activity of GOD immobilized with BSA, gelatin and lysozyme with increase in glutaraldehyde concentration probably owing to the leakage of enzyme during washing stage of immobilization or during measurement of enzyme activity. 5 to 15 µl of glutaraldehyde may not be sufficient to crosslink the enzyme and 6 mg of PBSA. A sharp decrease in the relative activity of GOD with increased concentration of glutaraldehyde may be attributed to the excessive modification of the enzyme that leads to deterioration of enzyme activity. From this study, it can be concluded that the optimal concentration of glutaraldehyde is essential to retain the maximum activity of the immobilized enzyme.

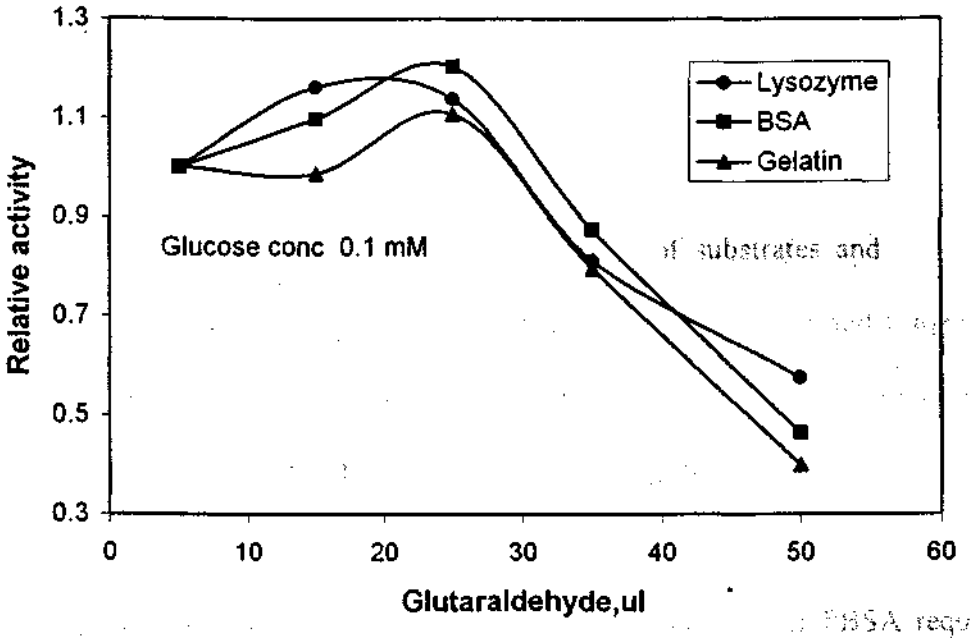


Figure 3.3.1 Effect of glutaraldehyde concentration on the biosensor response for the GOD immobilized with various PBSAs.

3.4 Effect of PBSA concentration on the biosensor response.

In order to optimize the PBSA concentration, glutaraldehyde and enzyme concentrations were kept constant and PBSA concentrations varied from 0 to 10 mg during the immobilization procedure. Then the activity of immobilized enzyme was measured as response in volts for 0.1 mM glucose solution and the results are shown in figure 3.4.1. As seen in figure, maximum activity of enzyme was observed for GOD immobilized with 4 mg of lysozyme and 6 mg of each BSA and gelatin as PBSA. With further increase in PBSA concentration, the relative activity of the immobilized GOD decreased sharply probably owing to the increase in the thickness of enzyme membrane. This in turn may lead to the limitations of diffusion of substrates and cosubstrates. Variation in the activity of the GOD immobilized with BSA gelatin and lysozyme can be attributed to the physico-chemical nature of the PBSA. It is known that the glutaraldehyde modifies only the ε-amino group of the lysine. Depending on the % lysine content of PBSA glutaraldehyde modifies the enzyme. As the % available amino group for glutaraldehyde decreased in PBSA the concentration of PBSA required to reach the maximum activity decreased at fixed concentration of glutaraldehyde. From our results it is clear that the % available amino group content is low in lysozyme when compared to gelatin and BSA accordingly, the maximum activity was observed for the lower concentration of glutaraldehyde (figure. 3.4.1).

From these studies, it is surmized that the 6 mg of PBSA and 25 µl of 5 % glutaraldehyde are optimal for the immobilization of GOD to obtain relatively higher activity after immobilization. Similar studies were conducted for the immobilization of the multienzyme system. Since there was no significant difference in the overall activity

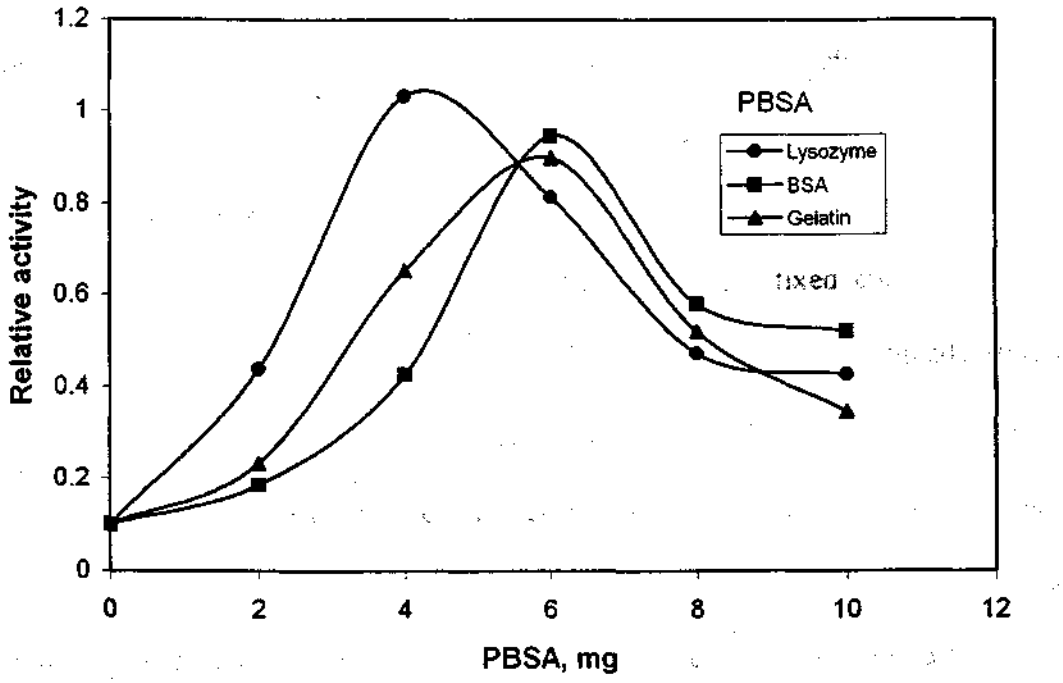


Figure 3.4.1 Effect of PBSA concentration on the biosensor response for glucose(O.lmM)

of the multienzyme system when compared to above results, same concentration of PBSA (6 mg) and glutaraldehyde (25 µl of 5 % solution) were retained for further studies on immobilized single and multienzyme system.

3.5 Effect of GOD loading on the glucose biosensor.

The purpose of enzyme concentration optimization for biosensor construction is to determine the minimum concentration of enzyme required for maximum performance in terms of response time and sensitivity in the desired range of detection. Since our aim was to construct the biosensor for food and fermentation analysis, optimization of GOD was carried out for the glucose detection range of 1-10 %. Various concentration (IU) of GOD containing enzyme membranes were prepared by using fixed concentrations of PBSA (6 mg) and glutaraldehyde (25 µl of 5 % solution) and then the steady state (3 minutes) response in volts was determined by injecting 25 µl of various concentrations of glucose solutions. The results plotted in figure 3.5.1 demonstrates that the enzyme concentration increased from 2 to 70 IU, response in volts increased linearly with concentration of glucose. Although, 1 and 5 IU GOD containing enzyme membrane shows a fair linearity, better sensitivity was observed with 10 IU GOD. Another interesting observation from the figure is that the sensitivity of the biosensor increased, at higher enzyme loading. However, since the upper detection limit (voltage) of the biosensor was around 2.5 V, the range of measurable glucose concentration decreased. This is due to the limitation of the base sensor electronics signal detection system. It is known that as the enzyme concentration increases the rate of enzymatic reaction increases linearly. Thus an increase in the enzyme concentration in the immobilized enzyme membrane, result in a linear increase in biosensor response in volts but limited

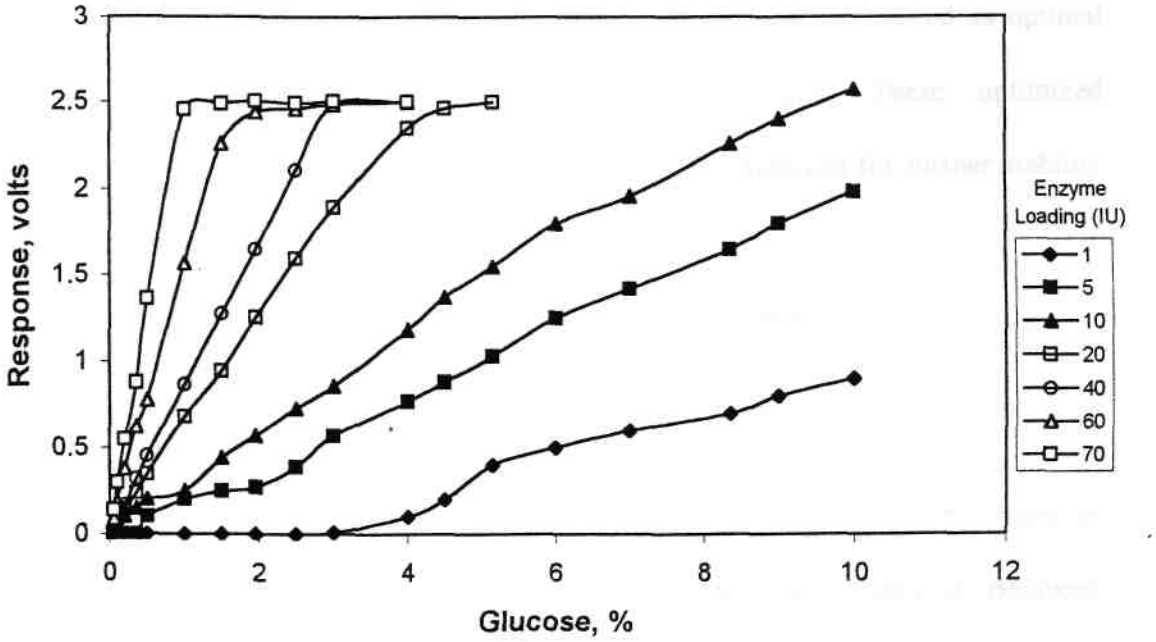


Figure 3.5.1 Effect of enzyme loading (IU) on the biosensor response for glucose

by the upper level of 2.5 V (figure 3.5.1). Loss of linearity below 0.05 % of glucose even at higher concentration of GOD (70 IU) can be attributed to the lower detection limit of the base sensor. Carr and Bowers (1980) reported that the biosensors based on oxygen depletion have poor lower detection limit when compared to H2O2 based biosensors. However , since our aim was to construct the biosensor for food and fermentation sample analysis, detection limit (volts) of the biosensor has not shown any influence on the desired detection range (1-10 %). On the basis of above optimization studies, 10 IU of GOD, 6 mg of lysozyme and 25 µl of 5 % glutaraldehyde were considered as optimal concentrations for the preparation of enzyme membranes. These optimized concentrations of enzyme, PBSA and glutaraldehyde were employed for further stability investigations of the immobilized GOD.

3.6 Optimization of the multienzyme system using Response Surface methodology.

Experimental design

Optimization of multienzyme concentrations for rapid response was attempted by using three factor, three level Box-Behnken design of experiments (Box & Behnken. 1960). It involves the investigation of the linear, quadratic, and cross-product effects of the three factors (invertase, mutarotase and GOD-concentrations), each varied at three levels and three center points for replication. Although other factors like concentrations of the protein based stabilizing agent (PBSA) may play some role in biosensor response, our observations reported earlier in this section an optimum PBSA concentration of 6 mg has been selected and only enzyme concentrations are considered as important variables. The factors and the levels at which the experiments were carried out are given in table 3.6.1. The design of experiments employed is presented in table 3.6.2. The optimization

of the multienzyme concentration for a minimum response time of the biosensor was carried out using response surface methodology (RSM). Measurements were carried out using 50 µl of 8 % sucrose solution. The time required to reach 90 % of steady state value was taken as the response time. For creating response surfaces, the experimental data obtained based on the above design was fitted to a second order polynomial equation of the form

$$Y = A_0 + A_1.X_1 + A_2.X_2 + A_3.X_3 + A_4.X_1^2 + A_5.X_2^2 + A_6.X_3^2 + A_7.X_1.X_2 + A_8 X_1.X_3 + A_9.X_2.X_3 \quad (3.6.1)$$

Where Y is the response time; X1, X_2 and X3 represent the concentrations (IU) of glucose oxidase, invertase and mutarotase respectively; Ao is a constant; A1, A2, A_3 are the linear coefficients; A4, A5, A6 are the quadratic coefficients; A7, A_8 A_9 are the cross product coefficients. The regression analyses, statistical significances and response surfaces were obtained using Microsoft Excel software (Version 5.0). Optimization of the reaction parameters was carried out through a software package Microsoft Excel - solver program (version 5.0) which used Newton's search method.

Table 3.6.1 Coded and actual variables taken for Box-Behnken design of experiments.

		Coded level variables		
Variables	**Units**	**-1**	**0**	**1**
X_1: GOD	**IU***	**1**	**5**	**9**
X_2: Invertase	**IU**	**10**	**30**	**50**
X_3: Mutarotase	**IU**	**5**	**55**	**105**

*** International Unit**

Table 3.6.2 Coded level combinations for a three-level, three-variable experimental design.

Sl. No	X1	X2	X3	Response time, Minutes	
				Predicted	observed
1	1	1	0	2.8875	3.00
2	1	-1	0	2.3250	2.36
3	-1	1	0	2.7083	2.66
4	-1	-1	0	3.8625	3.75
5	1	0	1	3.3145	3.50
6	1	0	-1	3.0062	2.66
7	-1	0	1	3.4937	3.83
8	-1	0	-1	4.1854	4.00
9	0	1	1	2.9645	2.66
10	0	1	-1	3.6229	3.85
11	0	-1	1	3.7270	3.50
12	0	-1	-1	3.4520	3.75
13	0	0	0	2.7261	2.73
14	0	0	0	2.7261	2.81
15	0	0	0	2.7261	2.63

The coefficients of the response surface model as given by equation (3.6.1) were evaluated by regression analysis and tested for their significance. The values of the coefficients **and** the ANOVA presented in table 3.6.3 indicate that the F_{model} value (3.47) is slightly low compared to $F_{9,5}$ value (4.77) at $P = 0.05$. This is probably because all the coefficients are retained in the equation. However, the lack of fit was observed to be insignificant, indicating that the model is adequate to represent the experimental data. The coefficient of determination (R^2) of the model was 0.862 and Average Absolute Relative Deviation (AARD) is 5.27 %, which indicate that the model is suitable to

adequately represent the real relationships among the reaction parameters used. The final predictive equation obtained is given by

$$\mathbf{Y = 2.7261 - 0.33958X_1 - 0.14792X_2 - 0.09583X_3 + 0.139011\ Xi^2 + 0.080678.X_2^2 +}$$

$$\mathbf{0.634844.X_3^2 + 0.429167}\ (X_1.X_2) + \mathbf{0.25\ (X_1.X_3) - 0.2333}\ (X_2.X_3) \qquad (3.6.2)$$

In the present study, the concentrations of the enzymes *viz.* Invertase, mutarotase and glucose oxidase were varied and the biosensor response was measured. The results of the experiments carried out indicate that while all the three enzyme concentrations influence the biosensor response, it is the concentration of mutarotase, that is probably a more important parameter to control the biosensor response.

Model validation.

The empirical model given by equation (3.6.2) was verified by conducting a set of separate independent experiments and the results are shown in table 3.6.4. The theoretically calculated response time values using equation (3.6.2) were found to be in good agreement with experimentally determined values.

Table 3.6.3 Coefficients of the model and analysis of Variance (ANOVA).

Coefficients*	Value	Standard Error	t Stat	P-value
A_0	2.7261	0.2040	13.3602	4.2E-05
A_1	-0.3395	0.1249	-2.7176	0.0418
A_2	-0.1479	0.1249	-1.1837	0.2897
A_3	-0.0958	0.1249	-0.7669	0.4777
A_4	0.1390	0.1839	0.7557	0.4838
A_5	0.0806	0.1839	0.4386	0.6792
A_6	0.6348	0.1839	3.4515	0.0182
A_7	0.4291	0.1767	2.4286	0.0594
A_8	0.2500	0.1767	1.4147	0.2162
A_9	-0.2333	0.1767	-1.3204	0.2439

Only coefficients significance at $P < 0.01$ presented

Regression Statistics	
Multiple R	0.9283
R^2	0.8618
Adjusted R^2	0.6131
Standard Error	0.3534
Observations	15

ANOVA

	Degree of freedom	Sum of Squares	Mean Sum of squares	F ratio	P- value
Regression	9	3.8962	0.4329	3.4658	0.0922
Residual	5	0.6245	0.1249		
Lack of fit	3	0.6088	0.2029	25.8122	N.S.*
Pure error	2	0.0157	0.0078		
Total	14	4.5208			

* Not significant at $P = 0.01$

Table 3.6.4. Model validation experiments.

Sl. No	X_1	X_2	X_3	Response time, minutes	
				Exptl.	Predicted
1	1	10	105	4.40	4.53
2	9	30	5	3.00	3.15
3	9	10	63	2.40	2.35
4	5	50	40	2.80	2.92
5	7	10	40	2.60	2.57
6	9	10	40	2.26	2.35
7	8	10	5	2.80	2.75
8	7	20	40	2.60	2.74

AARD = 3.33 %

3.7 Response surface plots.

The typical response surfaces are shown in figure 3.7.1-3.7.3. As seen in figure 3.7.1, at lower concentrations of mutarotase (5 IU) and invertase (10 IU), the response time was relatively higher. A moderately high concentration of mutarotase (55-75 IU) and higher concentration of invertase (50 IU) favored minimal response time at 1 IU of GOD using 50 mM phosphate buffer pH 7.0 and 27±1°C. As the invertase concentration increased, response time decreased at all the concentrations of mutarotase. Similarly increasing mutarotase concentration (5 to 75 IU) resulted in a decrease in response time at any given concentration of invertase. This was probably due to increase in the conversion rate of α-D-glucose to β- D-glucose by the action of mutarotase. Further increase in mutarotase concentration from 75 to 105 IU resulted in an increase in response time at all the concentration of invertase probably due to inhibition of mutarotase by its product, β-D- glucose. Similar observation of increased response time at higher concentrations of mutarotase has been reported (Xu et al., 1989).

As seen from figure 3.7.2, the behaviour of the response time going through a maximum with increasing mutarotase concentration has been observed at all GOD concentrations, again probably owing to inhibition of the enzyme mutarotase by its product, β-D-glucose. Increase in GOD concentration, however, resulted in a decrease in response time at all concentrations of mutarotase. This may be due to reduction in the accumulation of β-D- glucose in the vicinity of the immobilized enzyme membrane. A similar trend of increase in response time with higher concentrations of mutarotase (75-105 IU) was observed at all the concentrations of GOD and invertase. Although the effect of glucose concentration on the mutarotase activity has been reported (Bentley &

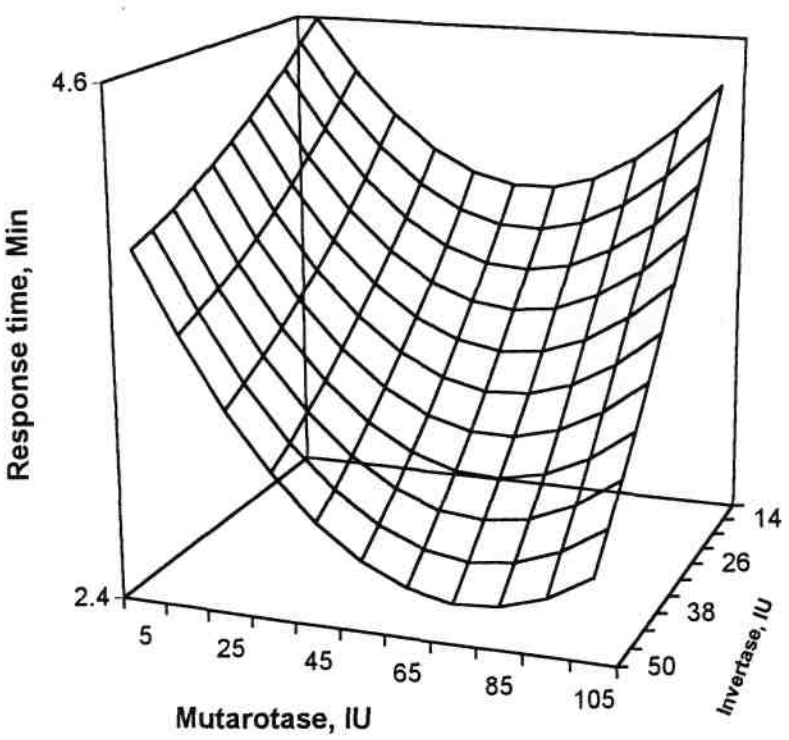


Figure 3.7.1 Response surface plot showing the effect of mutarotase and invertase on the response time. GOD is constant at 1 IU level.

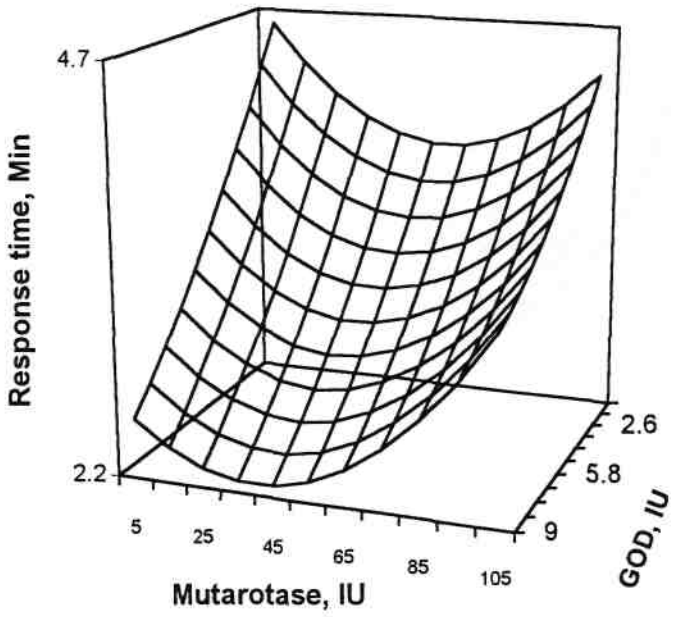


Figure 3.7.2 Response surface plot showing the effect of mutarotase and GOD on the response time. Invertase is constant at 30 IU level.

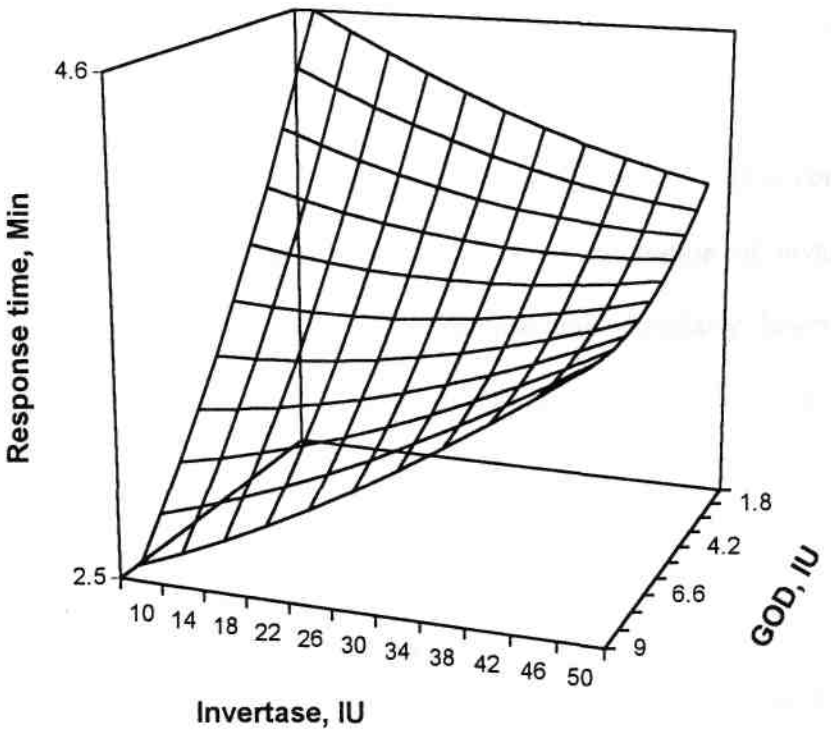


Figure 3.7.3 Response surface plot showing the effect of invertase and GOD the response time. Mutarotase is constant at 5 IU. level.

Bhate. 1960), it has not been specified whether the inhibition of mutarotase is due to α-D- glucose or β-D- glucose. Our results indicate that the increase in response time with higher concentrations of mutarotase (75-105 IU) as seen in figure 3.7.1 and 3.7.2 and a decrease in response time with increase in GOD concentration (figure 3.7.2) may in fact be due to reduction in the accumulation of β-D-glucose in the vicinity of immobilized enzyme membrane. The inhibition of mutarotase probably occurs due to excess of β-D-glucose and not α-D- glucose.

The effect of varying concentrations of invertase and GOD at constant mutarotase concentration is shown in figure 3.7.3. Lower concentration of invertase and higher concentration of GOD resulted in lower response time. Similarly lower concentration of invertase and GOD at constant mutarotase concentration resulted in higher response time. The decrease in response time with increasing GOD concentration at any given concentration of invertase may be due to reduction in the build up of β-D-glucose in the vicinity of the multienzyme membrane.

The most efficient and economical conditions would be to use the lowest concentrations of all the enzymes and yet achieve minimal response time. The optimal enzyme loading conditions to obtain lowest response time were predicted by using Microsoft Excel software (version 5.0). Figure 3.7.4 shows the contour plot of equal response time for different invertase and GOD concentrations at constant mutarotase concentration. By using response surface methodology, an attempt to understand the relationship between the important variables (enzyme concentrations) on the response time was found to be quite useful to arrive at economical loading of enzymes to obtain rapid response.

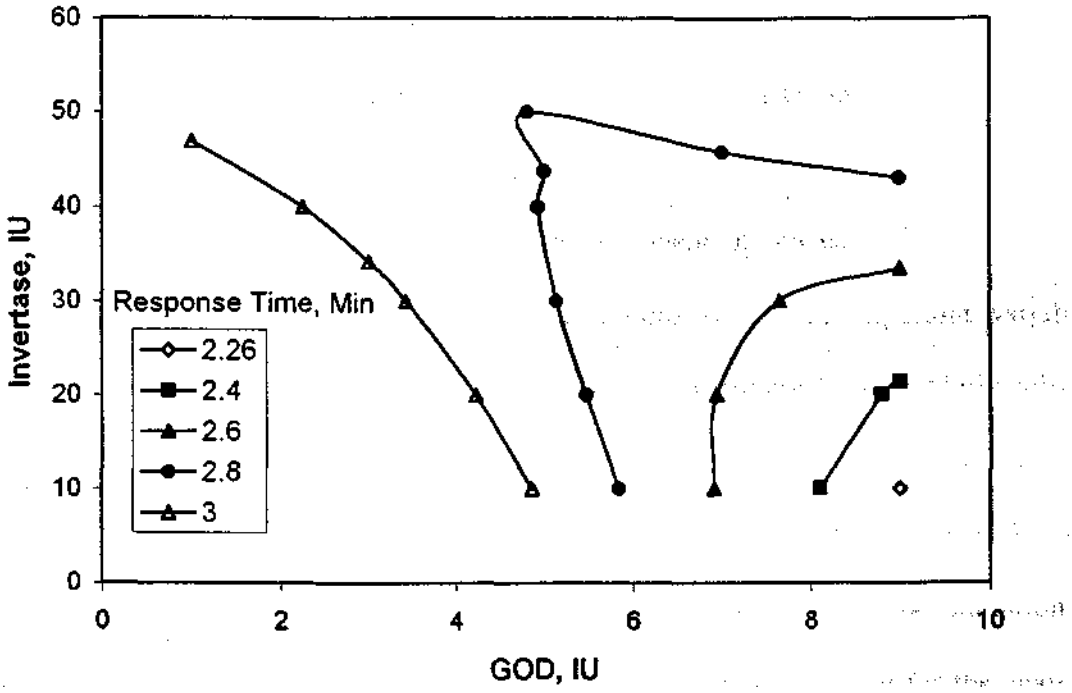


Figure 3.7.4 Contour plot showing the ranges of invertase and GOD to obtain various constant response time values. Mutarotase value is constant at 40 IU.

3.8 Chapter summary.

Cathodic amperometry based biosensor device was constructed for the estimation of glucose and sucrose in food and fermentation samples. For the estimation of glucose, GOD and for sucrose estimation, multienzyme system comprising invertase, mutarotase and GOD were immobilized by glutaraldehyde cross linking using various PBSAs. Operating parameters of the biosensor for the estimation of glucose and sucrose in the range 1-10 % were standardized. Steady state response time for the estimation of glucose and sucrose was found 3 and 4 minutes respectively. For better process economy in terms of glutaraldehyde, PBSA, enzyme loading and pH were optimized for estimation of glucose and sucrose. The multienzyme concentrations which are important variables influencing the biosensor response were optimized by Response Surface Methodology. In the range of parameters studied, response time decreased with decrease in invertase and with increase in mutarotase and GOD. Mutarotase concentration above 75 IU was found to result in an increased response time which was attributed to the inhibition of mutarotase by its product β-D-glucose. The optimal conditions achieved for the analysis of sucrose are; Invertase 10 IU; mutarotase 40 IU; GOD 9 IU. With these conditions, the predicted and actual experimental response time values were 2.26 and 2.35 minutes respectively, showing a good agreement.

Chapter 4

Estimation of sucrose in presence of glucose

ESTIMATION OF SUCROSE IN PRESENCE OF GLUCOSE

Although enzyme based biosensors have been extensively used for applications in clinical and pharmaceutical area, food and fermentation processes and environmental monitoring, since not all enzyme catalysed reactions involve transducer-active compounds such as hydrogen ions, oxygen, or hydrogen peroxide, only a limited number of analytes can be determined by using mono-enzyme sensors. In such cases, single enzyme systems using calorimetry based transducer have been reported (Hundeck et al., 1992; Danielsson. 1994). In the case of sucrose estimation, although these biosensors require single enzyme (invertase), disadvantages like drift in base line due to change in sample temperature exist. In such cases, coupled enzyme reactions for analyte conversion upto the stage of the evolution or consumption of the electroactive species provide an alternative. The primary product of the conversion of the first analyte is further converted enzymatically with the formation of measurable secondary product (Xu et al., 1989; Scheller and Renneberg. 1983). One of the major problems in multienzyme based biosensors is the interference of indicator enzyme substrates while analysing real samples. For example, in the case of a sucrose biosensor, interference of glucose limits the practical application of the biosensor for real samples containing glucose. Several attempts have been reportedly made to eliminate the interference of glucose while estimating the sucrose using the multienzyme biosensor (Cordonner et al., 1975; Satoh et al., 1976; Scheller and Renneberg. 1983; Mohammed et al., 1987; Matsumoto et al., 1988; Xu et al, 1989; Riche et al., 1995;).

In one of the methods reported, an additional immobilized glucose oxidase membrane was used to eliminate glucose (Scheller and Renneberg. 1983). This method being based on anodic amperometry, suffers from the electrochemical interference of ascorbic acid, uric acid and NADH while analysing real samples (Scheller & Schubert. 1992). Also, excess loading of glucose oxidase and catalase in the glucose eliminating layer, leads to increase in the response time. Another attempt reported, involves the insertion of a reactor containing immobilized invertase and mutarotase after the steady state response is reached for glucose using the glucose oxidase electrode (Scheller and Karsten. 1983). Though it was possible to analyse the sucrose in presence of low glucose concentration (0.1-0.6 mM), this method is not suitable at higher concentration of glucose. The use of a two electrode system has been reported as a simple way to eliminate the interference of glucose in the estimation of sucrose, (Xu et al., 1989). One electrode with only immobilized glucose oxidase estimates glucose and the other electrode containing three enzymes invertase, mutarotase and glucose oxidase determines both glucose and sucrose together. The net sucrose content was calculated as a difference of the two values. However, due to the interference of glucose on the response behavior of sucrose biosensor, the direct calculation as a difference leads to erroneous results (Riche et al, 1995). This problem has not yet been solved satisfactorily.

The present work aims at developing an effective methodology to account for the interference of glucose and estimate sucrose in the presence of glucose using the two electrode system with a knowledge of the response behaviour of the sucrose electrode for pure glucose and pure sucrose.

4.1 Estimation of sucrose in presence of glucose by using the two electrode system.

In order to examine whether the presence of glucose leads to any significant errors in the measurement of sucrose using the sucrose electrode, experiments were conducted with prepared standard solutions containing glucose and sucrose with varying concentrations of sucrose but the total sugar being 6 % (60 g/L). The calculations were done using calibration line for pure sucrose and assuming that since the sucrose electrode has the multienzyme system converting sucrose to glucose, the measurements should give the total sugars comprising sucrose as well as glucose. Therefore, subtracting the known glucose concentration from the total, should give the sucrose concentration. However, the results shown in table 4.1.1 indicate clearly that there are substantial errors in the sucrose analysis and the error increases with glucose percentage in the mixture. Therefore the methodology involving two electrodes for glucose and sucrose leads to substantial and unacceptable errors. This clear analysis has not been shown earlier and reported for the first time here.

4.1.2 Reasons for the erroneous results.

Why does this happen?. To answer this question, we examine the voltage response of the sucrose electrode for glucose as well as sucrose in terms of the linear calibration graph shown in figure 4.1.2.1. It is clearly seen that for the same % glucose and sucrose, the voltage response given by the electrode is very different, the signal for pure glucose being much higher. What are the reasons for this?

Table 4.1.1 Estimation of sucrose in presence of glucose by using two electrode system.

Total sugar (glucose + sucrose) = 6 %. (by wt.)

Glucose fraction f_g	Glucose %, (G)	Sucrose %, (S_a)	Expected voltage, Volts	Obtained voltage, Volts	Total sugar, S_T calculated %	Sucrose calculated $S_c = S_T - G$	Difference in sucrose % $S_c - S_a$	% error
0	0	6.0	1.2	1.2	6.0	6.0	0	0
0.1	0.6	5.4	1.0	1.4	7.2	6.6	1.2	22
0.3	1.8	4.2	0.8	1.5	8.0	6.2	2.0	48
0.5	3.0	3.0	0.55	1.55	8.2	5.2	2.2	74

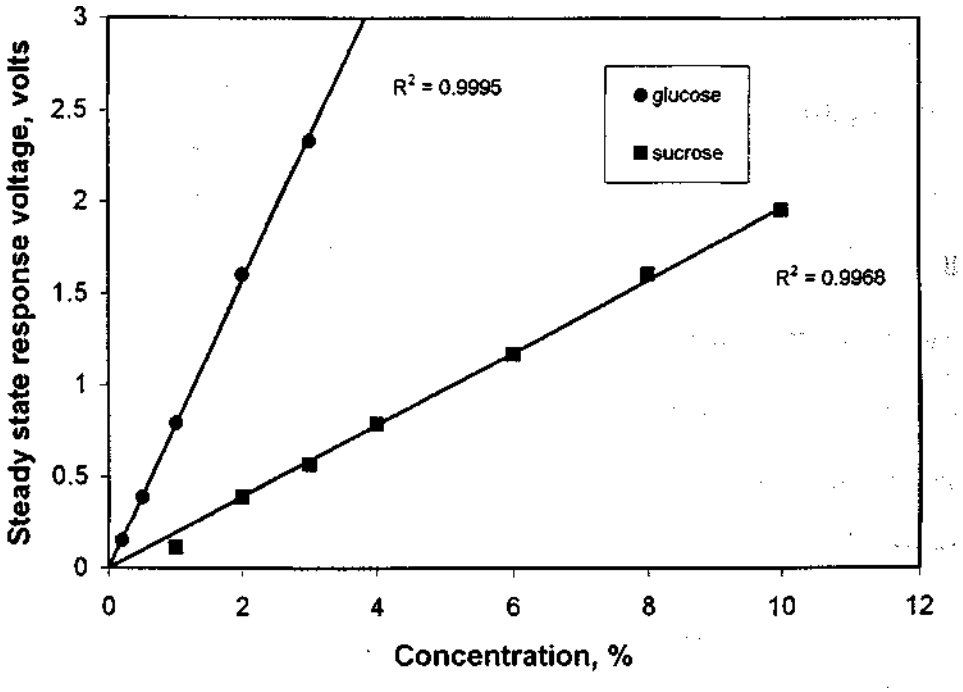
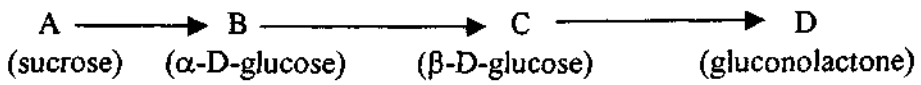


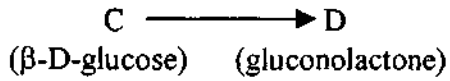
Figure 4.1.2.1 Voltage response of the sucrose electrode for pure glucose and sucrose.

The ultimate biochemical reaction that causes a depletion of oxygen in the vicinity of the sensing element of the dissolved oxygen electrode is the oxidation of glucose by GOD. In the case of glucose as the substrate, this involves first diffusion of the glucose molecules across the semipermiable outer membrane to the immobilized GOD membrane and then the reaction. In the case of sucrose, it involves the diffusion of the sucrose to the immobilized multienzyme membrane followed by the sequential enzyme reaction of the inversion of sucrose, mutarotation to β-D-glucose and finally glucose oxidation by GOD. All these steps cause additional resistances, delaying the dynamics of the crucial final reaction of glucose oxidation to gluconolactone. This in turn affects not only the rate of oxygen depletion around the dissolved oxygen (DO) sensing membrane but also steady state concentration and therefore the electrochemical signal produced. Thus for the same concentration of the sugar in terms of glucose and sucrose, the response voltage of the electrode is different, that for glucose being much higher. The slope of the calibration line for sucrose is 0.2025 giving a detection range of 1-10 % whereas that for glucose is much higher at 0.7845, giving a lower detection range of 0.2 %-3 %. It is noted that the lower detection range for glucose with the sucrose electrode is because the injection volume is 50 µl (necessitated by the need for adequate response for sucrose measurements in the range 1-10 %) whereas the injection volume for glucose electrode is lower at 25 µl only.

The reaction phenomenon occurring at the sensing element of the DO electrode for the sucrose biosensor for the two situations involving sucrose and glucose as the substrate can be visualized in another way. For sucrose, the mechanism will be sequential reaction.



whereas for glucose, it will be only the last of the above reactions



Assuming that the diffusional resistance for the transport of glucose and sucrose molecules across the semipermiable membrane are not different, for the same concentration of glucose and sucrose in the bulk, the net concentration of glucose in the vicinity of the enzyme membrane is lower for sucrose, leading to a lower steady state voltage. This translates into a calibration line of a lower slope value. When there is a glucose sucrose mixture, for the same total sugar concentration the voltage response will be in between that for pure glucose or pure sucrose, the upper limit of course being set by the electronic circuitry of the detector system.

Another possible reason for the difference in the calibration lines of glucose and sucrose as shown in figure 4.1.2.1 is the difference in molecular weights of the two, resulting in higher molarity (by the ratio of molecular weight of sucrose to that of glucose (342/180)) for the glucose for the same concentration by weight. For example, 1 % glucose by weight corresponds to 55 mM whereas 1 % sucrose corresponds to 29.2 mM only. However, even if we take sucrose corresponding to 55 mM (58.4 mM), still the voltage response for sucrose is lesser than that for 55 mM glucose. This is also seen by a plot of voltage responses for glucose and sucrose in terms of molar concentrations where

the lines corresponding to glucose and sucrose do not coincide and are quite different. Therefore this line of reasoning is not of much help.

One approach to overcome the problem of the interference of glucose in the analysis of sucrose in a glucose-sucrose mixture first by the two electrode method is to totally eliminate glucose from the mixture first and then measure sucrose by the sucrose electrode. This can be done by adding GOD to completely oxidize glucose. However, this will require time (1-2 hours) as well as consumption of GOD enzyme, one can visualize an immobilized GOD reactor column through which the sample is passed for converting glucose to gluconic acid but it will be an additional step. Further, such a reactor will need residence time of 30-60 minutes. A soluble GOD reactor will involve consumption of GOD. Therefore there is a need for an effective alternate method.

Another possible method is to use invertase to convert sucrose to glucose and fructose first and then measure glucose with GOD electrode (Mason. 1983). Having measured the glucose in the original mixture (without inversion) using the same GOD electrode, the measure of glucose belonging to the inverted sucrose is available and therefore the total sucrose in the mixture can be calculated. However this is an indirect method involving only glucose sensor and no sucrose sensor. Further, external inversion of sucrose is involved taking time (30-60 minutes) as well as consumption of the enzyme invertase, or an immobilized invertase reactor. The above reasoning points to the need for an effective alternate method for sucrose estimation in the presence of glucose. **4.2 Empirical formula for sucrose in presence of glucose.**

Under such a situation of differing calibration lines for glucose and sucrose for the sucrose electrode, how do we estimate sucrose in presence of (known concentration)

glucose ?. It was felt that the ratio of the slopes for pure glucose and pure sucrose in the calibration graph of the sucrose electrode should be the crucial factor in this and the following empirical formula was arrived at

$$\text{Net sucrose concentration} \quad \theta = x_2 - \left(x_1 * S_g / S_s \right) \qquad (4.1)$$

Where *X2* = total sugar (glucose and sucrose) weight % as measured by the sucrose electrode, using the calibration line for pure sucrose (slope S_s); x_1 = glucose concentration (weight %) as measured by the glucose electrode (single enzyme GOD) or any other method and S_g,S_s = slopes of the calibration lines for glucose and sucrose respectively for the sucrose electrode.

The applicability of equation 4.1 was examined by preparing standard mixtures of glucose and sucrose of known compositions and analysing the same using the biosensor with sucrose electrode. The results given in table 4.2.1 indicate clearly that equation (4.1) works very well. Figure 4.2.1 shows a plot of the sucrose concentration calculated by the equation (4.1) against the actual concentration initially taken. An excellent correlation is indicated by a 45° line with an equation y = 0.9971x + 0.0122 with a correlation coefficient $R^2 = 0.9957$.

Table 4.2.1 Calculation of net sucrose concentration in standard mixtures containing known concentrations of glucose and sucrose by using glucose and sucrose biosensor using equation 4.1.

Glucose and sucrose wt. % in standard mixtures		Wt. % glucose from glucose biosensor	% error	Total sugar content (sucrose and glucose) in wt. % from sucrose biosensor using calibration line for pure sucrose	Net sucrose wt. % calculated by equation 1.	% error
Glucose	Sucrose					
0.1	0.9	0.12	20.00	1.15	0.95	5.55
0.3	0.7	0.29	3.33	1.25	0.73	4.28
0.6	1.4	0.62	3.33	1.72	1.37	2.14
0.2	1.8	0.19	5.00	2.11	1.79	0.55
0.4	3.6	0.38	5.00	4.32	3.68	2.22
0.8	7.2	0.82	2.50	8.60	7.2	0.00
1.2	2.8	1.12	1.66	4.73	2.82	0.71
0.5	0.5	0.6	20.00	1.62	0.60	20.00
1.0	1.0	1.1	10.00	2.84	0.96	4.10
2.0	2.0	2.08	4.00	5.54	1.99	0.50
2.5	2.5	2.61	4.40	6.99	2.67	6.80

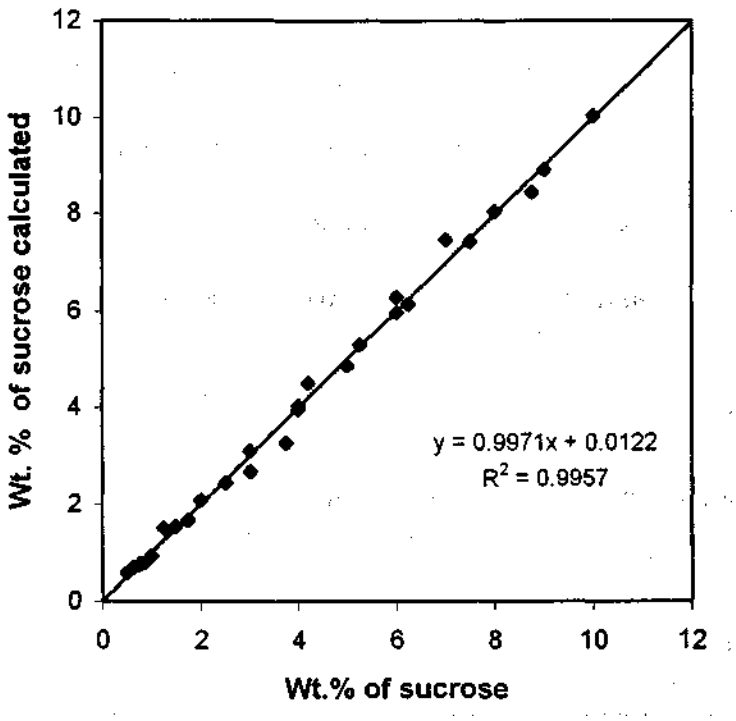


Figure 4.2.1 Sucrose concentration calculated by equation (4.1) against the actual concentration initially taken in the glucose and sucrose mixture.

4.3 Estimation of sugar in real samples and comparison with HPLC.

Using equation (4.1), and the two electrode method, now we can estimate both glucose and sucrose in real samples conveniently and with reasonable accuracy. The results of such an analysis for a number of fruit juice samples such as mango, grapes, orange etc are shown in table 4.3.1. The values predicted by the biosensor plotted against those determined by HPLC method for both glucose and sucrose (figure 4.3.1 and 4.3.2) neatly fall into a 45° straight line with equation $y = 0.9933x$ and ($R^2 = 0.9982$) and $y = 1.0096x$ ($R^2 = 0.9888$) respectively. These results indicate that the empirical formula given by equation (4.1) is very effective for application in real systems.

4.4 A theoretical analysis and elimination of empiricism of equation (4.1).

We attempted to plot the biosensor steady state response (in volts) against sugar concentrations in several ways to arrive at a theoretical basis. Figure 4.4.1 shows a plot of voltage response versus glucose concentration in a mixture containing different percentage of sucrose. This plot is not useful to predict the sucrose concentration

Figure 4.4.2 shows the plot of voltage response against sucrose concentrations for varying concentrations of glucose in the sample. While these plots are linear both slopes and intercepts appear to vary with the percentage of glucose in the mixture and an attempt at predicting the sucrose in presence of glucose was unsuccessful.

Table 4.3.1 Comparison of Biosensor results with HPLC method for real samples.

Sample (Juices)	Glucose, wt. %			Sucrose wt. %		
	HPLC	Biosensor	% error	HPLC	Biosensor	% error
Sugar cane	6.9	6.7	2.89	9.2	9.15	0.54
Mango	4.6	4.5	2.17	4.5	4.7	4.44
Apple	3.9	4.0	2.56	1.2	1.35	12.50
Grapes	6.9	6.5	5.79	1.2	1.19	0.83
Orange	2.9	3.0	3.44	5.0	5.15	3.00
Sapota	8.2	8.2	0.00	1.2	1.1	8.33
Banana	6.5	6.7	3.07	5.1	5.05	0.98

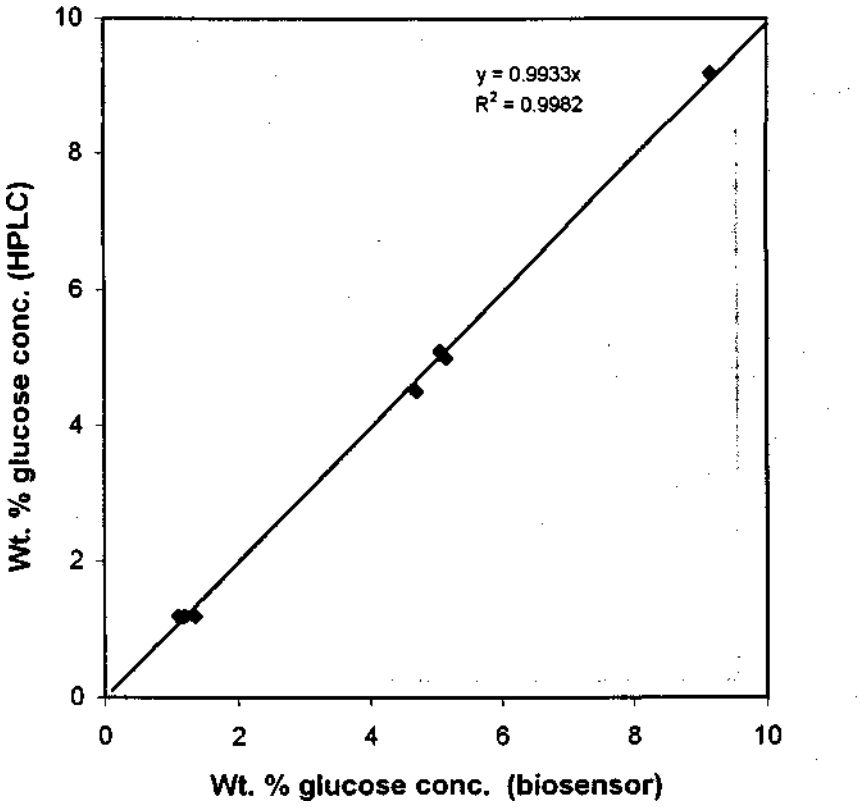


Figure 4.3.1 Glucose concentration values predicted by using the glucose biosensor for various food samples plotted against those obtained by HPLC method.

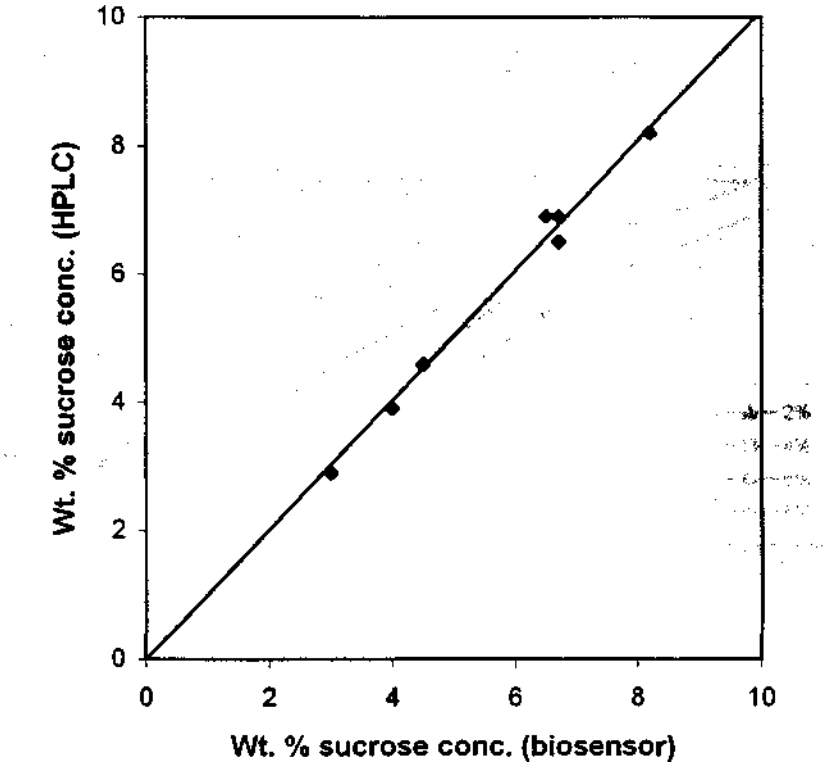


Figure 4.3.2 Sucrose concentration values predicted by equation (4.1) using the biosensor for various food samples plotted against those obtained by HPLC method.

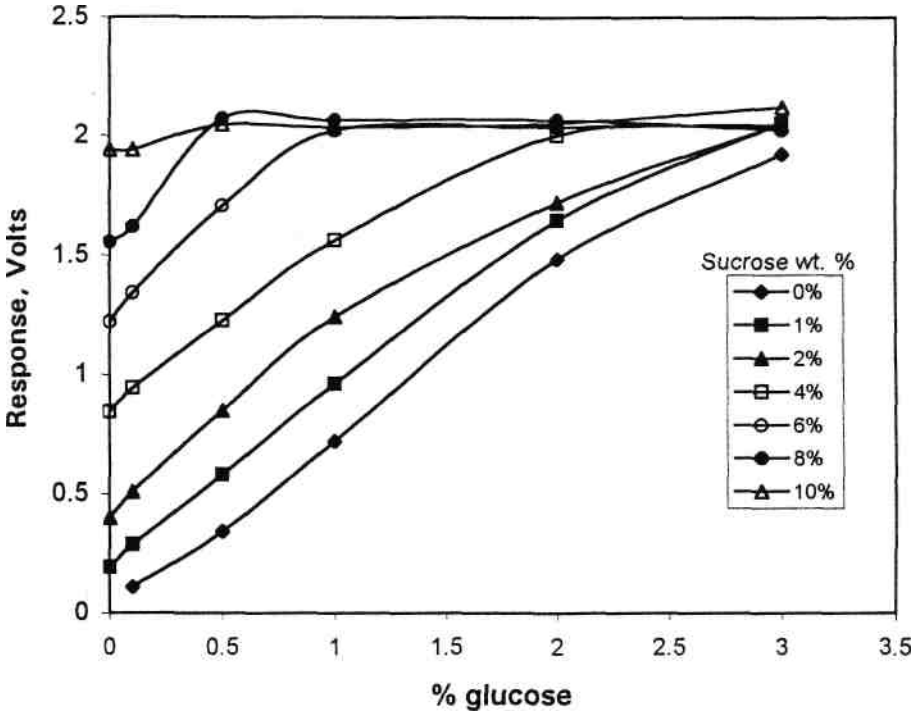


Figure 4.4.1 Steady state voltage response versus glucose concentration in a mixture containing different percentages of sucrose.

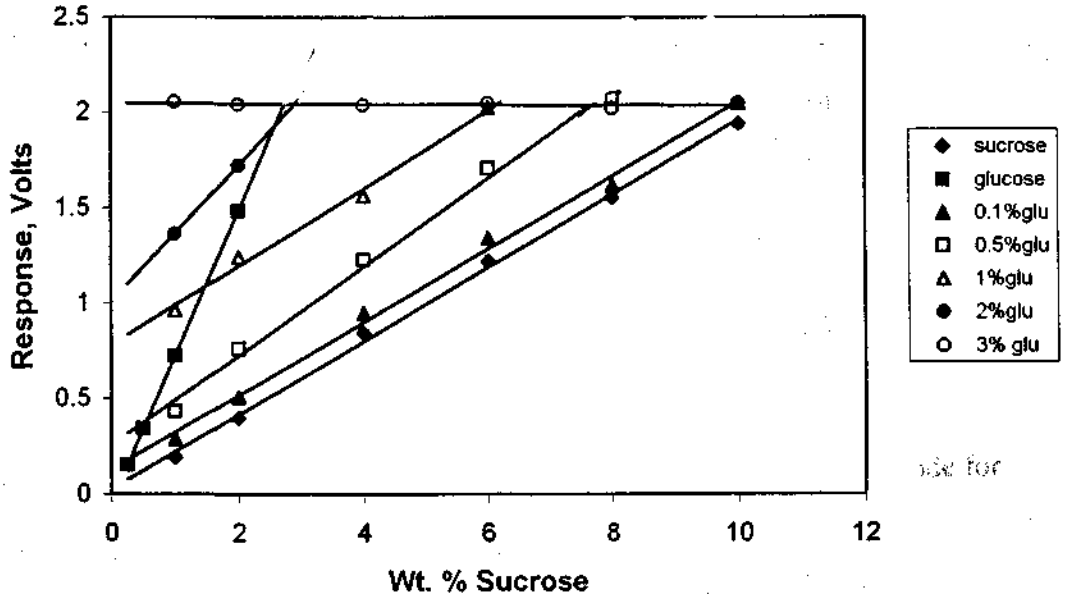


Figure 4.4.2 Steady state voltage response against sucrose concentration in mixtures containing varying concentrations of glucose.

The approach that finally succeeded was as follows.

Figure 4.4.3 shows the plot of steady state voltage response of the sucrose electrode against total sugar concentration for different weight fractions of glucose in the glucose sucrose mixture. This plot clearly shows a linearity at all compositions. While all the straight lines pass through the origin (0,0), the slope increases as the weight fraction of glucose increases. Can we predict this slope value for a given composition of glucose? This is tested by plotting the slope (y) values versus the weight fraction (fg) of glucose which is shown in figure 4.4.4 and indicates an excellent linearity with an equation.

$$Y = 0.580f_g + 0.0204, \qquad R^2 = 0.9992 \qquad (4.2)$$

Equation (4.1) can be written in terms of the individual slopes as

$$S_{gS} = S_S + (S_g - S_S)f_g \qquad (4.3)$$

where S_g,Ss and S_{gS} are the slopes of the calibration lines of sucrose electrode for pure glucose, sucrose, and glucose-sucrose mixtures respectively.

However since we do not knowf_g, we cannot predict the slope Sgs and therefore cannot immediately calculate the sucrose composition by knowing the voltage response of the sucrose electrode. In order to do this, we have to derive the formula in terms of the weight percentage of glucose in the glucose sucrose mixture measured with glucose electrode, this is accomplished as follows. Consider the schematic diagram shown in figure 4.4.5 of the voltage response of the sucrose electrode versus total sugar (in weight %) for pure glucose, pure sucrose and a glucose sucrose mixture with glucose weight fraction fg.

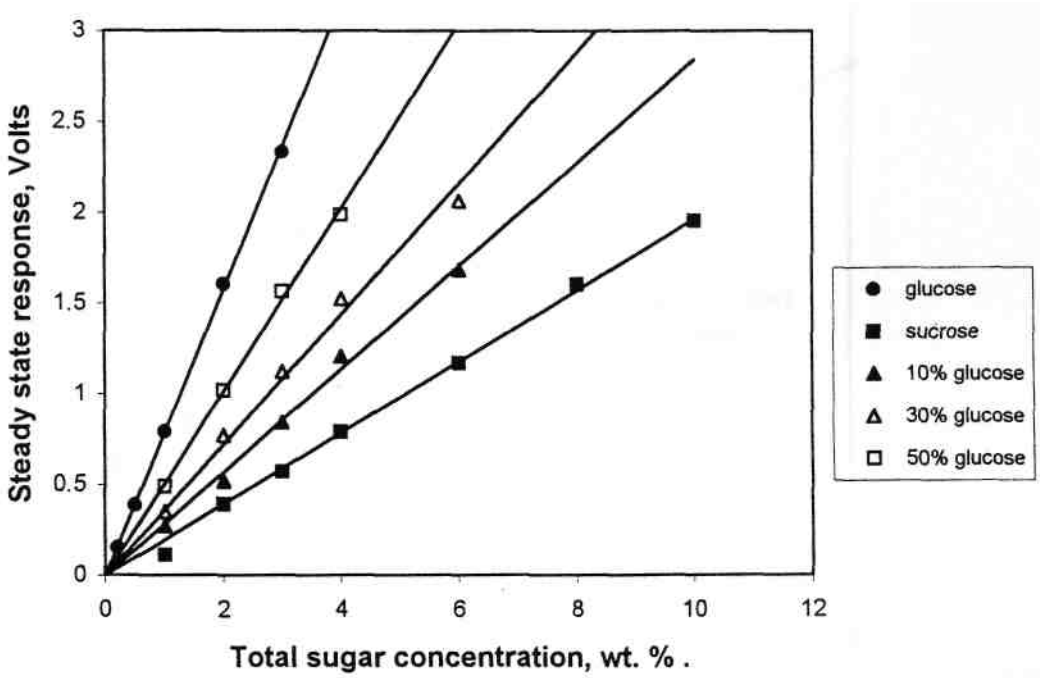


Figure 4.4.3 Steady state voltage response of the sucrose electrode against total sugar concentration for different weight fractions of glucose in the glucose and sucrose mixture.

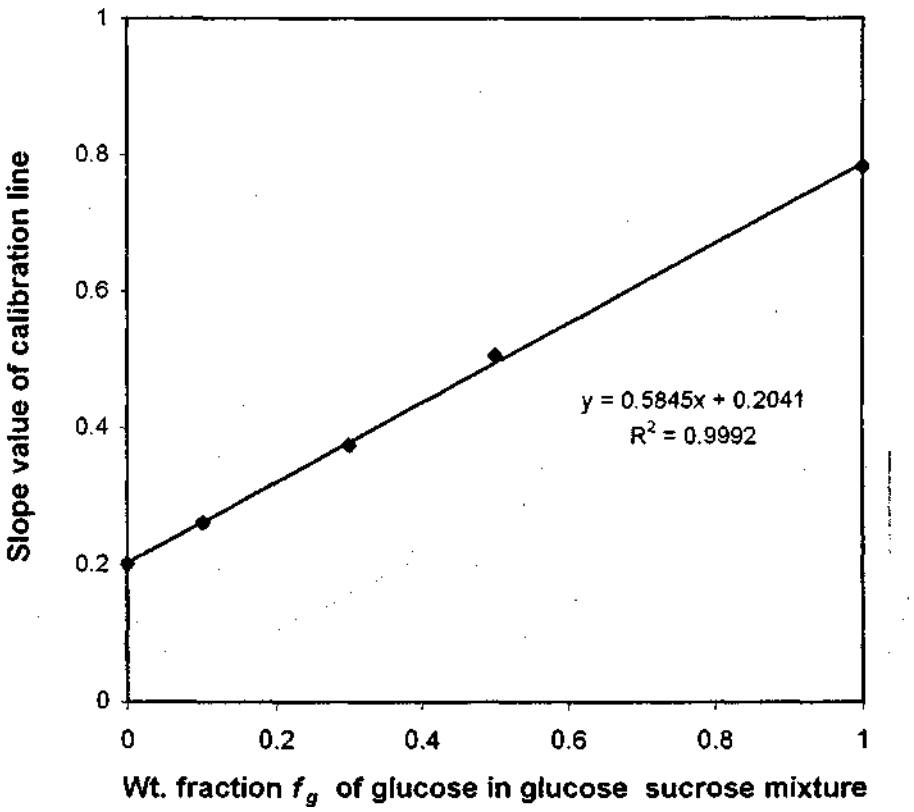


Figure 4.4.4 A plot of slope values from fig. 4.4.3 versus the weight fraction (f_g) of glucose.

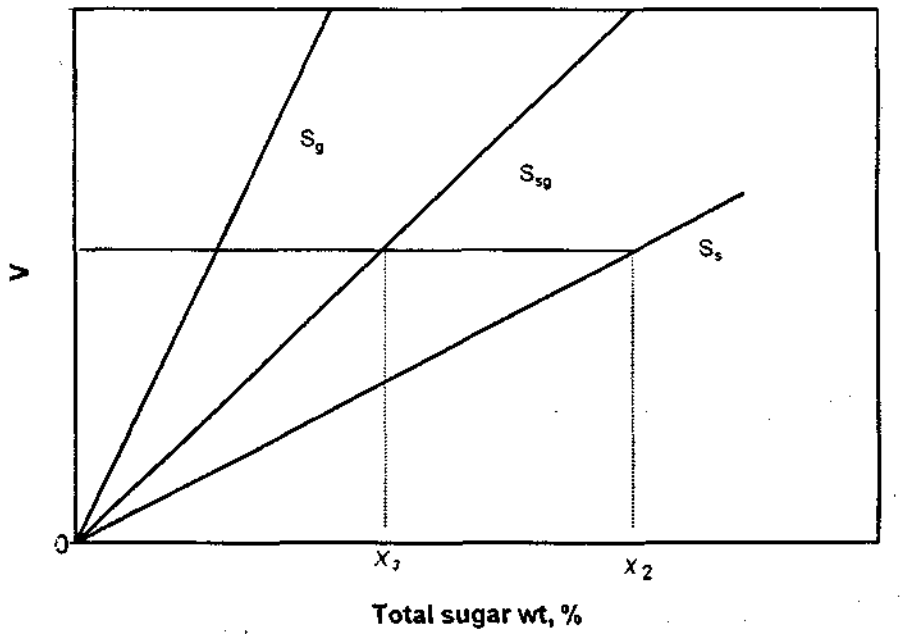


Figure 4.4.5 Schematic plot of voltage response of the sucrose electrode against various concentrations of glucose (S_g), mixture of glucose and sucrose (S_{Sg}) and sucrose (S_s).

Let the following be designated

V- Voltage response with sucrose electrode for the glucose sucrose mixture

X_1,- Actual weight % glucose (as measured by glucose electrode)

x_2- Weight % sucrose as measured by the sucrose electrode as if the mixture were pure sucrose using the calibration line of slope Ss

X_3- Total sugar as measured by the sucrose electrode as if the mixture were pure using the calibration line of slope Ss_g

θ - Net sucrose content in weight % in the mixture

$$= x_2/[1 + (\lambda - 1)/(1 + \theta/ x_1)]- x_1 \qquad (4.8)$$

where $\lambda = S_g/S_S$

Rearranging equation (4.8) we have

$$\theta\,[1 + (\lambda -1)/(1+\theta/x_1) = x_2-x_1[1 + (\lambda -1)/(1+\theta/x_1)$$

or $\theta[(\lambda + \theta/x_1)/(1+\theta/x_1)] = x_2-x_1\,[(\lambda+\theta/x_1)/(1 + \theta/x_1)$

Rearranging

$$(\theta + x_1)\,(\lambda +\theta/ x_1)/\,(1 + \theta/ x_1) = x_2$$

or $x_1(\lambda + \theta/ x_1) = x_2$

$\therefore$ $\theta/ x_1 = x_2/ x_1- \lambda$

or $\theta = x_2 -\lambda\, x_1$

$$\theta = x_2 - x_1 \cdot S_g/S_S \qquad (4.9)$$

we have

We can see that equation (4.9) is identical with equation (4.1) showing that equation (4.1)

which was empirically usable can in fact be theoretically derived.

$$= x_2/[1 + (\lambda - 1)/(1 + \theta/ x_1)]- x_1 \qquad (4.8)$$

where $\lambda = S_g/S_S$

Rearranging equation (4.8) we have

$$\theta\ [1 + (\lambda -1)/(1+\theta/x_1) = x_2-x_1[1 + (\lambda -1)/(1+\theta/x_1)$$

or $\theta[(\lambda + \theta/x_1)/(1+\theta/x_1)] = x_2-x_1\ [(\lambda+\theta/x_1)/(1 + \theta/x_1)$

Rearranging

$$(\theta + x_1)\ (\lambda +\theta/ x_1)/\ (1 + \theta/ x_1) = x_2$$

or $x_1(\lambda + \theta/ x_1) = x_2$

$\therefore$ $\theta/ x_1 = x_2/ x_1- \lambda$

or $\theta = x_2 -\lambda x_1$

$$\theta = x_2 - x_1 * S_g/S_S \qquad (4.9)$$

We can see that equation (4.9) is identical with equation (4.1) showing that equation (4.1) which was empirically usable can in fact be theoretically derived.

4.5 Chapter Summary

The problem of glucose interference during sucrose estimation by using the multienzyme based biosensor was effectively overcome, using firstly an empirical equation based on the response behaviour of the sucrose biosensor separately for pure glucose and sucrose. By using this empirical formula sucrose could be accurately estimated in prepared solutions containing pure glucose and sucrose as well as various fruit juice samples by using the sucrose biosensor in conjunction with the glucose biosensor. This was verified by the HPLC method and the agreement was excellent. With a theoretical understanding of the response behaviour of the sucrose electrode in the presence of glucose, the empiricism of the formula could be successfully eliminated leading to a practically effective method for determination of sucrose in real food samples using the biosensor.

Chapter 5

Stability behaviour of immobilized single and multienzyme systems – effect of stabilizing agents.

STABILITY BEHAVIOUR OF IMMOBILIZED SINGLE AND MULTIENZYME SYSTEMS - EFFECT OF STABILIZING AGENTS.

Stability of enzymes is one of the important considerations for their biotechnological applications (Farahdiba et al., 1993; Gupta. 1991; Klibanov. 1979). In order to increase the stability of enzymes and thus to improve the process economy various attempts have been made (Chen et al., 1998; Heller and Heller. 1998; Minagawa et al., 1998; Wang et al., 1992). Often the stability of enzymes can be increased by immobilization. There are different methods of immobilization, each having its own advantages and disadvantages. One of the popular enzyme immobilization methods, particularly for biosensor applications uses, crosslinking by glutaraldehyde. Glutaraldehyde, being a strong bifunctional reagent, modifies the enzyme drastically, leads to conformational changes and loss of activity (Broun. 1976). This deleterious effect can be minimized by using inert proteins like BSA, gelatin, thrombin and lysine. These proteins avoid excessive intramolecular crosslinkages within the enzyme and enhance the intermolecular linkages between the enzyme and inert proteins (Broun et al., 1973). While the complementary surface (Chang and Mohany. 1995) and durability of matrix proteins (Gabel et al., 1970; Gabel. 1973) are reported to play an important role in the stabilization of immobilized enzymes, in practice, the application of matrix proteins during the process of immobilization has been limited to BSA and gelatin. It is quite possible that stable proteins other than BSA and gelatin showing better complementarity may have beneficial effect on the stability of free as well as immobilized enzyme preparations. The stabilization of desired enzyme may be achieved by using proteins, which may be catalytically active or inactive and may be referred to a protein based

stabilizing agents (PBSA). However, one should keep in mind that, if the PBSA is an enzyme, its catalytic effect should not interfere with the biochemical reaction of the desired enzyme electrode.

Stability of immobilized enzyme can be assessed by exposing the preparation to varying temperature , pH and denaturant concentrations and examining the operational and storage stability. Several attempts have been reportedly made to investigate the stability of immobilized enzymes as a function of heat, pH and various solvents by using CD , NMR, FS and DSC (Jurgen.1976; Lane et al., 1971). However, these methods have several constraints with respect to their application to immobilized enzymes. The available literature on the thermal denaturation studies of immobilized enzymes is still scanty. It has been reported in literature that use of enzyme based biosensors can be applied for the fundamental biochemical studies involving assessment of stability of immobilized enzymes (Mosbach and Mattiasson. 1976). However due to poisoning and deactivation of the biosensor electrode at higher temperature and electrochemical interference of denaturants like urea, the use of biosensor was restricted only for the analytical purposes.

In order to understand the stabilization of enzymes against heat, pH and also operational stability, which is important for the commercial viability of any biosensor, single (GOD) and multienzyme system (invertase, mutarotase and GOD) were immobilized by crosslinking method using BSA, gelatin and lysozyme as PBSAs. The amperometric biosensor developed in this work using a simple Clark type oxygen electrode is used for the investigations on enzymes immobilized with various PBSAs

with temperature, pH and operational stability as variables and the results are reported in this section.

5.1 Stability of GOD immobilized with various PBSAs.

5.1.1 Operational stability of immobilized GOD.

Figure 5.1.1 demonstrates the operational stability of the enzyme electrode for glucose, containing GOD immobilized with different PBSAs in 50 mM phosphate buffer pH 6.0. In order to quantify the operational stability of GOD immobilized with various PBSAs, repeated measurements with 100 µl of 3 % glucose solution were carried out. As shown in the figure, as the number of analyses was increased to 200, activity of GOD (in terms of biosensor response) immobilized with gelatin, BSA and lysozyme increased to 118, 121 and 130 % respectively. This increase in activity of immobilized GOD is probably owing to the decreased diffusional barriers for the glucose. Mohammed et al. (1987) reported that initial increase in the activity of immobilized enzyme during operational stability studies is due to decreased diffusional resistance for the analyte. Further usage of immobilized enzyme for repeated analysis of glucose leads to sharp decrease to less than 40 % initial activity after 300 and 520 analyses respectively for GOD immobilized with gelatin and BSA (figure 5.1.1). However, GOD immobilized with lysozyme retained a stable high activity upto 475 analyses and thereafter declined to reach 50 % activity around 700 analyses. Thus, lysozyme was found to be the best for the stabilization of GOD among the PBSAs tested, followed by BSA and gelatin. For GOD immobilized with lysozyme, it was possible to analyze 700 samples during 230 days of operation, at the end of which 50 % of the initial activity of the immobilized GOD was retained. With BSA as PBSA it was possible to analyze slightly reduced number of 520

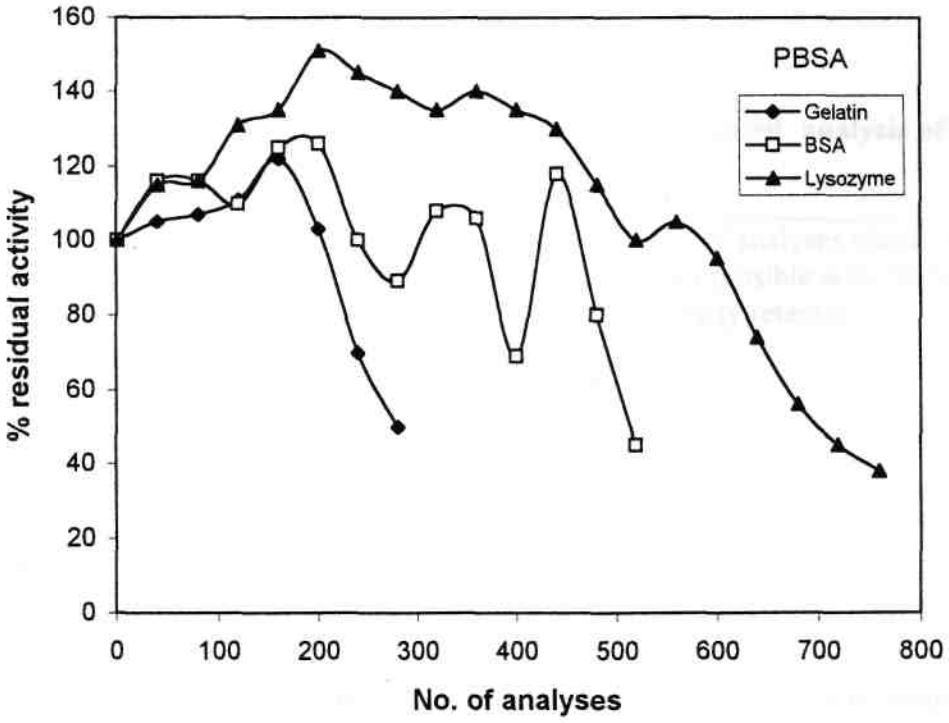


Figure 5.1.1 Residual activity after repeated glucose (3%) analysis with biosensor using GOD immobilized with different PBSA.

samples during 150 days of operation and with gelatin as PBSA it was possible to analyse only 245 samples during 60 days of operation (Table 5.1.1). Another significant observation from figure 5.1.1 is that the reproducibility of the biosensor signal for GOD immobilized with BSA is poor. Corresponding behaviour of GOD immobilized with lysozyme as PBSA was very stable.

Table 5.1.1 Comparison of % activity retained after repeated analysis of glucose with different protein based stabilizing agents.

PBSA	% activity after no. of analyses for 3 % glucose								No. of analyses (days of use) possible with 50 % activity retention.
	100	200	300	400	500	600	700	800	
Gelatin	110	103	38	--	--	--	--	--	250(60)
BSA	126	111	69	65	32	--	--	--	450(90)
Lysozyme	151	140	135	105	105	100	50	43	750(230)

In order to further understand the increased operational stability of GOD immobilized with lysozyme when compared to BSA and gelatin as PBSA, detailed investigations have been carried out on stability as a function of heat and pH for the GOD immobilized with various PBSAs.

5.1.2 Thermal stability of immobilized GOD.

Figures 5.1.2.la-lc and table 5.1.2.1 demonstrate the thermal deactivation behaviour of immobilized glucose oxidase with various PBSAs (gelatin, BSA and lysozyme) at different pH values. Thermal stability studies on the GOD immobilized with various PBSAs were carried out by measuring the activity of immobilized GOD at 27+l°C before and after incubation at desired temperature for 15 minutes. As the pH

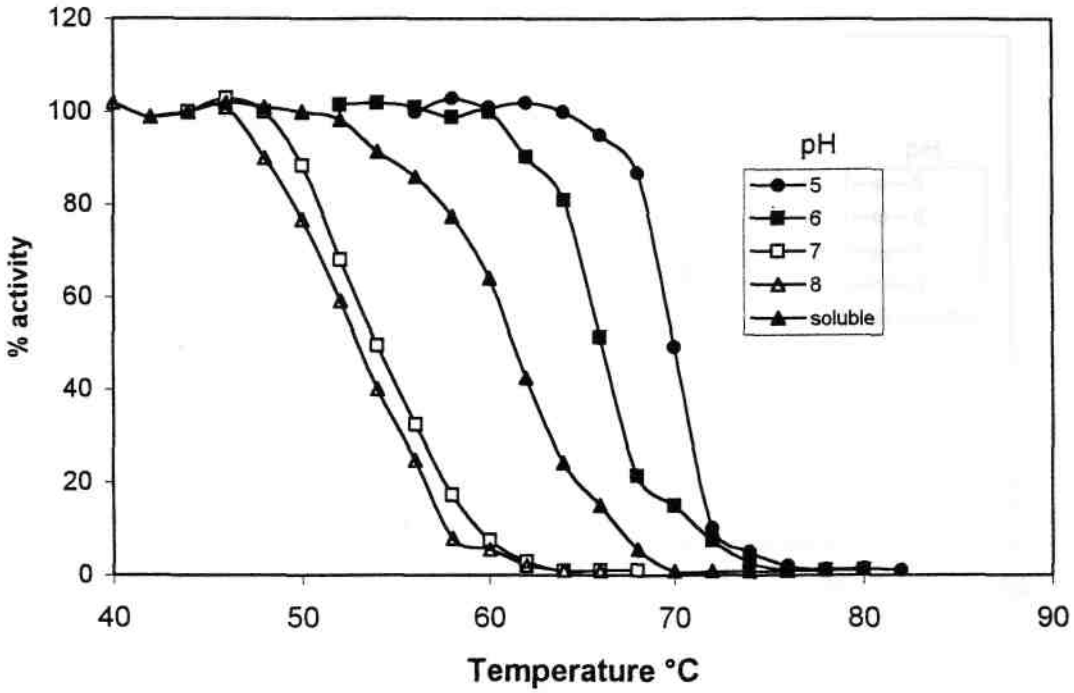


Figure 5.1.2.1a Thermal inactivation of GOD immobilized with gelatin as PBSA at various pH values.

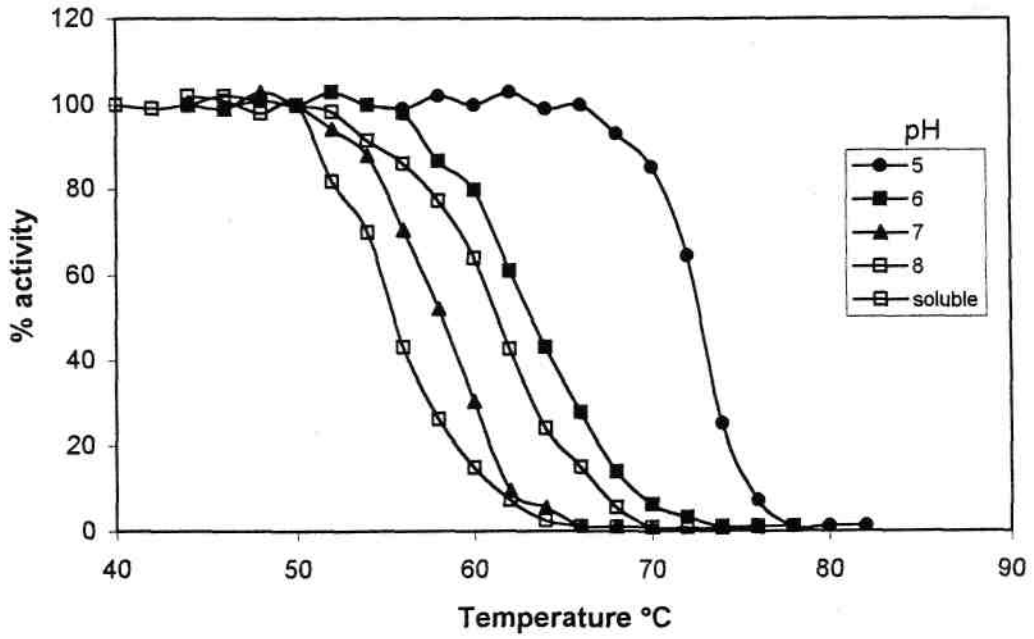


Figure 5.1.2.1b: Thermal inactivation of GOD immobilized with BSA as PBSA at various pH values.

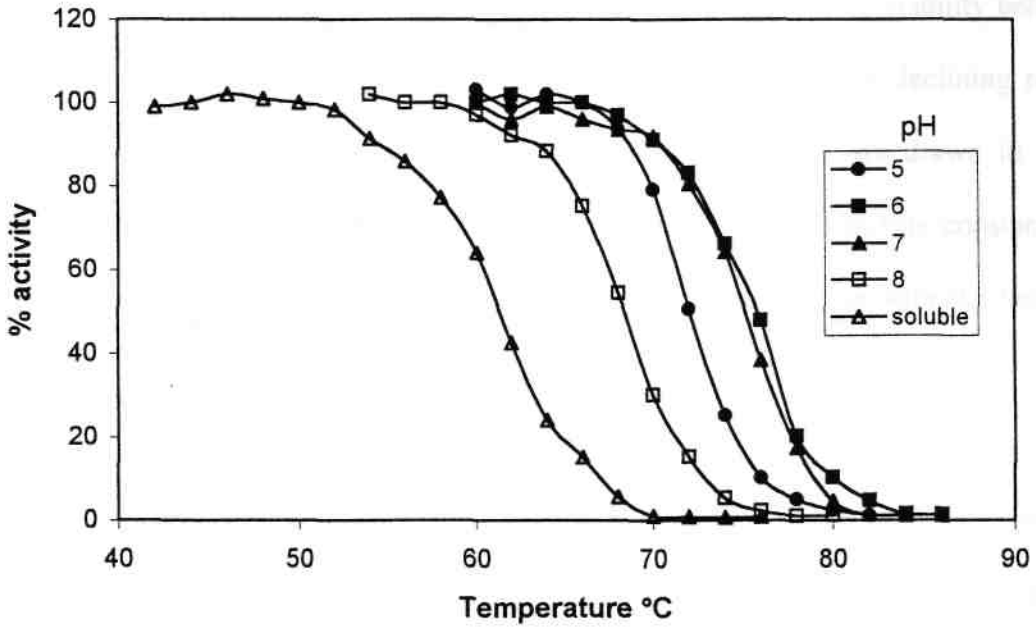


Figure 5.1.2.1c Thermal inactivation of GOD immobilized with Lysozyme as PBSA at various pH values.

increases, thermal deactivation of the immobilized GOD with BSA and gelatin increases but with lysozyme as PBSA, stability actually increased from pH 5.0 to 6.0, stayed without change in the range pH 6.0 to 7.0 but declined sharply from pH 7.0 to 8.0. Corresponding deactivation of soluble enzyme is also shown in figures 5.1.2. la-lc at pH 5.0 at which its activity is maximum and the activity of the enzyme decreases steeply on either side of this pH (as seen in figure 5.1.3.1.). Therefore thermal deactivation of soluble GOD was carried out at pH 5.0 only, for comparison with the stability behaviour of GOD immobilized with different PBSAs. The semi log plots of declining residual activity versus temperature corresponding to figures 5.1.2.1a-lc are drawn in figures 5.1.2.2a-2c, showing a linear behaviour. The calculated inactivation rate constants as a function of temperature are given in table 5.1.1.1, which are in tune with the behaviour exhibited in figures 5.1.2.1a-lc.

Figure 5.1.2.3 demonstrates the effect of pH on the Transition temperature (Tm) of GOD immobilized with various PBSAs. As the pH increased from 5 to 8, transition temperature of GOD immobilized with BSA and gelatin as PBSAs decreased sharply. However, in case of GOD immobilized with lysozyme, Tm increased from pH 5 to 6, remained steady between 6 and 7 and then decreased sharply from pH 7 to 8. A probable explanation for this observed temperature stability behaviour of GOD immobilized with different PBSAs as attempted is follows.

Increase in the thermostability of immobilized GOD compared to that of soluble enzyme can be attributed to the glutaraldehyde crosslinkages between the enzyme and PBSAs. This results in streric hindrance of active site of enzyme in the 3-dimensional lattice of the insolubilized protein and hence the enzyme configuration is

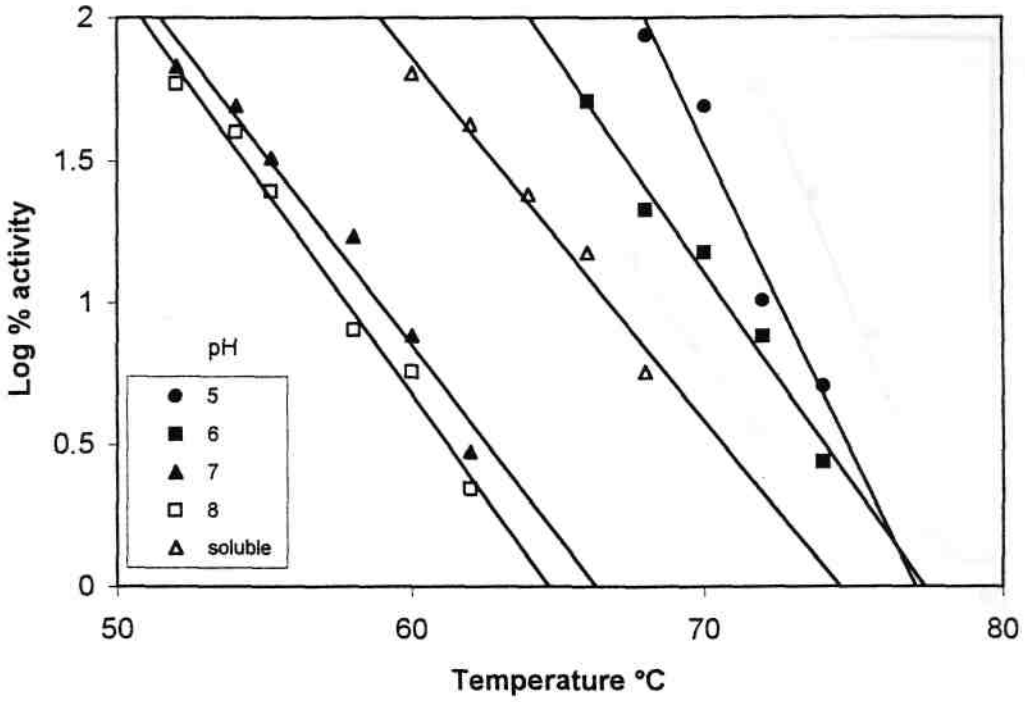


Figure 5.1.2.2a Semilog plot for the thermal inactivation of GOD immobilized with gelatin

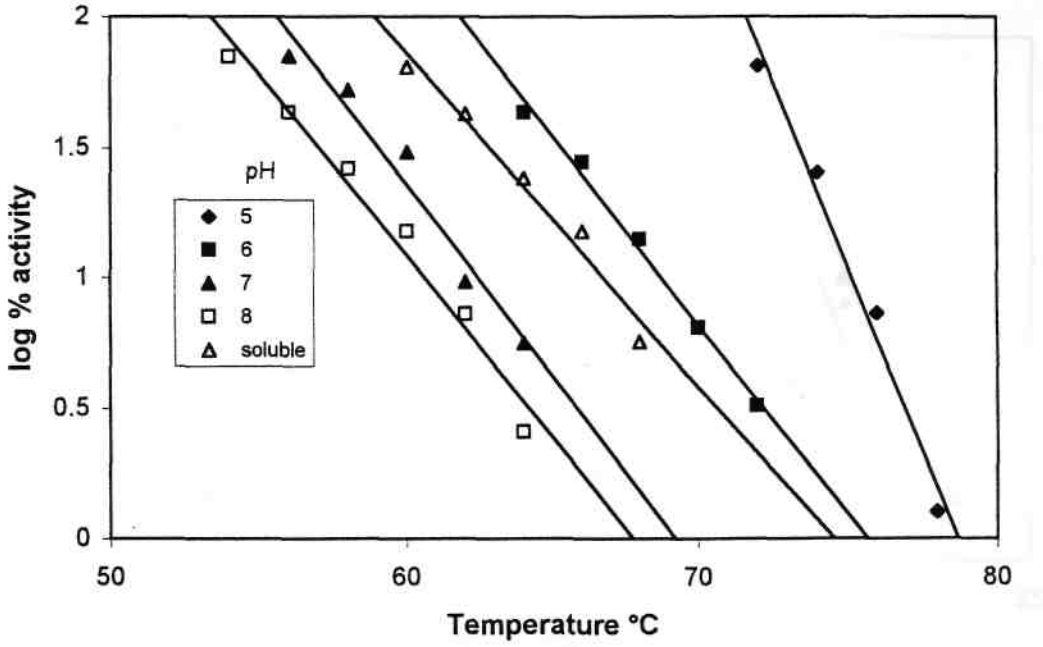


Figure 5.1.2.2b Semilog plot for the thermal inactivation of GOD immobilized with BSA

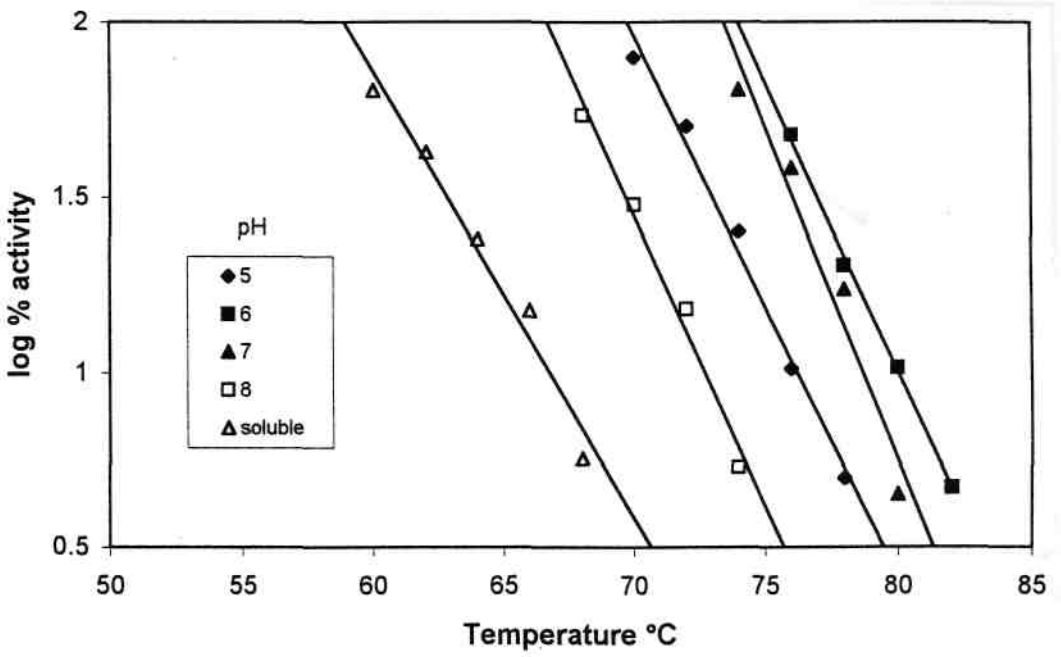


Figure 5.1.2.2c Semilog plot for the thermal inactivation of GOD immobilized with lysozyme

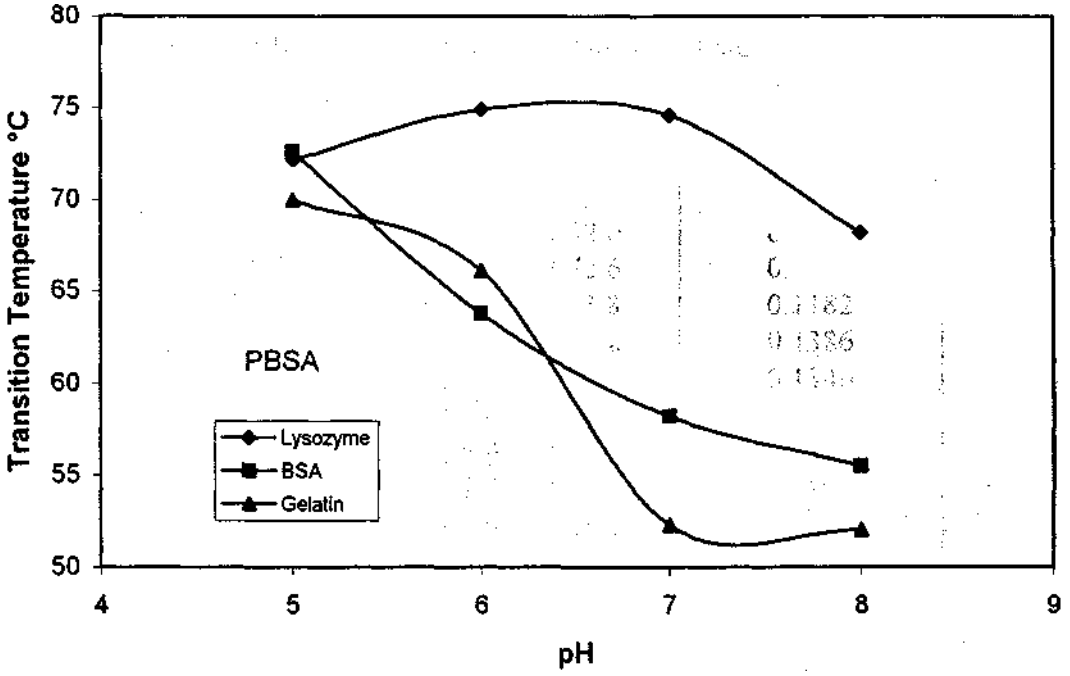


Figure 5.1.2.3 Effect of pH on the Transition temperature (Tm)of the GOD immobilized with different PBSAs.

thermodynamically favourable. Variation in stability of immobilized enzyme with different of PBSAs (figure 5.1.2.1a-lc & table 5.1.2.1) can probably be attributed to (1) the influence of the physicochemical properties of PBSAs and (2) the strength of crosslinkages between enzyme and PBSA.

Table 5.1.2.1 Transition temperature (Tm) of soluble and immobilized GOD at different pH with various PBSAs.

PBSA	pH	Tm °C	Inactivation rate constant, k, $°C^{-1}$
Gelatin	5.0	70.0	0.1579
	6.0	66.1	0.1070
	7.0	52.3	0.1180
	8.0	52.0	0.1209
BSA	5.0	72.6	0.2301
	6.0	63.8	0.1182
	7.0	58.2	0.1386
	8.0	55.5	0.1340
Lysozyme	5.0	72.2	0.1560
	6.0	74.9	0.1645
	7.0	74.6	0.1900
	8.0	68.2	0.1740
Soluble GOD	5.0	61.7	0.1065

Physicochemical properties of PBSA play a major role in determining the stability and complementarity of the carrier protein to the enzyme. If the carrier itself is unstable compared to that of enzyme, then the stability of the enzyme preparation is adversely affected. In our experiments, thermal stability of GOD immobilized with gelatin and BSA sharply decreased as the pH increased while the usage of lysozyme as PBSA enabled the retention of better stability even at higher pH values (figures5.1.2.1a-lc& 5.1.2.3).

The surface properties (hydrophilíc or hydrophobic) of the desired enzyme to be stabilized, and the PBSAs can be an important factor in the extent of stabilization. If both enzyme and PBSA have complementarity surface properties it may lead to favourable interactions and consequently a better stabilization (Chang and mohany. 1995). In our studies, soluble GOD, in presence of lysozyme as additive showed a 3-fold increase in the thermal stability (discussed further in chapter 6). Same was not true with other PBSAs as additives. Also, the negative effect of NaCl on the stability of the enzyme in the presence of lysozyme as additive, is a clear indication of the role of the ionic interactions between the GOD and lysozyme on the stabilization of GOD, which is adversely affected by NaCl. This ionic interaction can also be explained by considering the isoelectric pH of GOD and lysozyme. Since GOD is an acidic protein with an isoelectric pH of 4.3 (Hamaguchi and Hayashi. 1972), it carries a net negative charge and lysozyme being a strong basic protein with an isoelectric pH of 10.6 (Canfield and Anne. 1963), carries a net positive charge at the working of pH 6.0. The result may be a favourable ionic interaction between GOD and lysozyme, which might contribute to the stabilization of GOD in the presence of lysozyme as additive. The ability of NaCl to weaken the ionic interactions may account for the observed adverse effect on the thermal stability of GOD.

For the same reason, GOD immobilized with lysozyme showed consistently higher transition temperatures under varying pH conditions (maximum being at pH 6-7) compared to that of BSA and gelatin as PBSAs as seen in figure 5.1.2.3. This accounts for the favourable ionic interactions between GOD and lysozyme which are maximum between pH 6-7, and the decreases on either side of this pH range. In case of BSA and

gelatin as PBSAs, with their isoelectric point being 5.13 (Whitney et al., 1976) and 4.19 (Leobel. 1928) respectively, as the pH increases, negative charge on both GOD and PBSA increases, resulting in an electrostatic repulsion between GOD and PBSA, which explains the steeply decreased transition temperatures at higher pH values.

5.1.3 pH stability of immobilized GOD.

Figure 5.1.3.1 shows the pH stability of soluble and immobilized GOD with various PBSAs. These experiments were carried out by measuring the activity in 50 mM phosphate buffer, pH 6.0 before and after treatment with desired pH values for 30 minutes at 27±l°C. Activity at pH 6.0 is considered as 100 %. Neither the immobilized GOD with different PBSAs nor the soluble GOD are stable below the pH 4.0, although soluble GOD is more stable at pH 4.0 than any of the immobilized preparations. At pH 1.5 soluble and immobilized GOD with BSA lost their activity completely but the immobilized preparation with lysozyme and gelatin retained 20 and 15 % activity respectively. A sharp decrease in the activity of the soluble and immobilized GOD below pH 4.0 can be attributed to the leaching of the tightly bound FAD to the enzyme molecule (David and Robert. 1983). Above pH 6.0 soluble GOD shows a steep decline in activity and a complete inactivation at pH 11. Corresponding behavior of the immobilized enzyme preparations showed much better stability. While immobilized enzyme with BSA and gelatin showed a slightly increased activity till a pH 8 and 9 respectively before declining at higher pH values, the immobilized GOD stabilized with lysozyme showed a highly remarkable stability. In fact it showed a slight but continuous increase in activity till a pH 11.0.

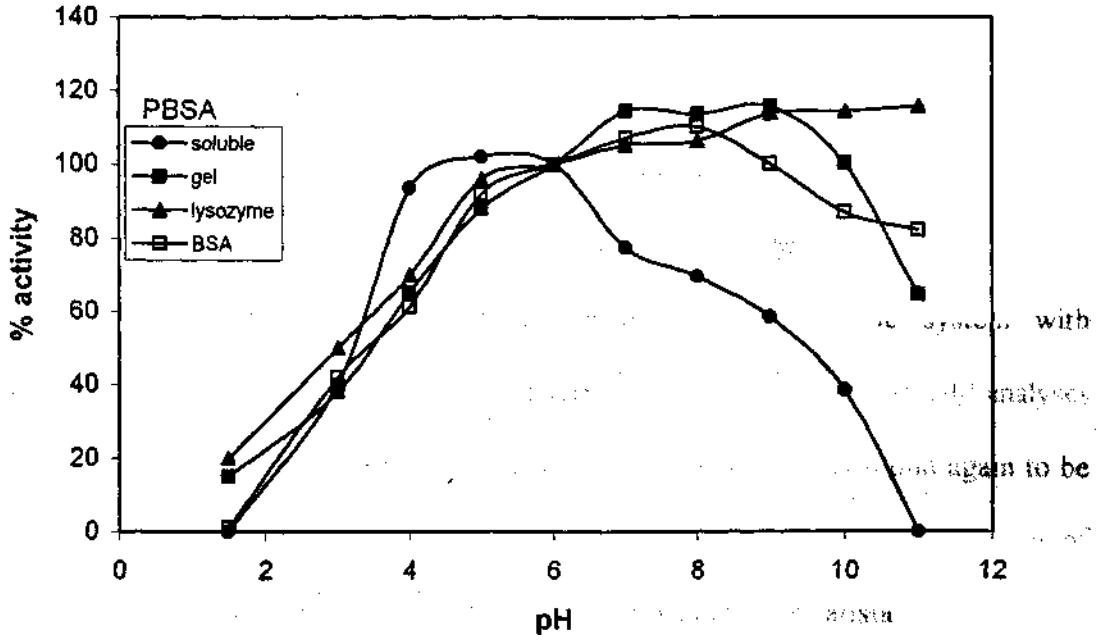


Figure 5.1.3.1 pH stability of soluble and immobilized GOD with different PBSAs.

5.2 Stability of the multienzyme system immobilized with various PBSAs.

5.2.1 Operational stability.

Figure 5.2.1.1 demonstrates the operational stability of multienzyme system immobilized with different PBSAs in 50 mM phosphate buffer pH 7.0 for the repeated measurements with 100 µl of 10 % sucrose. As shown in figure, as the number of analysis increased from 1 to 75, activity (in terms of biosensor response) of the multienzyme system immobilized with gelatin increased to 150 %, whereas for the multienzyme system immobilized with BSA and lysozyme, no significant increase in activity was observed. Further usage of the system leads to a sharp decrease to less than 50 % initial activity for 240 and 150 analyses in the case of gelatin and BSA as PBSA respectively (figure 5.2.1.1). Under similar conditions multienzyme system with lysozyme as PBSA enabled the retention of much more stable activity upto 240 analyses reaching 50 % activity only around 400 analyses. Thus lysozyme was found again to be the best among the PBSAs tested, followed by gelatin and BSA. In terms of the time of operation, as shown in table 5.2.1.1, lysozyme as PBSA allowed a satisfactory operation for 400 samples during 40 days at the end of which 50 % of the initial activity remained. Correspondingly, gelatin as PBSA enabled the analysis of a lesser number of 230 samples during 30 days of operation and with BSA as PBSA it was possible to analyse only 150 samples during 25 days of operation. Another significant observation is that though the stability of multienzyme system did increase by incorporating lysozyme during immobilization, when compared to the single enzyme system the stability of the multienzyme system was lower (see figure 5.2.1.1). This may be probably due to the complexity of the multienzyme system, wherein the inactivation of any one of the

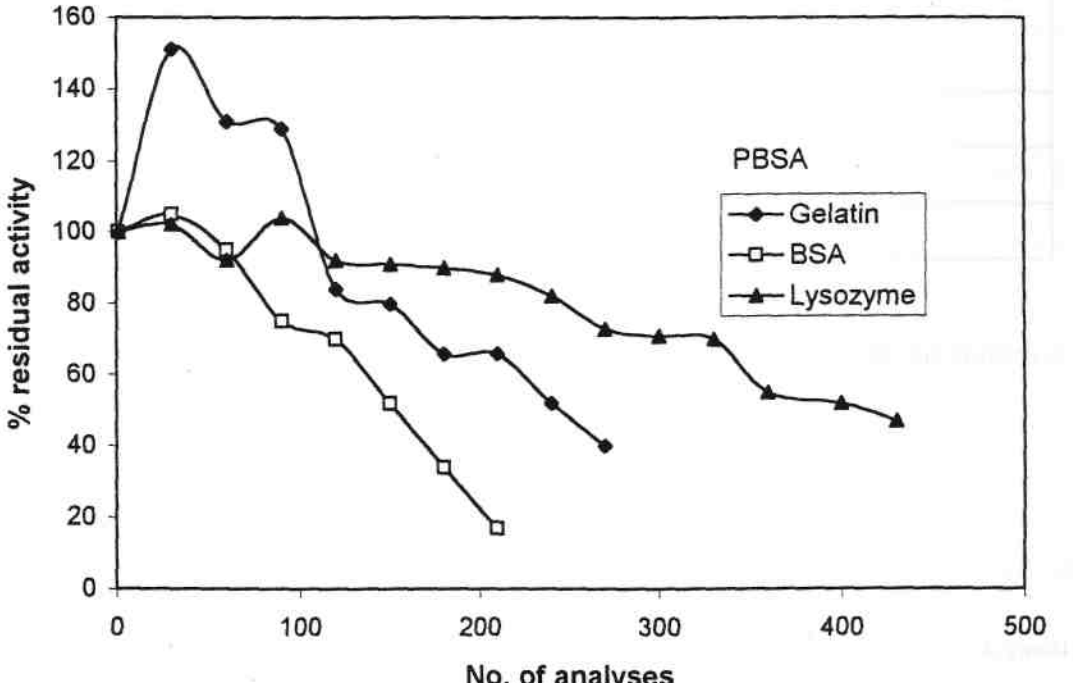


Figure 5.2.1.1 Residual activity after repeated sucrose (10 %) analysis with biosensor using GOD immobilized with different PBSA at pH 6.0.

enzymes may lead to loss of overall activity. The inactivation of the multienzyme membrane is probably owing to the inactivation of the invertase at pH 7.0, as discussed in section 5.2.2.

Table 5.2.1.1 Comparison of % activity retained after repeated analysis of sucrose with different stabilizing agents

PBSA	% activity after No. of analysis for 10 % sucrose				No of analyses (days of use) possible with 50 % activity retention.
	120	210	300	400	
Gelatin	84	67	--	--	250(30)
BSA	70	17	--	--	150(25)
Lysozyme	92	88	71	52	400(40)

An important significance of the notable increase in stability in our studies is that it has been achieved at relatively high analyte concentrations. Although, it has been claimed (Guilbault and Montalvo. (1970): Matsumoto et al., (1988): Xu et al., (1989): Mohammed et al., (1987)) that higher operational stability (500-1000 analyses) of the single and multienzyme systems for the estimation of glucose and sucrose respectively has been obtained, the concentrations of the substrates employed for the quantification of the operational stability were very low. Carr and Bowers (1980) reported that the concentration of substrate employed is a decisive factor for operational stability of the biosensor, lower substrate concentrations of substrate giving higher operational stability and vice versa. In most of the reported studies, operational stability of the single/multienzyme membrane was quantified by injecting relatively very low concentrations of the glucose and sucrose solution (1-5 mM). On the other hand, in our studies, we have employed much higher substrate concentrations of 160 mM (3 %) for

glucose and 290 mM (10 %) for sucrose to quantify the operational stability of single and multienzyme system respectively, since our aim is towards application in food and fermentation samples, wherein higher concentrations of glucose and sucrose solutions are expected in actual use. At the low substrate concentrations, incorporation of lysozyme as PBSA is expected to give much higher operational life.

5.2.2 Thermal stability of the immobilized multienzyme system.

Figure 5.2.2.1 demonstrates the thermal stability behaviour of the soluble and immobilized multienzyme system with various PBSAs. Overall activity of the multienzyme system was measured by using sucrose as substrate. Thermal stability of multienzyme system was carried out by measuring the activity at 27±1 °C before and after incubation at desired temperature values. As shown in the figure, while the soluble multienzyme system has lost 50 % activity at 43.5 °C, when immobilized with PBSAs, a considerable increase in thermal stability was observed. With BSA, gelatin and lysozyme the transition temperature, Tm at which 50 % activity loss took place was found to be 53, 53.5 and 64°C respectively (see table 5.2.2.1). As explained for the single enzyme (GOD) system, this increase in stability for the multienzyme system immobilized with various PBSAs can be attributed to the glutaraldehyde crosslinkages between the enzymes and PBSA as also the physicochemical properties of PBSA. In the case of the single enzyme system as mentioned earlier, ionic interactions between lysozyme and glucose oxidase appear to play a crucial role in the thermal stabilization of soluble and immobilized GOD. Although, we have observed during the studies on thermal stability of multienzyme system that there is no ionic interactions between the multienzyme system and lysozyme, increase in the thermal stability observed in the case of the multienzyme system with

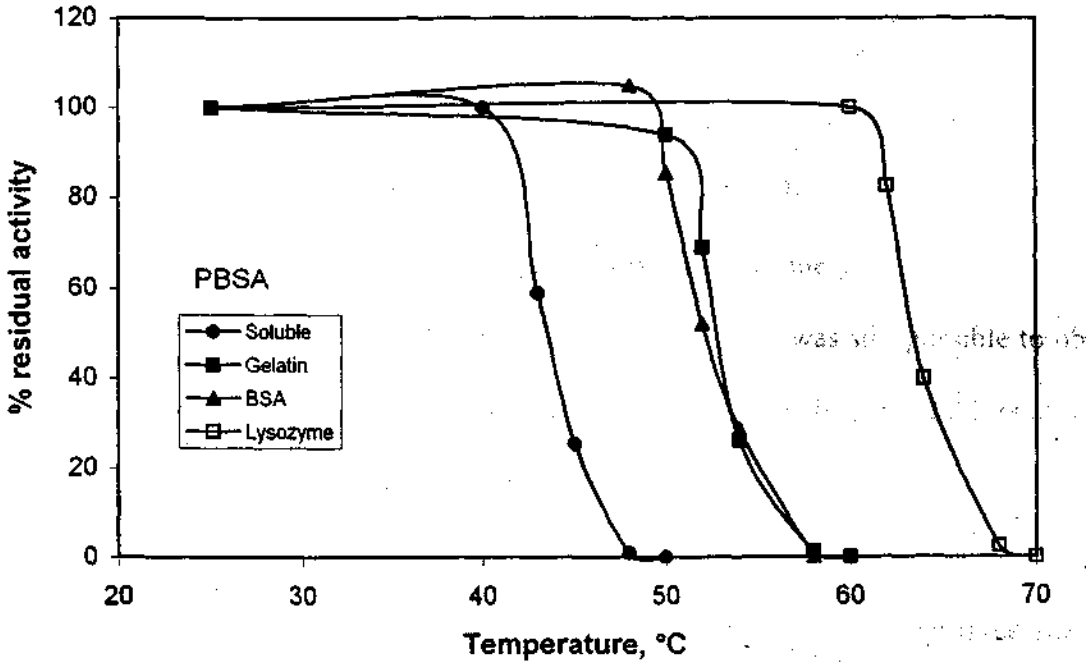


Figure 5.2.2.1 Thermal inactivation behaviour of soluble and immobilized multienzyme system with various PBSAs at pH 7.0.

lysozyme as the PBSA is probably owing to the higher thermal stability of lysozyme itself compared to BSA and gelatin.

As shown earlier in table 5.1.2.1, GOD alone immobilized with gelatin, BSA and lysozyme at pH 7.0 showed transition temperatures Tm of 52.3, 58.2 and 74.6°C respectively, which are considerably higher than the Tm values observed by us for the three enzyme system, particularly with BSA and lysozyme as PBSA. In order to pinpoint which of the three enzymes may be causing the reduced thermal stability of the multienzyme system in comparison with single enzyme GOD, we carried out the thermal stability studies on the bienzyme system (invertase and GOD; mutarotase and GOD) immobilized with various PBSAs. Though the combined enzyme action of invertase and GOD on sucrose was low due to the absence of mutarotase, it was still possible to obtain a quantifiable signal from the electrode. The results shown in figure 5.2.2.2 (also table 5.2.2.1), indicate that the transition temperature, Tm of the bienzyme system (invertase and GOD) immobilized with BSA gelatin and lysozyme were 53, 55 and 63°C respectively, significantly very close to the corresponding values for the three enzyme system observed earlier. However, for the bienzyme system (mutarotase and GOD) immobilized with gelatin, BSA and lysozyme, Tm values were 54, 59.5 and 66.5°C respectively, (as shown in figure 5.2.2.3 table 5.2.2.1) which are closer to those of GOD alone and indicate an appreciably higher thermal stability. Thus from the observed thermal stability behaviour of single- (GOD), bi- (invertase and GOD; mutarotase and GOD) and three enzyme (invertase, mutarotase and GOD) systems, it can be inferred that it is the stability of the invertase that is crucial for the thermal stabilization of multienzyme system. It is quite possible that by stabilizing the invertase against

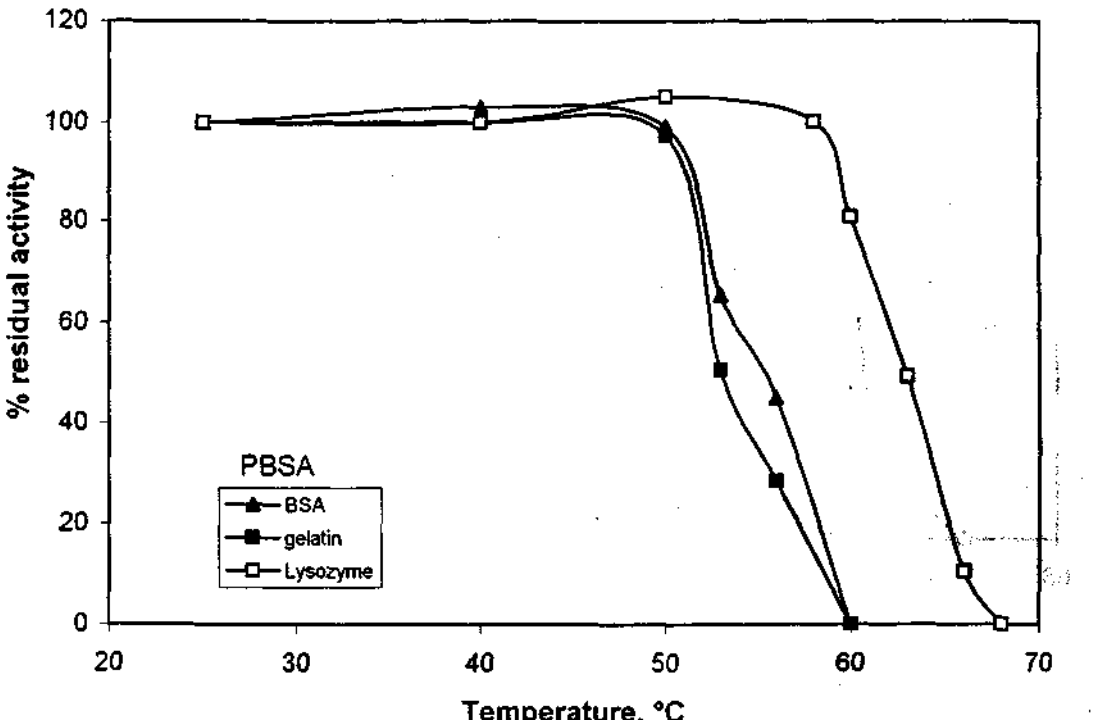


Figure 5.2.2.2 Thermal inactivation behaviour of the bienzyme system (invertase and GOD) immobilized with various PBSAs at pH 7.0.

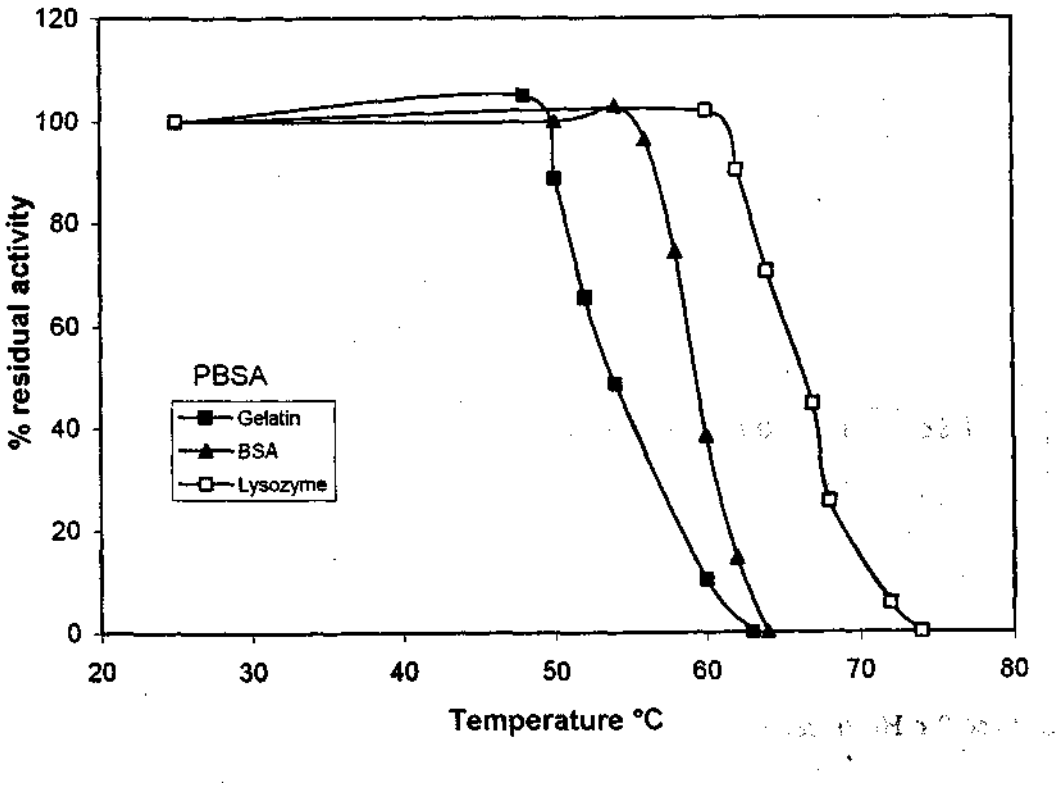


Figure 5.2.2.3 Thermal inactivation behaviour of the bienzyme system (mutarotase and GOD) immobilized with various PBSAs at pH 7.0

temperature, thermal stability of the multienzyme system can be increased further. As a support for this statement (see table 5.2.2.1) that the stability of invertase immobilized with lysozyme is remarkably better than with BSA and gelatin and thus the stability of multienzyme system. These results clearly indicate that the stability of multienzyme system for sucrose analysis can be enhanced by improving the stability of invertase.

Table 5.2.2.1 Transition temperature, Tm values for the three enzyme system and two bienzyme systems immobilized with various PBSAs.

PBSA	Three enzyme system	Bienzyme system (Invertase &GOD)	Bienzyme system (Mutarotase & GOD)	Single enzyme GOD
Gelatin	53.5	55.0	54.0	52.3
BSA	53.0	53.0	59.5	58.2
Lysozyme	64.0	63.0	66.5	74.6

Though a relatively high thermal stability of soluble invertase at pH 5.0 has been reported, it significantly decreased as the pH increased from 5.0 to 7.0 (Wang et al., 1996). Since the optimum pH for the multienzyme system immobilized with various PBSAs is in the range of 6.2-7.2, thermal stability of invertase around this pH is crucial for the stabilization of multienzyme system for better usage.

Our observations during these investigations indicate that by selecting a suitable PBSA, the thermal stability of the single and multienzyme systems can be considerably enhanced for their better biotechnological applications. Another advantage of using

lysozyme as PBSA is that although it is an enzyme, its reaction products will not interfere with the biochemical and electrochemical reactions at the electrode.

5.2.3 pH stability of the immobilized multienzyme system.

Figure 5.2.3.1 shows the pH stability of soluble and immobilized multienzyme system with various PBSAs. These studies were carried out by measuring the activity in 50 mM phosphate buffer pH 7.0 before and after treatment with desired pH values for 30 minutes at 27±1 °C. Activity at pH 7.0 is considered as 100 %. As seen in the figure, as the pH increased from 1.5 to 5, the stability of immobilized multienzyme system increased. A sharp decrease in the activity of the soluble and immobilized multienzyme system below pH 4.0 can be attributed to the adverse effect of lower pH values on the GOD. As mentioned in the pH stability studies on GOD alone, at the lower pH values, GOD probably loses its activity due to leaching of the tightly bound FAD to the enzyme molecule. Above pH 7.0 soluble multienzyme system shows a steep and complete inactivation at pH 10. Corresponding behaviour of the immobilized multienzyme preparations showed much better stability.

5.3 Chapter summary.

It has been possible to significantly improve the stability of the immobilized enzymes by using a non-conventional protein based stabilizing agent like lysozyme, which is crucial for the biosensor application. Increase in the stability of single and multienzyme system immobilized with lysozyme was attributed to the ionic interactions (with GOD) and inherent stability of lysozyme when compared to the BSA and gelatin. By selecting suitable protein based stabilizing agents, enzymes can be stabilized for their more profitable use. Eventhough lysozyme is an enzyme, its reaction products will

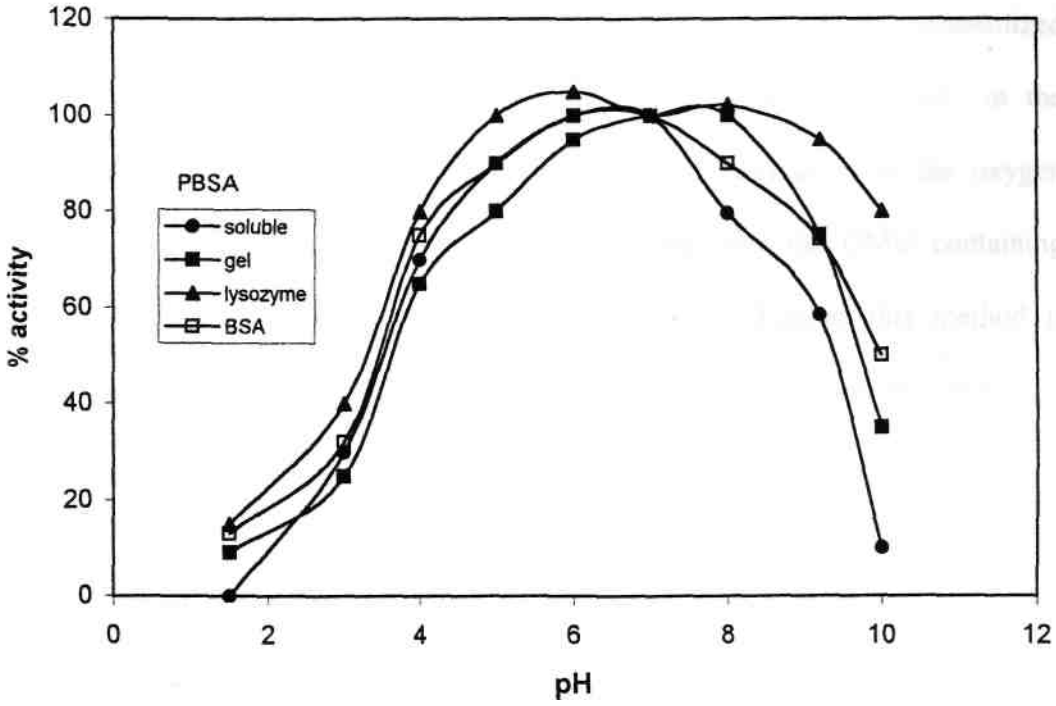


Figure 5.2.3.1 pH stability of soluble and immobilized multienzyme system with different PBSAs.

not interfere with the biochemical reactions of the single and multienzyme system (Bergmeyer. 1974) at the oxygen electrode (Scheller and Schubert. 1992). A further advantage could be that since lysozyme is an antimicrobial agent, it may also help to avoid microbial contamination of immobilized enzyme preparations, thus giving a better durability of the enzyme membrane.

It has been possible to use a simple amperometric principle based oxygen electrode biosensor for the fundamental stability investigations on the immobilized single and multienzyme system, which overcomes the constraints involved in the biochemical investigations of the immobilized enzymes. Deactivation of the oxygen electrode due to direct heating was avoided by exposing only the DMU containing immobilized enzyme membrane to the desired temperature. Further this method is expected to be applicable to study the stability of other immobilized enzymes, catalyzing reactions involving consumption of oxygen.

Chapter 6

Thermal inactivation of GOD: mechanism and stabilization using additives

THERMAL INACTIVATION OF GLUCOSE OXIDASE: MECHANISM AND STABILIZATION USING ADDITIVES

Glucose oxidase (GOD) from *A. niger* is a flavoprotein (β-D-glucose: oxygen-oxidoreductase, EC 1.1.3.4) which catalyses the oxidation of β-D-glucose by molecular oxygen to D-glucono-δ-lactone and hydrogen peroxide. This protein is a dimer (molecular weight 160 Kda) containing two identical subunits (Tsuge et al., 1975). The dimer contains two disulfide bonds and two free sulfhydryl groups (Tsuge et al., 1975) and two FADs tightly bound to the enzyme (Swoboda. 1965). GOD is an industrially important enzyme widely used in food processing and gluconic acid production (Róhr et al., 1983). It is also used in biosensors for medical applications to quantify the glucose in blood, food and fermentation industry (Turner et al., 1987) and environmental monitoring (Gouda et al., 1997). For practical applications, the stability of the enzyme is important and often a limiting factor. In order to increase the stability of GOD against thermal inactivation, use of additives like various salts, mono- and poly hydric alcohols and polyelectrolytes has been reported (Ye et al., 1988; Appleton et al., 1997). The effectiveness of stabilization by incorporation of additives would depend on the nature of enzyme, its hydrophobic/hydrophilic character and the degree of its interaction with the additives (Ye et al., 1988). Ye and Combes (1988) reported aggregation as the main cause for inactivation of glucose oxidase, which can be prevented by modifying the microenvironment of the enzyme. In chapter 5, it has been pointed out that the thermal stability of GOD at 60°C can be increased by incorporating lysozyme as an additive during immobilization. The role played by the complimentarity of surface between the enzyme and lysozyme appears to be crucial in the stabilization of GOD. Though several

attempts have been reportedly made to improve the stability of GOD (Ye et al., 1988; Ye and Combes. 1988; Appleton et al., 1997), information on the mechanism of thermal inactivation is not available. Furthermore, most of the thermal stability studies on GOD in the presence of additives are limited to measuring the residual activity after exposure to various temperatures for a specific time. In this context, an attempt at a better understanding of the thermal inactivation mechanism of GOD would be appropriate which can help enhance the thermal stability using appropriate additives. With this objective, experiments have been carried out on the effect of some selected additives on the thermal stability of GOD. In addition to lysozyme which was observed earlier by us to increase the stability of GOD, two other salts viz., NaCl and K2SO4 which are commonly reported to stabilize enzymes through ionic and hydrophobic interactions respectively, have been selected for the thermal stability studies reported in this work.

In order to assess the effect of additives on protein stability, the residual activity of the enzyme was measured as a function of time and kinetic constants were determined as reported by Devi and Rao (1998). Arrhenius plots were constructed and thermodynamic parameters such as free energy (ΔG*), enthalpy (ΔH*) and entropy (ΔS*) were calculated. These thermodynamic parameters, are useful to have a better understanding of the forces responsible for the thermal inactivation of enzyme and mechanism of enzyme stabilization by additives. In order to understand the role of oxidation of-SH group in the thermal stability of GOD, experiments were carried out at 60°C in the presence of β-mercapto ethanol (β-ME) and N-ethylmaleimide (NEM) separately as well as together. Along with these data, circular dichroism (CD) and SDS-

PAGE measurements were also used to understand the thermal inactivation mechanism and the protein stabilization by additives.

6.1 Thermal Inactivation Measurements.

Kinetics of thermal inactivation of GOD was studied at different temperatures, both in the absence and presence of various additives as follows. 100 µl (lmg) of enzyme solution was added to 0.9 ml of 20 mM phosphate buffer, pH 6.0 and kept in a constant temperature bath at desired temperatures. 10 µl (10 µg = 2 IU) of enzyme solution samples were withdrawn at periodic intervals, immediately cooled in ice bath prior to assay and the residual activity was measured and expressed as a percentage of initial activity at various time intervals. From a semilog plot of residual activity versus time, the inactivation rate constants *(k_r)* were calculated (from the slopes) and apparent half lives were estimated. **6.1.1 Activation energy calculations.**

Thermal stability of GOD in presence and absence of various specified additives was determined in terms of the inactivation rate constant *(k_r)* as a function of temperature in the range of 56-67°C. The temperature dependence of k_r was analysed according to Arrhenius plot (natural logarithm of k_r versus reciprocal of absolute temperature) and the activation energy (E_a) was obtained from the slope of the this plot. Activation enthalpy ΔH* was calculated according to the equation.

$$\Delta H^* = E_a - RT \qquad (6.1)$$

where R = universal gas constant; T is absolute temperature The values for free energy of inactivation (ΔG*) at different temperatures were calculated from the thermodynamic equation .

$$\Delta G^* = -RT\ln(K_r h/kT) \quad (6.2)$$

where *h* is the Planck's constant and *k* is the Boltzmann constant.

Activation entropy ΔS* was calculated as

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (6.3)$$

6.2 Thermal inactivation kinetics of GOD in the presence and absence of additives.

In order to avoid the interference of traces of catalase, (which is commonly present in the commercial preparation of GOD) during thermal inactivation studies, CD and SDS-PAGE measurements, commercially available GOD was purified as follows. Commercially available GOD was purified by passing through a G-250 column to remove traces of catalase present. 2 mL fractions were collected and then the protein concentration in each fraction was quantified by measuring OD at 280 nm. The GOD containing fractions were pooled, lyophilized and desiccated at 4°C. Figure 6.2.1 shows the native-PAGE of the purified and commercial preparation of GOD.

6.2.1 Thermal inactivation Kinetics of GOD alone.

Thermal inactivation kinetics of native GOD carried out in the temperature range 57-67°C in 20 mM phosphate buffer, pH 6.0 is plotted in figure 6.2.1.1 in terms of residual activity as a function of time at fixed temperatures. At all the temperature studied, the inactivation is observed to follow an exponential decay, indicating that the inactivation of GOD followed first order kinetics. The Arrhenius plot shown in figure 6.2.2.4 (corresponding to line with no additive) was linear in the temperature range studied. From this plot and making use of equations 6.1-6.3, the activation parameters

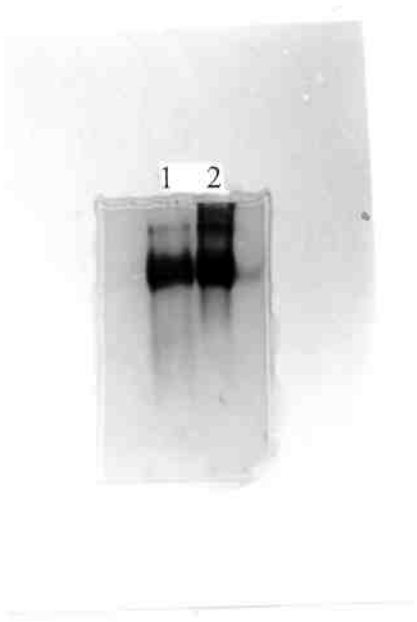


Figure 6.2.1. Native-PAGE of purified (lanel) and commercially available GOD (Lane2).

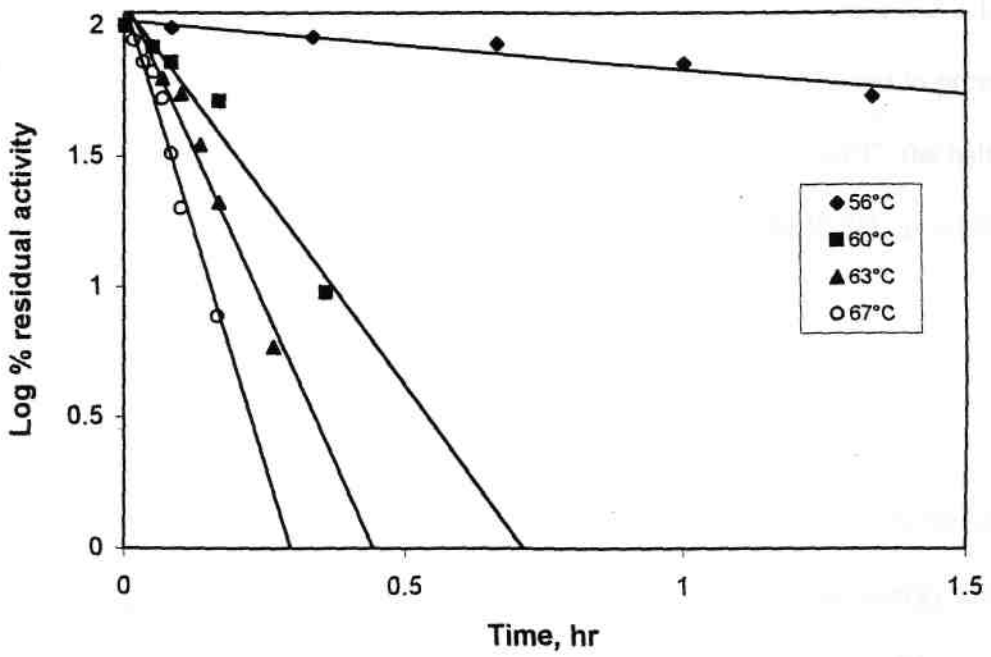


Figure 6.2.1.1 Thermal inactivation of GOD in 20 mM phosphate buffer, pH 6.0 at various temperatures.

viz., free energy of activation (ΔG*), enthalpy of activation (ΔH*), entropy of activation (ΔS*) are calculated and given in table 6.2.2.1.

6.2.2 Effect of additives on the thermal stability of GOD.

The effect of various additives viz., lysozyme, NaCl and K_2SO_4 on the thermal stability of the GOD was followed by measuring the residual activity with time. The semilog plot shown in figure 6.2.2.1-6.2.2.3 indicates first order thermal inactivation kinetics in all the cases. The activation parameters calculated through the Arrhenius plot (figure 6.2.2.4) in the presence and absence of additives are given in table 6.2.2.1. As shown in the table, at all the temperatures studied, half life of GOD increased in presence of each of the additives used. Taking a typical case, at a temperature of 60°C, the half life increased by 3, 33 and 23-fold with 7.1x $l0^{-4}$M, 1 M NaCl and 0.2 M K_2S0_4 as additives respectively. Correspondingly, at the same temperature the activation energy of GOD increased from 30 to 35, 38 and 44 Kcal mol^{-1}; activation entropy increased from 25 to 33, 41 and 61 cal deg^{-1} mol^{-1} in the presence of lysozyme, NaCl and K2SO4 respectively. Increase in half life, activation energy and activational entropy in the presence of additives indicates the increased thermal stability of GOD. The net free energy change ΔG* at 60°C was 0.89, 2.38 and 2.11 Kcal mol^{-1} respectively in presence of lysozyme, NaCl and K2SO4. The magnitude of standard free energy of activation reflects the effectiveness of relative stabilization by various additives. The difference in the slopes (activation energy) of Arrhenius plot (figure 6.2.4.4), in presence of the lysozyme, NaCl and the K2SO4 indicates the difference in mechanism of enzyme stabilization. Significant increase in activation energy, E_a (44.39 Kcal/mole) in the presence of only 0.2 M K_2SO_4

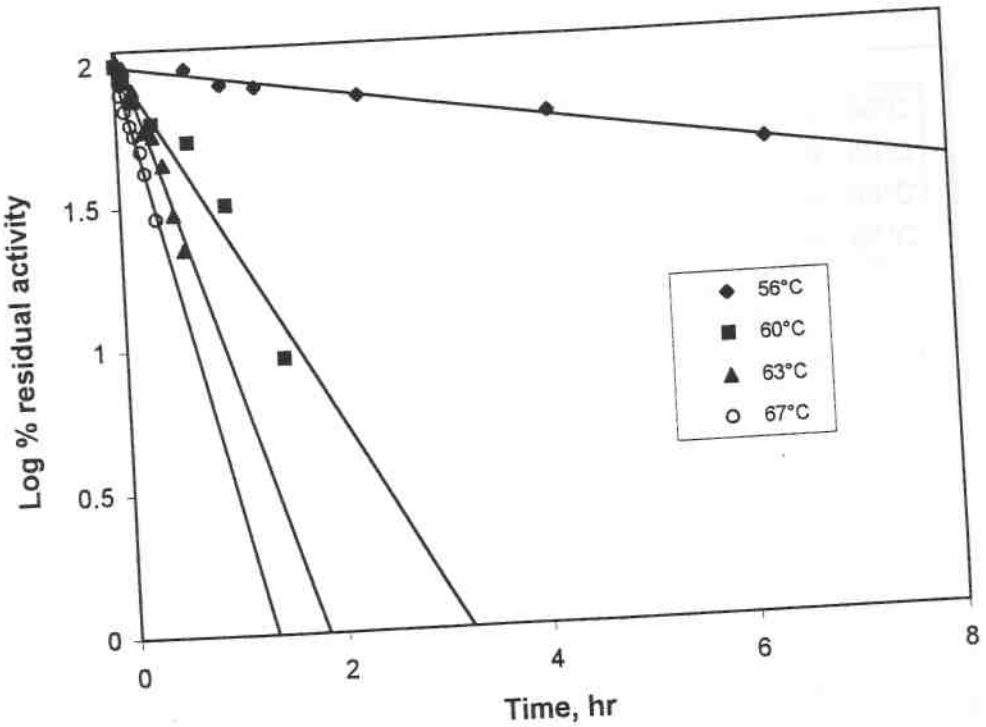
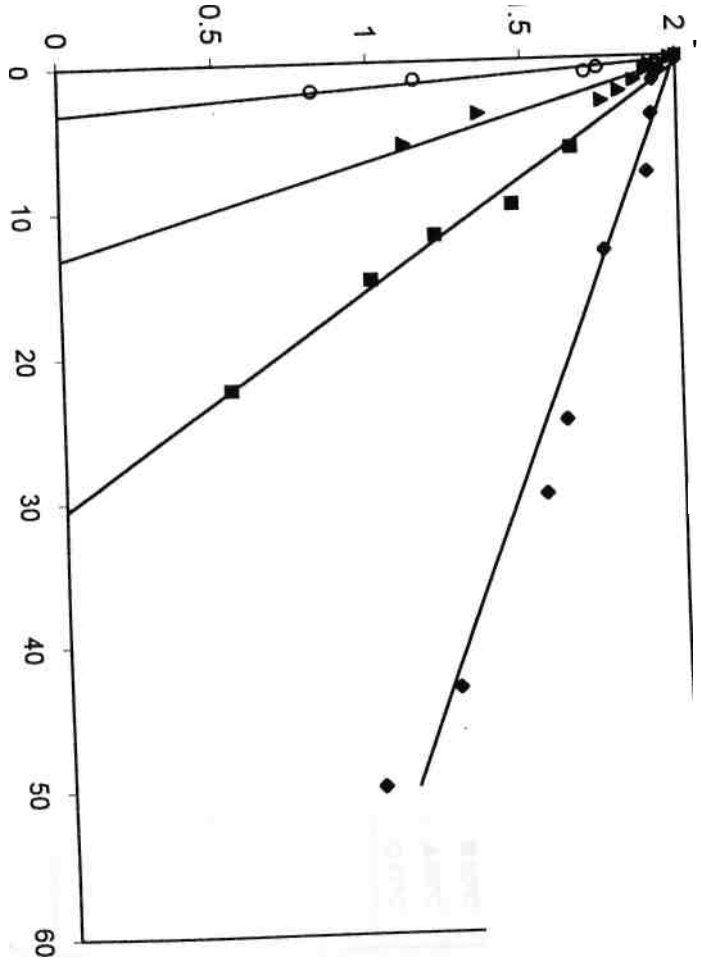


Figure 6.2.2.1 Thermal inactivation of GOD in presence of 7.1 x 10^{-4} M tysozyme

Log % residual activity



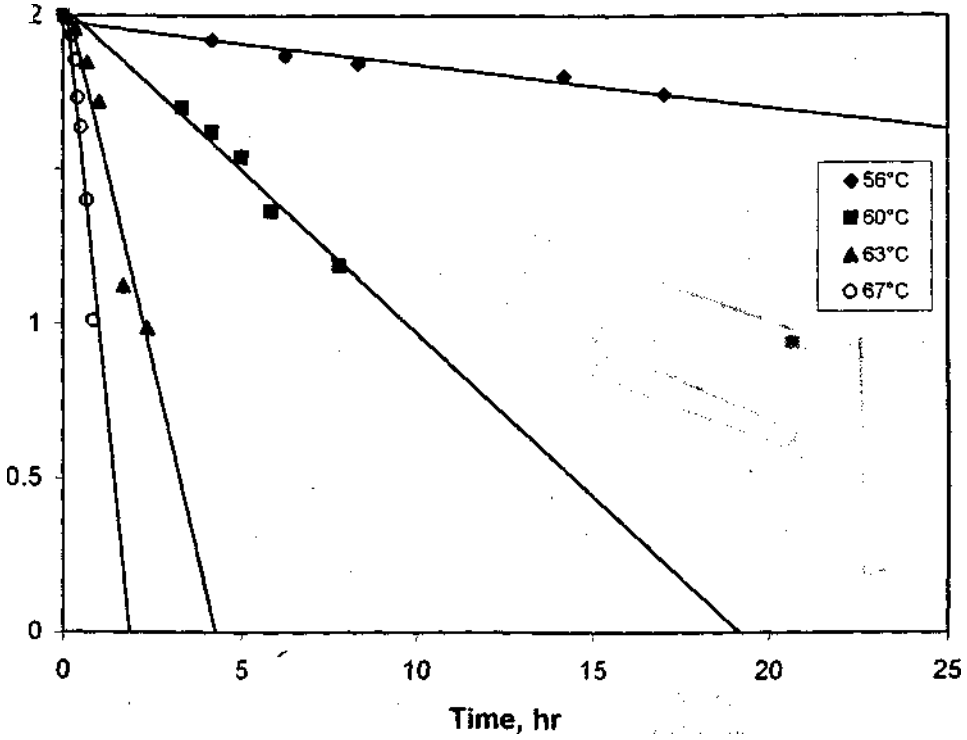


Figure 6.2.2.3 Thermal inactivation of GOD in presence of 0.2 M K_2S0_4.

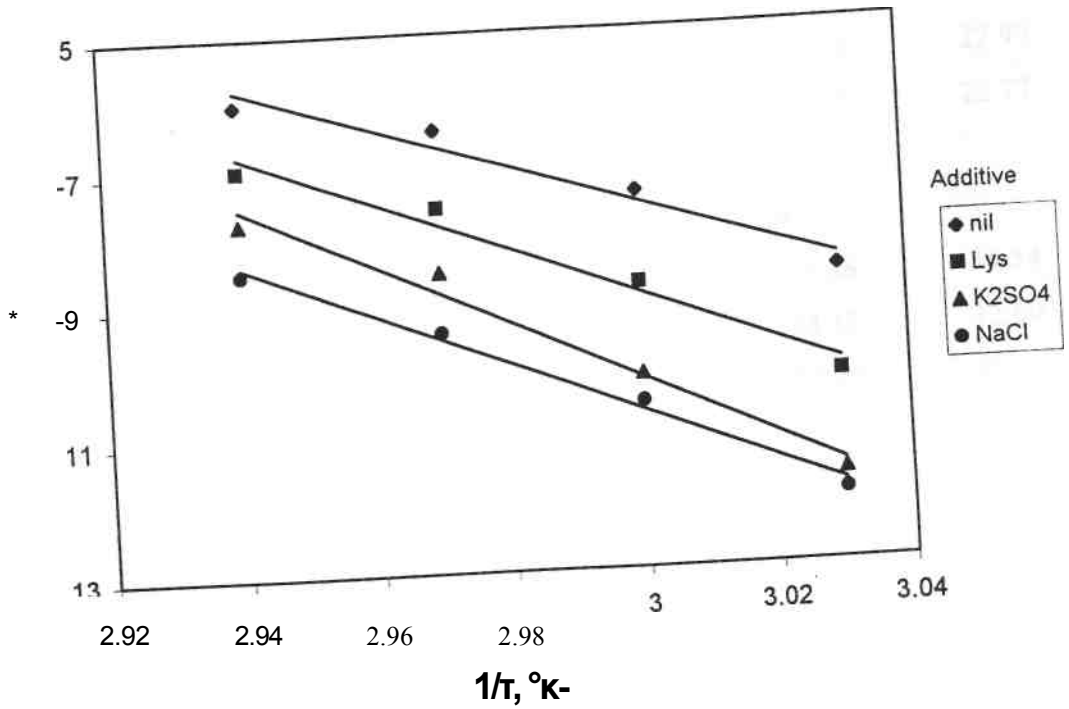


Figure 6.2.2.4 Arrhenius plot of thermal inactivation of GOD in the Presence of various additives.

Table 6.2.2.1 Activation parameters of GOD in the presence of additives.

Incubation temp. (°C)	Half-life (min)	Inactivation rate constant, (itrxioV)	ΔG* (Kcalmol^{1})	ΔH* (Kcalmol^{1})	ΔS* (car^{1} dcj; mo|1)
		GOD alone			
56	86	1.35	21.89	30.19	25.23
60	13	9.6	22.60	30.18	22.77
63	7.5	16	22.45	30.18	22.99
67	4.5	24.9	22.43	30.17	22.77
		E_a = 30.85 Kcalmol-^{1} GOD in presence of 7.1 x lO^M lysozyme			
56	322	0.36	22.68	34.38	35.54
60	46	2.5	23.49	34.37	32.69
63	24	5.0	23.22	37.34	33.11
67	12	9.5	23.08	34.36	33.19
		E_a = 35.04 Kcalmol-^{1} GOD in presence of 1 M NaCl			
56	1806	0.064	23.74	37.47	41.73
60	434	0.26	24.98	37.51	41.36
63	146	0.79	24.45	37.45	38.69
67	58	2.0	24.13	37.45	39.17
		E_a = 38.2 Kcalmol-^{1} GOD in presence of 0.2 M K_2S0_4			
56	1446	0.08	23.60	43.74	61.23
60	308	0.39	24.71	43.73	57.12
63	62	1.90	23.87	43.73	59.11
67	27.5	4.30	23.61	43.72	59.15
		E_a = 44.39 Kcal mol-^{1}			

compared to 1 M NaCl (38.2 Kcal/mole) and 7.1 x 10^{-4} lysozyme (35.04 Kcal/mole), indicates the stabilization of GOD by K_2S0_4 is of conformational origin through hydrophobic interaction. Stabilization of GOD (in terms of increased half life and activation parameters) by NaCl and lysozyme in acidic pH values indicates the role of ionic interaction between GOD and lysozyme or NaCl. However, activation energy, i.e., the energy required to denature the enzyme is higher in the presence of 0.2 M K_2S0_4 when compared to 1 M NaCl and 7.1x 10^{-4} lysozyme. This indicates that the hydrophobic interactions play a more predominant role in the stabilization of GOD than ionic interactions in this case. The relatively small value of ΔG^* (22.6 Kcal) for GOD at 60°C indicates the labile nature of enzyme. Over the temperature range studied the increase in the value of activation parameters, ΔG^* and ΔH^* in the presence of additives indicates the increased thermal stability. The change in the activation entropy, ΔS^* in the presence of additives can be explained in terms of an enhancement of the order and compactness of the structure, thus favouring intramolecular stabilizing force and consequently increasing the stability of enzyme. Significant change in the activation entropy and difference in the slopes of the Arrhenius plots in the presence of K2SO4, indicates the stabilization of GOD is conformational origin.

6.2.3 Effect of lysozyme concentration on the stability of GOD.

Since lysozyme was observed earlier to enhance the stability of immobilized GOD, more detailed studies on the thermal inactivation of GOD were carried out in the presence of various concentrations of lysozyme at 60°C in 20 mM phosphate buffer, pH 6.0. As the mole ratio of lysozyme to GOD increased from 0-110, half-life of GOD increased from 13 to 46 minutes (figure 6.2.3.1), thereafter showing no further

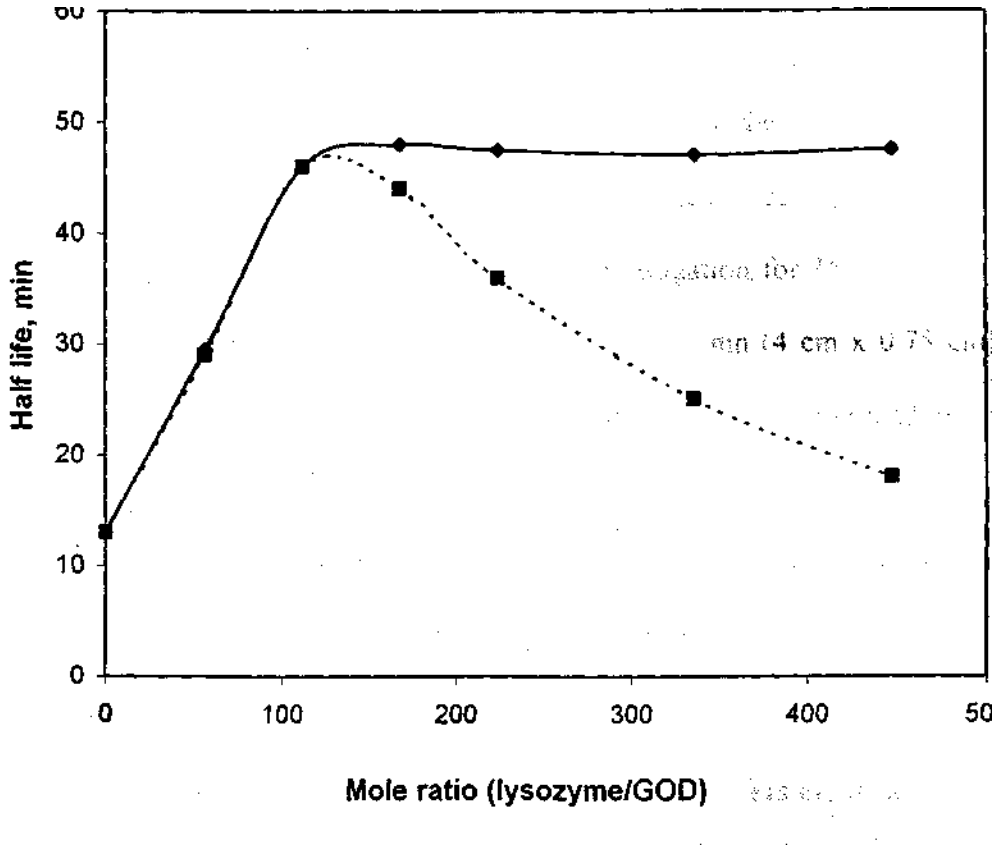


Figure 6.2.3.1 Effect of mole ratio of lysozyme to GOD on the half-life of GOD at 60°C in 20 mM phosphate buffer, pH 6.0. (■ ..... •) without coagulent removal and (♦—») coagulent removed by centrifugation-

improvement in the thermal stability of GOD. The requirement of a relatively high mole ratio (55-110) of lysozyme to stabilize the GOD suggests that the interaction between lysozyme and GOD is non-specific. Increased stability of GOD in the presence of lysozyme in the acidic pH range (pH 6.0) confirms the ionic interactions between lysozyme and GOD. A significant observation as seen in figure 6.2.3.1 was that in the presence of higher concentration of lysozyme (above 150 mole ratio) the half life of GOD decreased (seen as dotted line). This is due to the coagulation of lysozyme observed at higher concentrations. In order to avoid the interference of the coagulated lysozyme during residual activity measurement, after exposing to 60°C for the specified time, samples were withdrawn, coagulant separated by centrifugation for 15 minutes at 4000 rpm, and the supernatant was passed through the G-75 column (4 cm x 0.75 cm). The eluted sample was used to measure the residual activity of GOD. This procedure ensured that the half life did not decrease after reaching the maximum. Since it was observed that increasing the lysozyme to GOD mole ratio above 110 gave no further improvement in the thermal stability of GOD, the lysozyme concentration corresponding to this mole ratio was used for thermal inactivation studies.

6.2.4 Effect of lysozyme incorporation on the kinetic parameters of GOD.

In order to better understand the stabilization of GOD by lysozyme, kinetic parameters viz., Michaelis-Menten constant, K_M, and maximum reaction velocity, V_{max} were determined in the presence as well as absence of lysozyme. As shown in figure 6.2.4.1 and table 6.2.4.1, as the concentration of lysozyme increased from 0 to 1.1 mM, K_m and V_{max} of GOD for β-D-glucose increased from 7.46 to 36.4 mM and 0.33 to

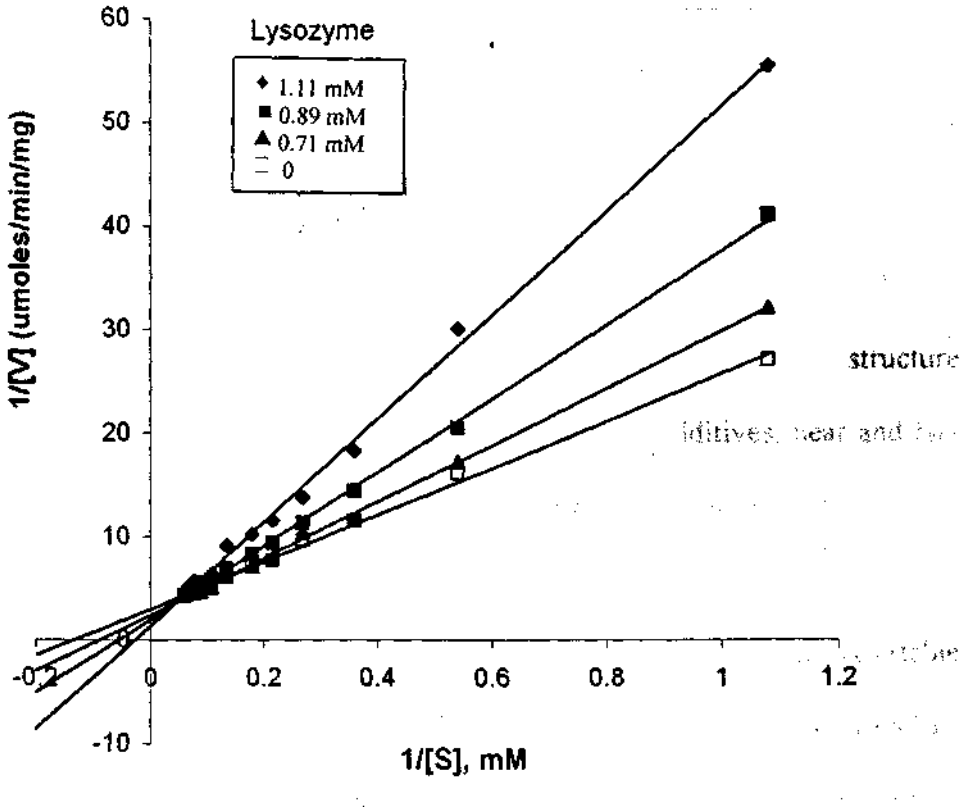


Figure 6.2.4,1 Lineweaver Burk plot determining the effect of lysozyme concentration on the kinetic parameters of GOD.

0.72 mM min-[1] respectively, which indicates the decreased affinity of GOD for β-D-glucose in the presence of lysozyme.

Table 6.2.4.1 Effect of lysozyme on K_m and V_{max} of GOD.

Concentration Of lysozyme (mM)	K_m (mM)	V_{max} (mM min^{-1})
0	7.46	0.33
0.71	11.28	0.41
0.89	17.78	0.50
1.11	36.4	0.73

6.3 Circular dichroism (CD) measurements.

In order to quantify the structural changes like tertiary and secondary structure during thermal inactivation of GOD and the stabilizing effect of additives, near and far-UV region CD spectra of the native enzyme before and after inactivation at 60°C for 15 minutes in the absence and presence of 0.2 M K2SO4 were measured and are shown in figures 6.3.1 - 6.3.4. Since the half life of GOD at 60°C in the absence of additives (table 6.2.2.1) is 13 minutes, in order to know the conformational changes of enzyme at its half life and stabilizing effect of K2SO4, thermal inactivation of GOD was carried out for 15 minutes at 60°C in the presence and absence of 0.2 M K2SO4. As shown in the figure 6.3.1, it appears that during thermal inactivation of GOD, the enzyme structure is unfolded as indicated from a significant loss in the tertiary structure. This unfolding of GOD was prevented by the addition of 0.2 M K_2S0_4 (figure 6.3.2) which indicates that the stabilization role of K2SO4 arises through its influence on the tertiary structure of enzyme. Further, under similar conditions, the far-UV region CD spectra, shown in figure 6.3.3 indicates that the α-helix (secondary structure) of the native protein was lost

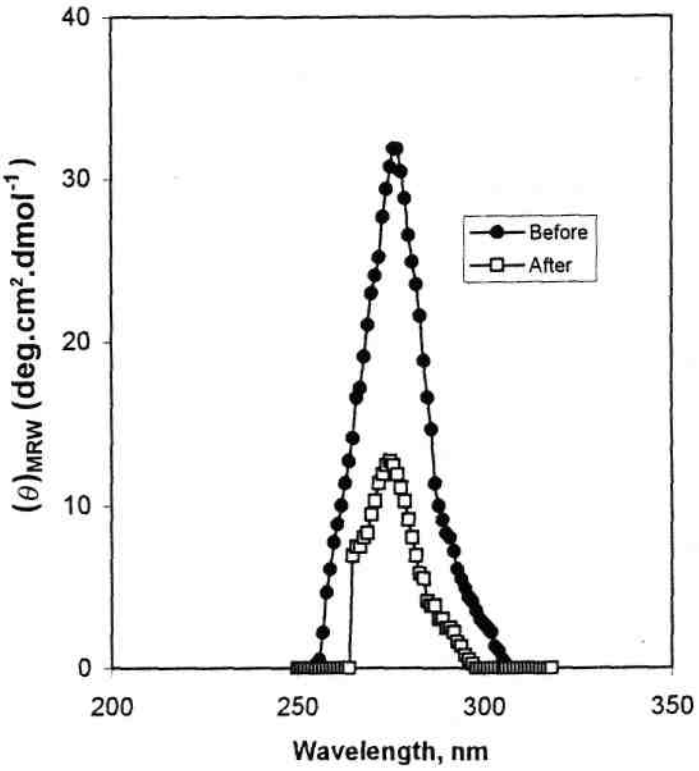


Figure 6.3.1 Near UV CD spectra of GOD before and after thermal inactivation for 15 minutes at 60°C.

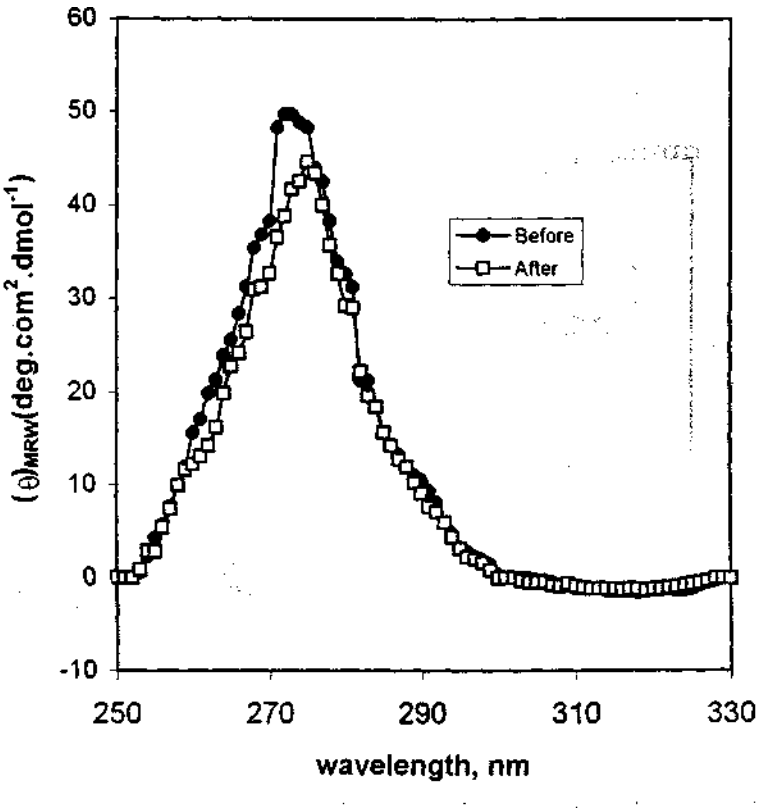


Figure 6.3.2. Near UV CD Spectra of GOD before and after thermal inactivation for 15 minutes at 60°C in presence of 0.2 M K_2S0_4.

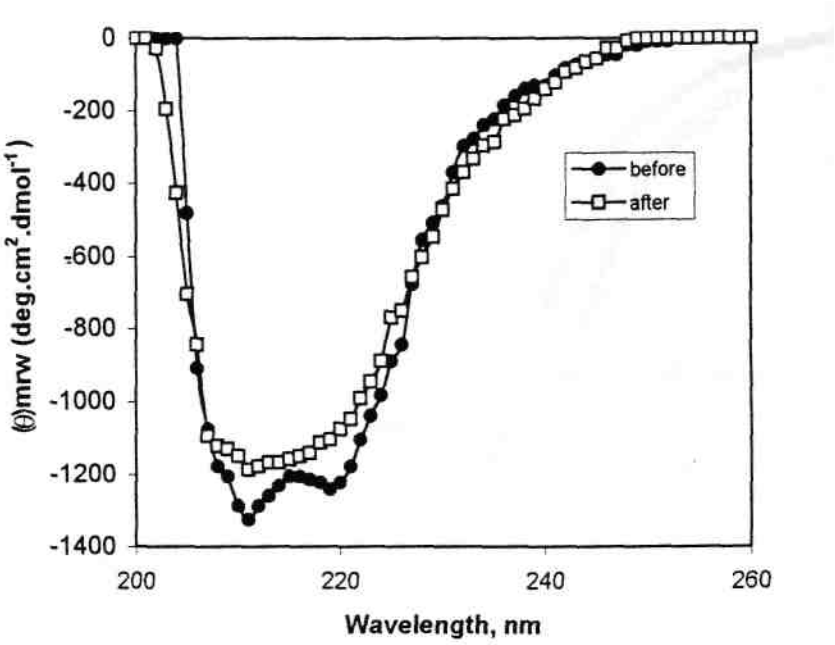


Figure 6.3.3 Far UV CD spectra of GOD before and after thermal inactivation for 15 minutes at 60°C.

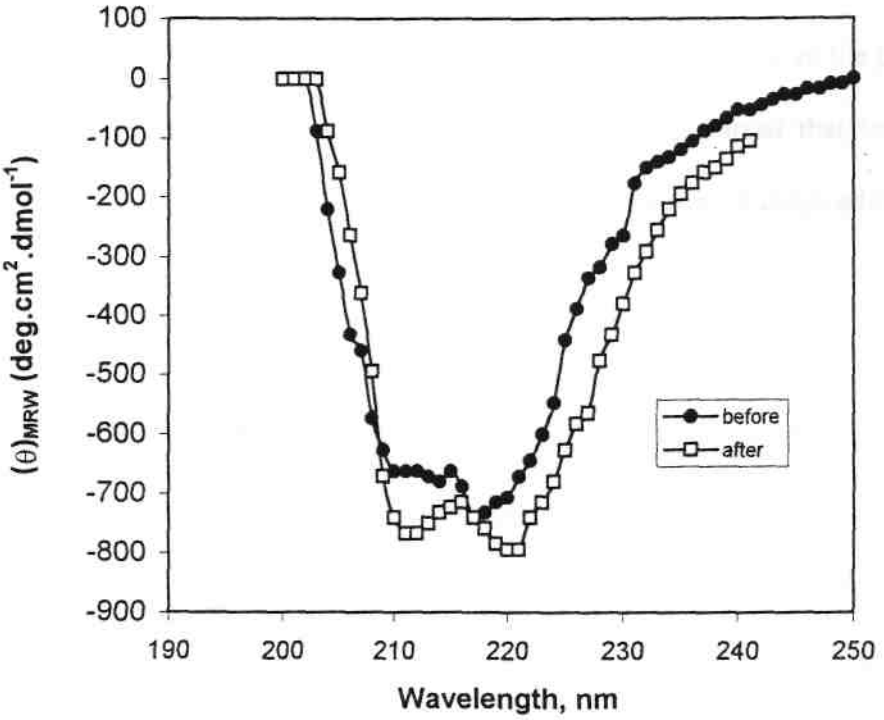


Figure 6.3.4 Far-UV CD Spectra of GOD before and after thermal inactivation for 15 minutes at 60°C in presence of 0.2 M K_2S0_4.

(on heat treatment at 60°C) as evidenced from the loss of peak at 208 nm. However, as seen from figure 6.3.4 in the presence of K_2S0_4 this peak is retained, indicating the stabilizing effect of K_2S0_4 through the protection of secondary structure of GOD. Thus the stability of enzyme appears to be enhanced by incorporation of $K_2S\theta4$ as an additive through a mechanism involving the protection of the tertiary as well as the secondary structure (α-helix) of the protein. Though the CD studies in the presence of NaCl and lysozyme were not carried out, based on the half lives of GOD at 60°C in the presence of NaCl (434 minutes) and lysozyme (46 minutes), it can be surmized that tertiary and secondary structure of GOD may be protected in the presence of these additives after heating at 60°C for 15 minutes.

6.4 SDS-PAGE electrophoresis.

The SDS-PAGE of native GOD before and after heat inactivation (60°C) and that of heat inactivation in the presence of the three additives, β-ME, NaCl and K2SO4 are shown in figure 6.4.1. From the mobility of the enzyme, it is evident that there are higher molecular weight non-specific aggregates in the heat inactivated sample (lane 1, figure 6.4.1). However, this non-specific aggregation of enzyme during heat treatment is seen to be avoided by using β-ME (lane 3), which is indicative of the involvement of oxidation of disulphide bonds and free -SH groups in the non-specific aggregation of enzyme. The stabilizing effect of β-ME was also confirmed by measuring the residual activity of enzyme during thermal inactivation at 60°C. In presence of β-ME, half-life of GOD increased from 13 to 25 minutes, indicating that the thermal inactivation of the native enzyme involve the intermolecular cross-linkage due to sulfhydryl (-SH) group oxidation, which can be prevented by β-ME. It is known that GOD contains a

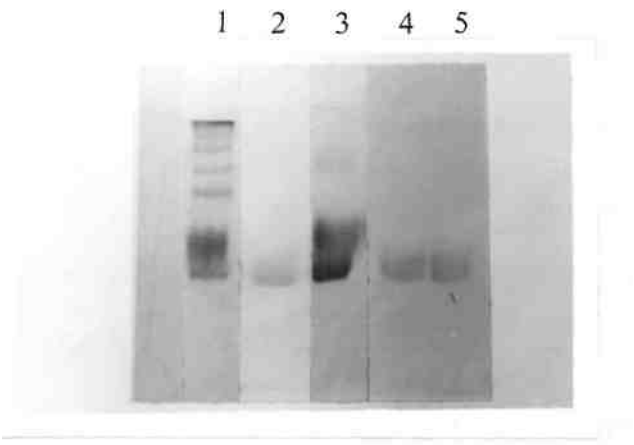


Figure 6.4.1 SDS-PAGE electrophoresis pattern of GOD before and after thermal inactivation. After heat inactivation (lane 1), native (lane 2) and heat inactivation of GOD in presence of β-ME (lane 3), NaCl (lane 4) and K_2SQ_4 (lane 5).

disulphide-bridge and a free -SH group for each of its two identical subunits (Ye and Combos. 1988). In order to know weather the free -SH or the -SH group involved in the disulphide bond formation is responsible for the inactivation of the enzyme, thermal inactivation of GOD was carried out in the presence of β-ME, NEM and both β-ME and NEM together. Increase in the half life of GOD in the presence of β-ME or NEM (25 minutes in each case) is much lower than in the presence of both β-ME and NEM together (46 minutes), indicating the importance of -SH group oxidation in the thermal inactivation of enzyme. Treatment with β-ME and NEM did not adversely influence the catalytic activity, indicating that neither the free -SH nor the -SH group involved in the disulphide bond formation is essential for the catalytic function of the enzyme. Reduction of the disulphide bond with β-ME followed by blocking of free -SH group with NEM (when both are used) leads to an increase in the thermal stability of GOD. These results, demonstrating the prevention of aggregation of GOD in the presence of β-ME and the increase in the thermal stability of GOD in the presence of both β-ME and NEM together indicate that the oxidation and reduction of-SH groups play a crucial role in the thermal inactivation and aggregation of GOD.

As shown in figure 6.4.1, non specific aggregation of enzyme can also be prevented by using salts like NaCl (lane 4) and K2SO4 (lane 5), indicating that the hydrophobic interactions of side chains during heat treatment are involved in the non specific aggregation of enzyme.

An important aim of this chapter is to throw more light on the role of lysozyme in the thermal stabilization of GOD from a basic angle through CD and SDS-PAGE measurements. However, due to the practical limitation of interference of lysozyme, in

the CD and SDS-PAGE measurements studies on the stability of GOD in the presence of lysozyme was limited only to the measurement of residual activity at various temperatures. However, based on the observed data on the half lives, kinetic and thermodynamic activation parameters in the presence of NaCl, K2SO4 and lysozyme, CD studies in the presence of K_2S0_4 and SDS-PAGE in the presence of NaCl, K_2S0_4 and β-ME, it can be inferred that the ionic interactions between lysozyme and GOD play a crucial role in the stabilization of GOD.

6.5 Chapter summary

Thermal inactivation behaviour of GOD at pH 6.0 followed a first order kinetics both in the absence and presence of the additives. Addition of lysozyme, NaCl and K_2S0_4 enhanced the half-life at 60°C by 3, 33 and 23-fold respectively. Based on the thermodynamic activation parameters, hydrophobic interactions appears to play a more predominant role than ionic interactions in the stabilization of GOD. CD and SDS-PAGE measurements, strongly indicate that thermal inactivation of GOD involves conformation changes, unfolding and non-specific aggregation of the enzyme molecule which are due to hydrophobic interactions of side chains and -SH group oxidation. Reduction of -SH group, which is not essential for the enzyme activity, by NEM, enhanced the thermal stability of GOD. Addition of lysozyme, NaCl and K2SO4 minimizes the unfolding and non-specific aggregation thus gives rise to enhanced thermal stability.

Chapter 7

Reversible denaturation of immobilized single and multienzyme system – role of lysozyme

REVERSIBLE DENATURATION OF IMMOBILIZED SINGLE AND MULTIENZYME SYSTEMS - ROLE OF LYSOZYME

In chapters 5 and 6, the rationale behind the stabilization of single and multienzyme system immobilized with various PBSAs was discussed. In this section attempts were made to explore the possibility of incorporating lysozyme during immobilization of enzymes as a renaturation tool for the denatured enzymes.

Denaturation of immobilized enzymes is a critical problem in industrial applications. A reversal of the denaturation to bring back the original activity is important from the application point of view. In the recent past several attempts have been made to facilitate protein folding in vitro by immobilization of enzymes. It has been reported that in order to prevent the autoproteolysis during refolding of reduced trypsin, the enzyme was covalently attached to the matrix. (Epstein and Anfinsen. 1962). Aggregation of trypsinogen during refolding was prevented by covalent coupling of protein to agarose beads (Sinha and Light. 1975). Furthermore, matrix assisted folding of denatured proteins was first demonstrated by Creighton (Creighton. 1985) using non-covalent immobilization on ion exchange resins. Ability to regain the functional state of the immobilized enzyme after undergoing denaturation has been known in some cases (Epstein and Anfinsen. 1962; Brown et al.,1970; Hayahi et al., 1994). In most of the cases a combination of intermolecular and intramolecular interactions responsible for the protein folding adversely affect the renaturation of immobilized enzymes (Kauzamann. 1959; Dill. 1990; Privolov and Gill. 1988). Most of the reported renaturation studies have been carried out on single protein immobilized on solid support. Trivedi et al (Trivedi et al., 1997) demonstrated the importance of heteromolecular interaction between the acidic

and basic proteins on the renaturation of lysozyme in solution. They have claimed that the renaturation of lysozyme (basic protein) was drastically affected due to the electrostatic interactions and aggregation by the addition of acidic proteins like BSA and alcohol dehydrogenase (ADH) to the renaturation buffer. In another study, the importance of electrostatic interaction between the barnase (basic protein) and chaperoning growl (acidic protein) during denaturation of barnase has been reported by Gray and Fershat (Gray and Fershat. 1993). It has been reported that aggregation of proteins during renaturation can be avoided by immobilization (Hayahi et al., 1994). While it is known that the electrostatic interactions between proteins which play a crucial role in the renaturation and aggregation of proteins can be avoided by immobilization, information on the renaturation of immobilized multiple proteins is not available. Also, while it is known that proteins like lysozyme can by themselves exhibit reversible denaturation with denaturants like guanidine hydrochloride (GdmCl) and urea (Goldberg et al., 1991; Perrauddin et al., 1976), that their incorporation during the enzyme immobilization may help the process of renaturation of the desired enzymes after undergoing denaturation has not been known. In this context, it would be interesting to study the denaturation-renaturation behaviour of immobilized multiproteins, where the role of ionic interactions and influence of refolding ability of the stabilizing agent on the desired enzyme can be investigated.

Denaturation/renaturation behaviour of proteins can be studied by using CD or NMR measurement. However these methods have several constraints with respect to their application to immobilized enzymes. However, the activity of enzymes like GOD in an immobilized form can be easily followed with the help of a dissolved oxygen electrode

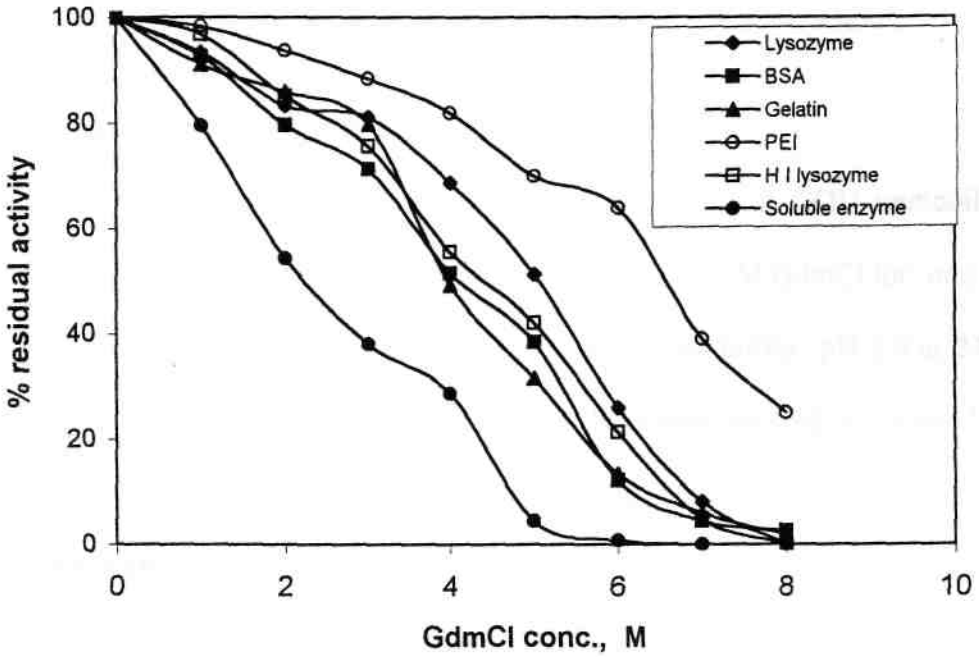


Figure 7.1.1.1 Denaturation behavior of the soluble and immobilized GOD (with different stabilizing agents) at various GdmCI concentrations.

to the denaturants compared to the enzyme, then the stability of enzyme preparation is adversely affected. As shown in figure 7.1.1.1, a remarkable stability of GOD immobilized with PEI against GdmCl concentrations was observed, probably owing to the inertness of PEI to GdmCl. Correspondingly, as the stability of stabilizing agent itself decreased as reported for lysozyme (Goldberg et al., 1991; Perrauddin et al., 1976) and BSA (Tanford. 1968) and gelatin (Courts. 1980), stability of GOD immobilized with these stabilizing agents decreased against GdmCl.

7.1.2 Renaturation behaviour of immobilized GOD.

Figure 7.1.2.1 demonstrates renaturation behaviour of GOD immobilized with various stabilizing agents after undergoing denaturation in 8 M GdmCl for one hour. The renaturation studies were carried out in 50 mM phosphate buffer pH 6.0 at 27±l°C. As shown in the figure, GOD immobilized with lysozyme showed a remarkably higher renaturation yield compared to other stabilizing agents. GOD immobilized with lysozyme regained 72 % of original activity, whereas GOD immobilized with PEI, BSA, heat inactivated lysozyme and gelatin regained 21, 39, 25 and 19 % activity respectively within 20 minutes of renaturation. Another observation from figure 7.1.2.1 is that the renaturation is almost complete in 20 minutes time. The significantly beneficial renaturation ability of GOD immobilized with lysozyme compared to other stabilizing agents may be attributed to the influence of refolding ability of the lysozyme on GOD through crosslinkages after undergoing denaturation in 8 M GdmCl. Glutaraldehyde crosslinkages between GOD and lysozyme thus appear to play an important role in the renaturation behaviour of the immobilized GOD. A significant evidence for the influence of refolding ability of the stabilizing agent on GOD after undergoing

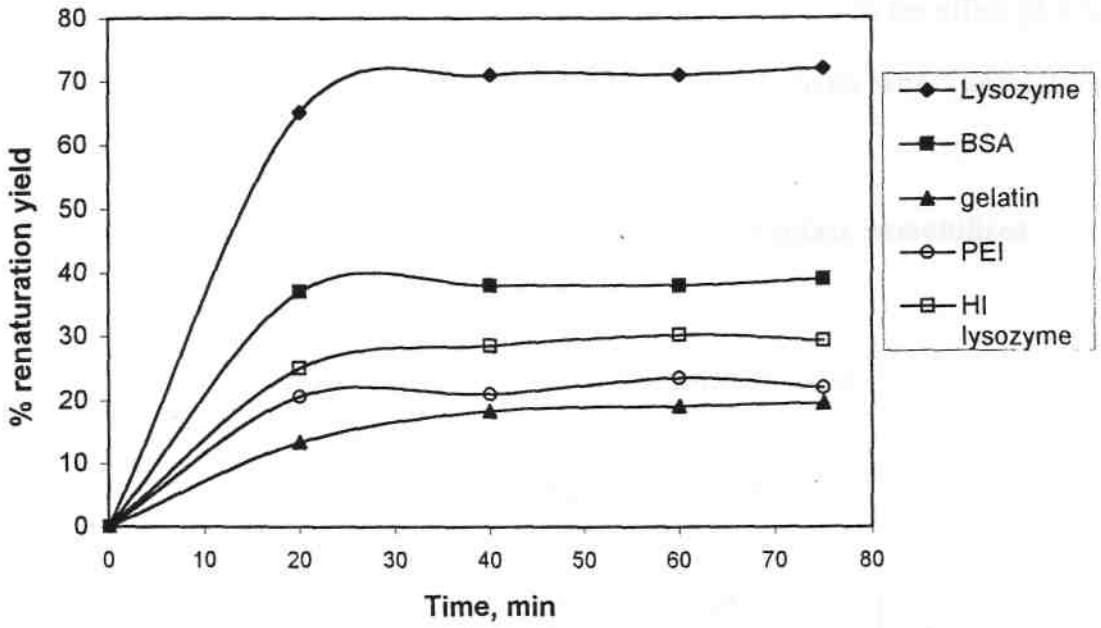


Figure 7.1.2.1 Renaturation behaviour of GOD immobilized with various stabilizing agents.

denaturation can perhaps be seen from the sharply decreased % renaturation of the GOD immobilized with heat inactivated lysozyme (figure 7.1.2.1). After heat inactivation, lysozyme loses the refolding ability and correspondingly, the renaturation influence on the immobilized GOD. Thus the refolding ability of the stabilizing agent appears to play a crucial role on the renaturation behavior of the immobilized enzyme.

7.1.3 Role of ionic interactions.

In a further support of the above hypothesis, table 7.1.3.1 shows the effect of 1 M sodium chloride on the renaturation of the immobilized GOD after undergoing denaturation in 8 M GdmCl for one hour.

Table7.1.3.1 Effect of NaCl on the reactivation of glucose oxidase immobilized with different stabilizing agents.

Stabilizing agent	% renaturation yield in presence of	
	None	1 M NaCl
PEI	20.41	20.26
BSA	38.36	39.21
Gelatin	20.41	21.26
Lysozyme	72.56	37.95
Heat inactivated lysozyme	25.13	18.59

It can be seen from table 7.1.3.1 the presence of 1 M sodium chloride in the renaturation buffer leads to a significant decrease in % renaturation only in the case of GOD immobilized with lysozyme as stabilizing agent and not in the case of other stabilizing agents *viz.,* BSA, gelatin, PEI and heat inactivated lysozyme. As mentioned in the 5.0 and 6.0 section that the increased thermal stability of soluble as well as immobilized GOD in

presence of lysozyme is attributable to the ionic interactions between them. The considerably decreased renaturation yield in the presence of 1 M sodium chloride for GOD immobilized with lysozyme supports this hypothesis. Addition of sodium chloride to the renaturation buffer may lead to a modification of the surface charges of denatured GOD and lysozyme by obstructing ionic interaction between them and thus adversely effect the renaturation process. Trivedi et al (Trivedi et al., 1997) reported that the heterochain interaction between acidic protein (BSA) and basic protein (lysozyme) resulted in a decrease in the renaturation of the lysozyme due to aggregation. This aggregation occurs by specific conformation and interaction of certain folding intermediates. The influence of electrostatic interactions between the folding protein, barnase and the chaperonin GroEL during renaturation of barnase has been demonstrated by Gray and Fershar (Gray and Fershat. 1993). Our results indicate that the glutaraldehyde crosslinkages between lysozyme and GOD, together with ionic interactions between them may play an important role in the renaturation behaviour of the immobilized enzyme.

7.1.4 Repeated denaturation/renaturation studies.

Figure 7.1.4.1 demonstrates the repeated denaturation (in 8 M GdmCl) for one hour and renaturation (in 50 mM phosphate buffer) of GOD immobilized with various stabilizing agents at pH 6.0 and at 27±l°C. In all the cases, there is a steep fall in the renaturation in the first cycle followed by a more gradual fall. However, as shown in figure 7.1.4.1, BSA and gelatin as stabilizing agents, gave negligible extent of renaturation at the end of fifth cycle itself. Whereas GOD immobilized with PEI and heat inactivated lysozyme resulted in a slower decrease in renaturation yields, reaching

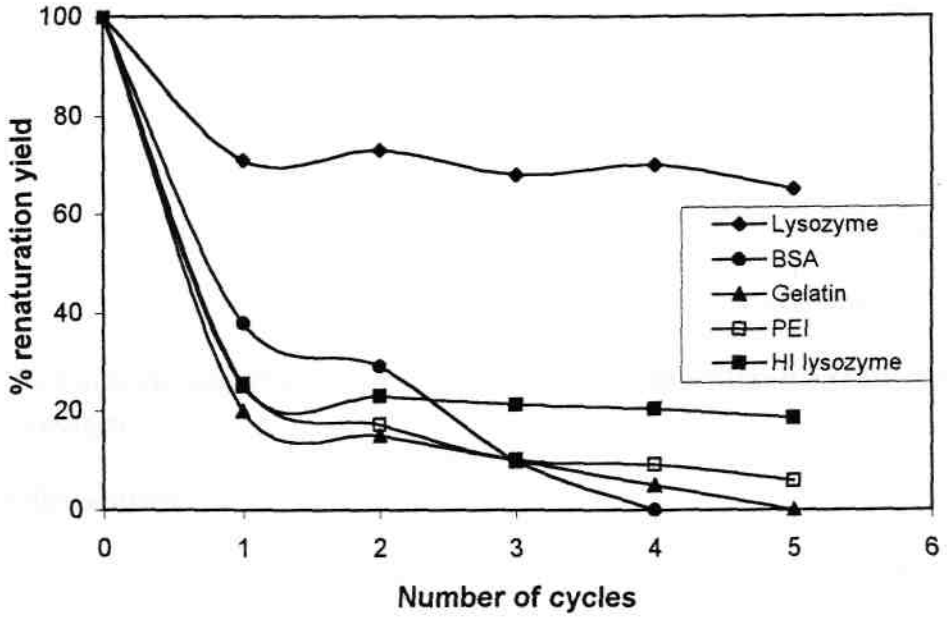


Figure 7.1.4.1 Repeated denaturation-renaturation of GOD immobilized with various stabilizing agents after undergoing denaturation in 8M GdmCl for one hour.

saturation values of 32 and 20 % respectively by the fifth cycle. However, remarkably, lysozyme as the stabilizing agent, after a steep fall (to 72 %) in the immobilized GOD activity in the first cycle, gave a stable and almost constant renaturation yields in the subsequent cycles, retaining 70 % renaturation yield at the end of fourth cycle. Decrease in activity in the first cycle to 72 % for the GOD immobilized with lysozyme may be due to the irreversible denaturation of loosely bound enzyme molecules on the surface of immobilized enzyme membrane during denaturation in 8 M GdmCl. This hypothesis of the irreversible denaturation of surface bound enzyme molecule is supported by the gradual decrease of the renaturation yield in the subsequent cycles (figure 7.1.4.1). These results indicate that the GOD immobilized with lysozyme can show almost quantitative reversible denaturation behavior after undergoing denaturation by GdmCl.

7.2 Reversible denaturation behaviour of immobilized multienzyme system.

7.2.1 Denaturation.

Figure 7.2.1.1 demonstrates the denaturation behaviour of immobilized multienzyme system comprising invertase, mutarotase and GOD with various PBSAs in various GdmCl concentrations at pH 7.0 for one hour at 27+l°C. The multienzyme system immobilized with BSA and gelatin lost their activity completely beyond 3 M GdmCl with midpoint transition value (Cm) of 1.8 and 1.3, whereas when immobilized with lysozyme and heat inactivated lysozyme, it lost its activity beyond 4 M with Cm of 2.8 and 2.4 respectively. As in the case of the single enzyme system (GOD), increase in the stability of the multienzyme system immobilized with lysozyme compared to that with gelatin and BSA as stabilizing agent can probably be ascribed to the physico-chemical properties of the stabilizing agent. If the carrier itself is unstable to the

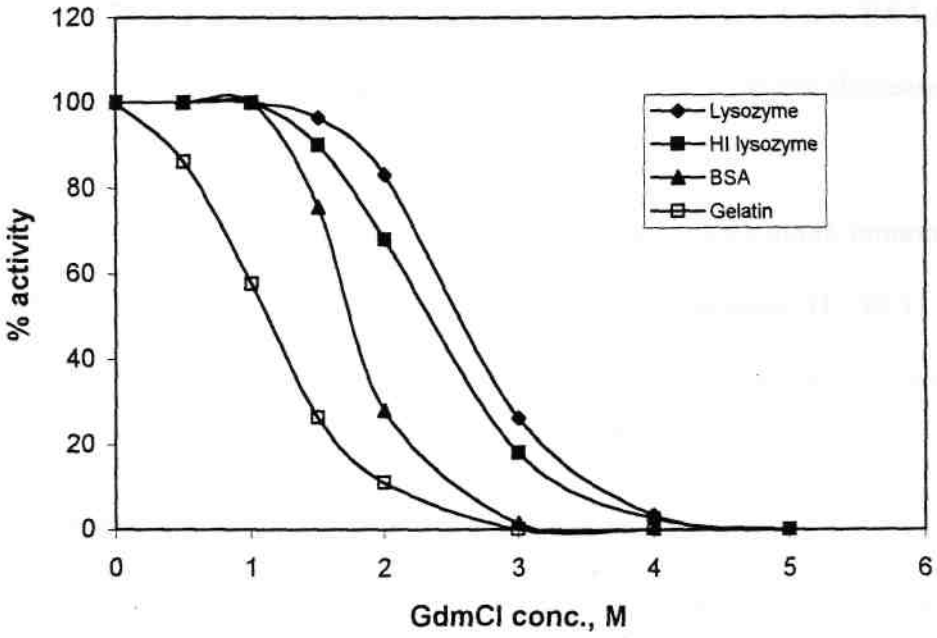


Figure 7.2.1.1 Denaturation behavior of the immobilized multienzyme system (with various stabilizing agents) at various GdmCl concentrations in 50 mM phosphate buffer pH 7.0.

denaturants compared to the enzyme, then the stability of enzyme preparation is adversely affected. As shown in figure 7.2.1.1, increase in the stability of multienzyme system immobilized with lysozyme against GdmCl was probably owing to the stability of stabilizing agent itself. Reported observations indicate that lysozyme (Goldberg et al., 1991; Perrauddin et al.,1976) is a more stable protein compared to BSA (Tanford. 1968) followed by gelatin (Courts. 1980), which is in support of our observations that the stability of the multienzyme system immobilized with lysozyme, BSA and gelatin decreased in the same order as the stability of stabilizing agent decreased against GdmCl.

It has earlier been reported in section 7.1 that GOD alone immobilized with gelatin, BSA lysozyme and heat inactivated lysozyme retained 31, 38,51 and 42 % activity after undergoing denaturation in 5 M GdmCl for one hour. These retained activities are considerably higher than those observed by us for the three enzyme system. In order to pinpoint which of the three enzymes in the multienzyme system could result in the reduced stability against GdmCl, we carried out the denaturation studies on the bienzyme system (invertase and GOD; mutarotase and GOD) immobilized with various PBSAs. Though the combined enzyme action of invertase and GOD on sucrose was low due to the absence of mutarotase, it was still possible to obtain a quantifiable signal from the electrode. The midpoint transition value (Cm) was calculated from a plot of % activity versus concentration of GdmCl (M) at 50 % activity and shown in the table 7.2.1.1. As seen in table, Cm values for the immobilized multienzyme system and bienzyme system (invertase and GOD) are closely related, whereas in the case of the immobilized bienzyme system (mutarotase and GOD) Cm values are relatively higher

than the multienzyme system values, indicating that the overall loss of multienzyme system activity is due to denaturation of invertase. Also, a comparison of the denaturation behaviour of immobilized multi- and single enzyme (GOD) systems, shows that while the multienzyme system immobilized with different PBSAs lost its complete activity beyond 4 M GdmCl, under similar conditions immobilized GOD alone lost its activity beyond 7 M GdmCl (see figure 7.1.1.1). This indicates that immobilized GOD alone is relatively much more stable than the multienzyme system. From these results (figure 7.1.1.1 and table 7.2.1.1) it was concluded that the overall loss of activity is first due to denaturation of invertase, followed by mutarotase and not due to GOD.

Table 7.2.1.1 Midpoint transition value (Cm) values for the multienzyme and two bienzyme system.

	Three enzyme system	Bienzyme system (Inveratse &GOD)	Bienzyme system (Mutarotase &GOD)
Lysozyme	2.8	2.7	3.1
Heat inactivated lysozyme	2.5	2.4	3.0
BSA	1.8	1.9	2.2
Gelatin	1.3	1.3	1.9

7.2.2 Renaturation behaviour of multienzyme system

Figure 7.2.2.1 demonstrates the renaturation behaviour of the multienzyme system immobilized with various PBSAs after undergoing denaturation in 5 M GdmCl for one hour. The renaturation studies were carried out in 50 mM phosphate buffer pH 7.0 at 27±l°C. As shown in the figure, with lysozyme, the renaturation yield was dramatic at 100 %, within 90 minutes, whereas BSA, gelatin and heat inactivated lysozyme resulted in regained activity of only 18, 15 and 22 % respectively. This remarkable beneficial effect of lysozyme in conferring 100 % renaturation compared to other stabilizing agents

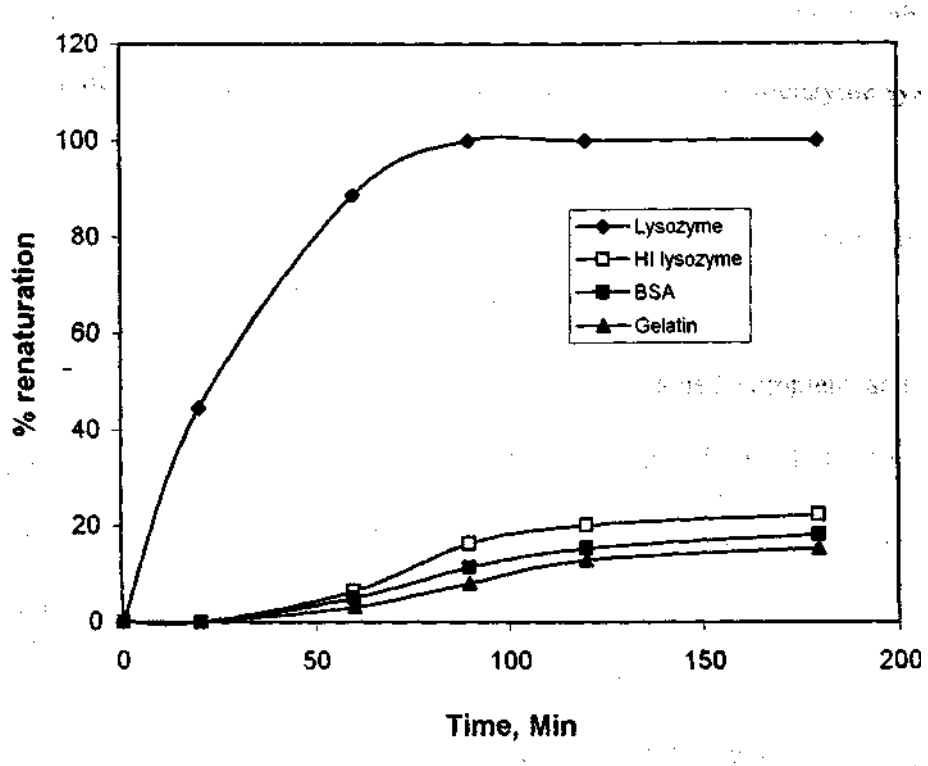


Figure 7.2.2.1 Renaturation behaviour of multienzyme system immobilized with various stabilizing agents.

may be attributed to the influence of refolding ability of the lysozyme through crosslinkages after undergoing denaturation in 5 M GdmCl. Glutaraldehyde crosslinkages between the enzymes and lysozyme thus appear to play an important role in this phenomenon. The sharply decreased % renaturation of the multienzyme system immobilized with heat inactivated lysozyme (figure 7.2.2.1) lends support to this hypothesis. After heat inactivation, lysozyme loses the refolding ability and correspondingly, the renaturation influence on the immobilized multienzyme system.

7.2.3 Effect of NaCl on the renaturation of multienzyme system.

Figure 7.2.3.1 demonstrates the effect of various NaCl concentrations on the renaturation of multienzyme system immobilized with lysozyme. As shown in the figure, multienzyme system immobilized with lysozyme has regained complete activity in 50 mM phosphate buffer pH 7.0 after undergoing denaturation in 5 M GdmCl for two hours. As the NaCl concentration increased (20, 40 and 60 mM) in the renaturation buffer, activity regain of multienzyme system was decreased to 90, 80 and 60 % respectively. Addition of sodium chloride to the renaturation buffer may lead to a modification of the surface charges of denatured GOD and lysozyme by a shielding effect and thus adversely affect the renaturation process. Günter et al (1996) reported that the decrease in renaturation yield of α-glucosidase in the presence of salt concentrations. This was attributed to the increased ionic strength in the renaturation buffer leading to the hydrophobic interactions of the folding intermediates (which leave part of the hydrophobic protein core exposed) with the matrix. From our results (figure 7.2.3.1) it can be observed that increasing concentration (ionic strength) in the renaturation buffer, led to a decrease in renaturation yield, probably be owing to the hydrophobic interactions

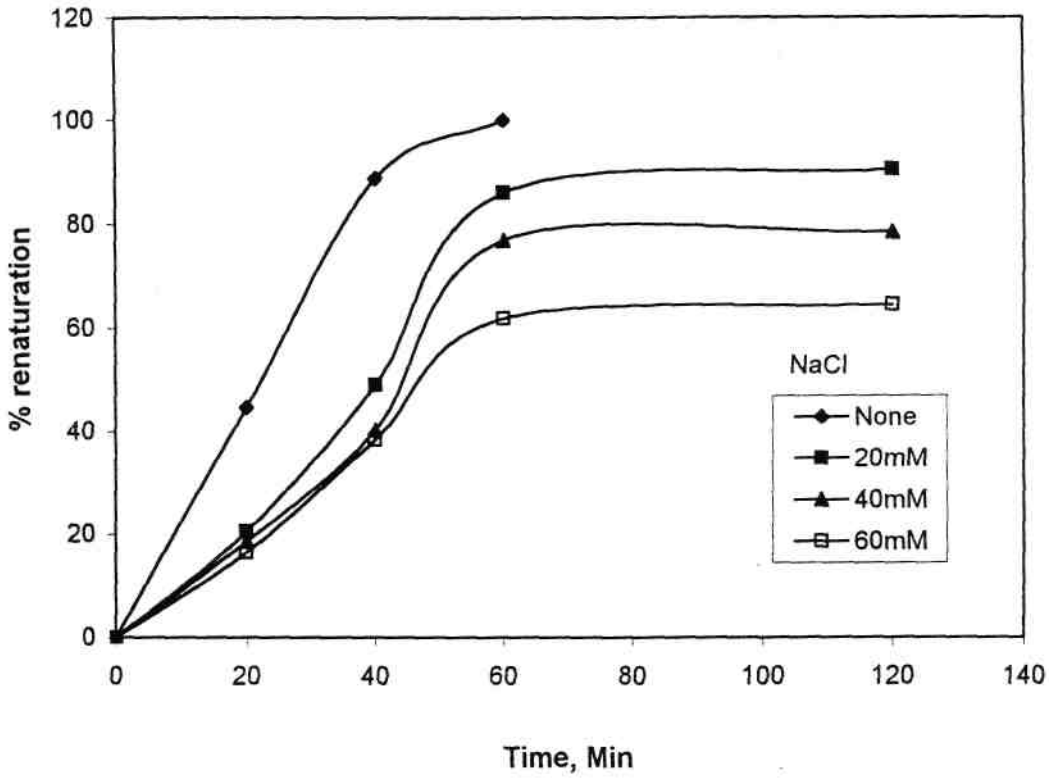


Figure 7.2.3.1 Effect of NaCl concentration on the renaturation of multienzyme system immobilized with lysozyme as PBSA after undergoing denaturation in 5 M GdmCl for two hours.

between the folding intermediates of immobilized proteins, which are exposed during denaturation with GdmCl.

7.2.4 Repeated denaturation/renaturation of the immobilized multienzyme system.

Figure 7.2.4.1 demonstrates the repeated denaturation (in 5 M GdmCl at pH 7.0 for two hours) and renaturation (in 50 mM phosphate buffer) of the multienzyme system immobilized with lysozyme as PBSA at pH 5.0 and at 27±l°C. After undergoing denaturation among the multienzymes, GOD immobilized with lysozyme has retained 10 % of activity, whereas that immobilized invertase and mutarotase lost their complete activity. As shown in figure 7.2.4.1, lysozyme as the PBSA, indicates a steady and almost constant renaturation yields in the subsequent cycles, retaining 100 % renaturation yield at the end of fourth cycle. Under similar conditions, in the very first cycle of renaturation, the multienzyme system immobilized with BSA, gelatin and heat inactivated lysozyme have shown less than 10 % of original activity and regaining ability was completely lost in the subsequent cycles. One of the major factors determining the yield of renaturation in vitro is the aggregation of denatured or partially denatured proteins. Aggregation of proteins during renaturation can be prevented by immobilization of proteins. However, even after immobilization, many proteins do not regain their complete activity (Kauzamann. 1959; Dill. 1990; Privolov and Gill. 1988). Our studies, here demonstrates the remarkable influence of refolding and consequent renaturation ability of lysozyme on the co-immobilized proteins. This significant influence of refolding ability of lysozyme on the immobilized enzymes after undergoing denaturation can probably be exploited for various biochemical investigations. For example, incorporation of lysozyme while

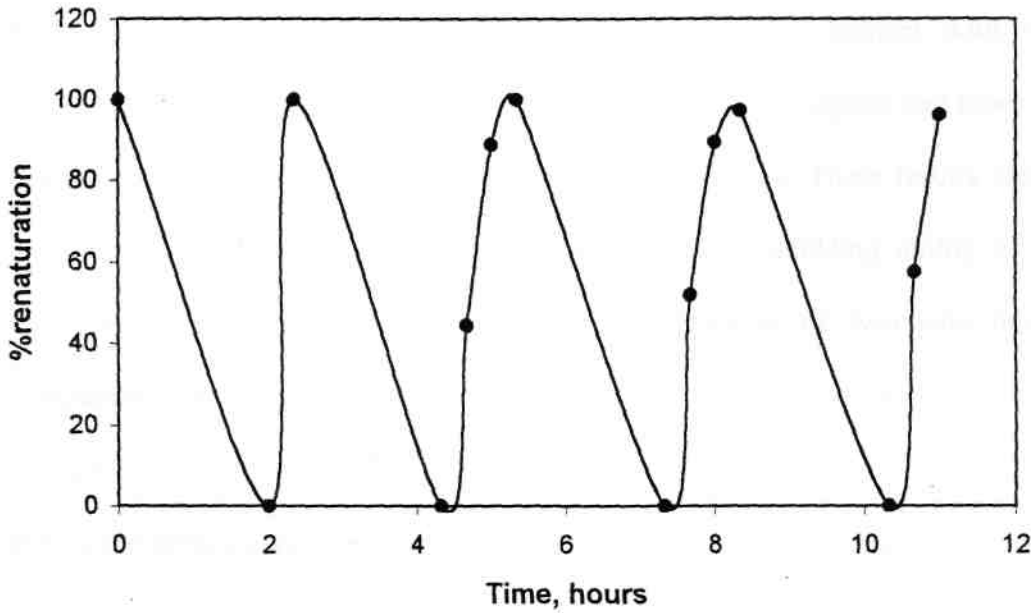


Figure 7.2.4.1 Repeated denaturation-renaturation of multienzyme system immobilized with lysozyme as PBSA after undergoing denaturation in 5 M GdmCl for two hours.

immobilizing the antibodies may help the process of regeneration of antibody surface, which is critical in the immunosensors.

7.3 Chapter summary.

The refolding ability of the lysozyme, glutaraldehyde crosslinkages between lysozyme and single and multienzyme system, appear to play an important role in the reversible denaturation behaviour of the immobilized single and multienzymes. Inability of renaturation of immobilized single and multienzymes immobilized with heat inactivated lysozyme suggest that the refolding ability of stabilizing agents can modulate the reversible denaturation behavior of the immobilized enzyme. These results should provide useful information in understanding the role of the refolding ability of the stabilizing agent on the immobilized enzyme. Incorporation of lysozyme during immobilization of enzymes can be employed as a useful tool for an intrinsic evaluation of the various refolding reagents by avoiding the aggregation which is a common problem in the denaturation study of soluble protein. By selecting a suitable stabilizing agent which has a refolding ability after undergoing denaturation with GdmCl, the desired enzyme can probably be reversibly denatured. Incorporation of lysozyme while immobilizing the antibodies may help the process of regeneration of antibody surface, which is critical in the immunosensors.

Chapter 8
Overall summary and highlights

SUMMARY AND HIGHLIGHTS.

In the context of the usage of immobilized enzymes for sugar biosensor applications, the present investigations attempt to a better understanding of the various aspects of the enzyme applications. The work has involved the construction of an immobilized enzyme based biosensor for glucose and sucrose in food and fermentation analysis, study of the effect of various parameters on the performance of the biosensor and also basic biochemical aspects of the immobilized single and multienzyme systems with respect to the effect of temperature, pH, denaturants as well as operational stability. Following are the summary and highlights of the present investigations.

1.Construction and optimization of biosensor

A reliable biosensor device based on immobilized enzyme and amperometric detection was constructed for the estimation of glucose and sucrose in food and fermentation samples. Biosensor operating parameters like response time, operating pH, concentrations of glutaraldehyde and a protein based stabilizing agents (PBSA) and enzyme loading were standardized. The multi enzyme concentrations which are important variables influencing the sucrose biosensor response were optimized by response surface methodology. Higher mutarotase concentration was found to result in an increased response time which was attributed to the inhibition of mutarotase by its product β-D-glucose.

2. Estimation of sucrose in the presence of glucose.

The problem of interference of glucose present in the sample, for sucrose estimation was effectively overcome by theoretical approach based on the response behaviour of the

sucrose biosensor for pure glucose and sucrose. This enabled a simple formula to accurately estimate sucrose in the presence of glucose using the two electrode system. This is being reported for the first time.

3. Stability of single and multienzyme systems.

The operational stability of the immobilized single and multienzyme systems, which is critical for practical applicability of the biosensor, has been significantly improved through incorporation of lysozyme as a PBS A. Thus it has been possible to increase the operating life of the single enzyme (GOD) sensing element from 245 to 700 analyses and multienzyme (Invertase, Mutarotase and GOD) system sensing element from 150 to 400 analyses. Similarly significant increase in stability against temperature and pH was also observed for both single and multienzyme system with lysozyme as a PBSA. Based on the thermal stability behavior of immobilized bi-(Invertase and GOD; Mutarotase and GOD) and the three enzyme system indicate the critical effect of the lower thermal stability of immobilized Invertase in the multienzyme environment. Increase in the stability of immobilized single and multienzyme system with lysozyme as PBSA has been attributed to the role of complementarily of surfaces between the enzyme and PBSA and the stability of PBSA itself. Even though lysozyme is an enzyme, its reaction products will not interfere with the biochemical reactions of the single and multi enzyme system at the oxygen electrode. Further advantage could be that since lysozyme is an anti microbial agent, it may also help avoid microbial contamination of immobilized enzyme preparations, thus giving a better durability of the enzyme membrane.

A significant increase in the thermal and operational stability of immobilized enzymes using lysozyme as PBSA is an important contribution to the practical aspects of biosensor applications as well as stabilization of enzymes in general.

4. Thermal stability studies of immobilized enzymes using biosensor.

The simple amperometric principle based dissolved oxygen electrode biosensor was used for the fundamental stability investigations on the immobilized single and multienzyme systems involving GOD, invertase and mutarotase. This method overcomes some of the constraints involved in the biochemical investigations of the immobilized enzymes by using spectrophotometer. Deactivation of the oxygen electrode due to direct heating at higher temperature was avoided by exposing only a detachable membrane unit (DMU) containing the immobilized enzyme membrane to the desired temperature. Further this method is expected to be applicable for the study of the thermal stability of other immobilized enzymes, catalyzing reactions involving consumption of oxygen.

5. Mechanism of thermal inactivation of GOD.

The mechanism of thermal inactivation of GOD and its stabilization by incorporation of appropriate additives has been examined from a fundamental angle. Thermal inactivation of enzyme involves conformation changes, unfolding and non-specific aggregation of the enzyme molecule which are due to hydrophobic interactions of side chains and -SH group oxidation. Unfolding and non-specific aggregation due to hydrophobic interaction of side chains can be prevented by additives such as K2SO4, NaCl and lysozyme. Non specific aggregation of enzyme can also be prevented by blocking the free -SH or the -SH group involved in the disulphide bond formation which are not essential for the catalytic function of the enzyme and thus enabling enhanced

thermal stability of enzyme. These observations were corroborated by CD and SDS-PAGE measurements. These studies throw more light on the mechanism of thermal inactivation of GOD as well as its minimization through the use of additives and the knowledge gained may be applicable in the case other similar enzymes.

6. Reversible denaturation of the immobilized single and multienzyme systems.

The denaturation/renatuiration behaviour of the immobilized single and multienzyme systems (GOD, invertase and mutarotase) against GdmCl has been investigated. Incorporation of lysozyme as PBSA has enabled a very significant renaturation. The refolding ability of the lysozyme, glutaraldehyde cross linkages between lysozyme and single and multienzyme system, appear to play an important role in the reversible denaturation behaviour of the immbilized single and multienzymes. Incorporation of lysozyme during immobilization of enzymes can be employed as a useful tool for an intrinsic evaluation of the various refolding reagents by avoiding the aggregation which is a common problem in the denaturation study of soluble protein. These observations may have potentially significant practical application for stabilization of immobilized enzymes.

By using simple dissolved oxygen electrode and the biosensor technique, difficulties encountered in the stability studies on immobilized enzymes in the presence of denaturants like urea using fluorescence spectroscopy and circular dichroism have been overcome. In the spectrophoptometric method in order to follow the denaturation behaviour of immobilized enzyme like GOD in the presence of denaturants, an indicator enzyme (peroxidase) is required to monitor the enzymatic reaction. Denaturation of the

indicator enzyme in the presence of denaturant leads to erroneous results. By using the Clark dissolved oxygen electrode the denaturation/renaturation behaviour of immbilized enzymes can be effeciently followed.

Some suggestions on scope for further research

Our studies on immobilized single (GOD) and multi enzyme (GOD, invertase and mutarotase) systems have shown that it is possible to achieve relatively high thermal as well as operational stability by incorporating lysozyme as PBSA during enzyme immobilization. Based on the results reported in this thesis, stability of other immobilized enzymes also can perhaps be improved by incorporation (during the immobiliztion step) of suitable PBSAs like carbonic anhydrase, cytochrome oxidase, aldolase which can provide a better complementarity of surface between the enzyme and PBSA.

Our attempt at understanding the mechanism of thermal inactivation of soluble GOD, has led us to conclude that an important cause for the thermal inactivation of GOD is non-specific aggregation due to hydrophobic interaction of side chains and - SH group oxidation, which can be prevented by blocking the -SH group using appropriate additives like K2SO4. Extrapolation of these studies to the immobilized enzyme system, where the -SH group is not essential for their catalytic activity could show significant improvement in the thermal stability of immobilized single and multi enzyme systems.

The biosensor method can be used as an efficient analytical tool to quantify the stability of immobilized enzymes catalyzing reactions involving consumption of oxygen. Denaturation/renaturation behaviour of immobilized enzymes could be followed by using a simple Clark type dissolved oxygen electrode as an efficient alternate analytical tool.

Reversible denaturation of immobilized single and multienzyme systems achieved in our study by utilizing the refolding ability of lysozyme can be used as an efficient method for an intrinsic evaluation of the various refolding reagents by avoiding the aggregation which is a common problem in the denaturation study of soluble protein. Further, this method of renaturation can probably be employed for the construction of biosensor for metal ion detection, where the preparatóion of apoenzyme is crucial but practically difficult.

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LIST OF PATENT, PUBLICATIONS AND POSTERS

PATENT:

1. **Gouda, M.D.,** Thakur, M.S. and Karanth, N.G. An improved process for the preparation of stabilized enzymes. 1454/DEL/1999.

PUBLICATIONS:

1. **Gouda, M.D.,** Thakur, M.S. and Karanth, N.G. Stability studies on immobilized glucose oxidase using an amperometric biosensor - Effect of protein based stabilizing agents *Electroanalysis*. Under revision.

2. **Gouda,** M.D., Kumar, M.A., Thakur, M.S. and Karanth, N.G. Biosensor for the extensive operational stability studies of immobilized enzymes. (Submitted to Biotechnology and Bioengineering).

3. **Gouda. M.D.,** Thakur, M.S. and Karanth, N.G. Reversible denaturation behaviour of immobilized glucose oxidase. (Submitted to Journal of Biochemistry- Tokyo).

4. **Gouda, M.D.,** Thakur, M.S. and Karanth, N.G. Optimization of multienzyme system for sucrose biosensor by response surface methodology. (Submitted to Journal of Agriculture and Food Chemistry).

5. **Gouda. M.D.,** Thakur, M.S. and Karanth, N.G. Thermal stability behaviour of immobilized multienzyme system - invertase, mutarotase and glucose oxidase -Effect of protein based stabilizing agents. (Submitted to FEBS Letters).

6. **Gouda. M.D.,** Appu Rao A.G., Thakur, M.S. and Karanth, N.G. Role of ionic interactions between glucose oxidase and lysozyme on the thermal stabilization of glucose oxidase (Submitted to Enzyme and Microbial Technology).

7. **Gouda. M.D.,** Thakur, M.S. and Karanth, N.G. Estimation of sucrose in presence of glucose by using multienzyme biosensor. (Submitted to Biotechnology and Bioengineering).

8. **Gouda. M.D.,** Thakur, M.S. and Karanth, N.G. Lysozyme assisted reversible denaturation behaviour of immobilized invertase, mutarotase and glucose oxidase (Submitted to FEBS Letters).

POSTERS PRESENTED AT SYMPOSIA:

1. **Gouda. M.D.,** Ashwini. R., Thakur. M.S., Kumar. M.A. and Karanth. N.G. (1998). Biosensor for glucose and sucrose. 4th International Food Convention (IFCON - 98) Nov. 23-27.
   Best Poster award.

2. **Gouda. M.D.,** Thakur. M.S. and Karanth. N.G. (1998). Enhanced stability of immobilized glucose oxidase for biosensor applications. 4th International Food Convention (IFCON - 98) Nov 23-27.

3. **Gouda.** M.D., Thakur. M.S. and Karanth. N.G (1999). Reversible denaturation of immobilized glucose oxidase. A poster presented at 68th annual meeting Society of Biological Chemists and Symposium on current trends in Biology at Indian Institute of Science, Bangalore, Dec 27-29.