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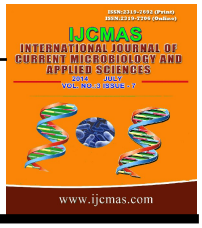



**Original Research Article**

# Enhancement of Functional Properties of Cholesterol Oxidase and Glucose Oxidase enzymes through Co-Immobilization on Organic Support

**Anamika Jha[1], Ajeet Singh[2] and Hemant K. Gautam[3]***

[1]Ashok and Rita Patel Institute of Integrated Study, Research in Biotechnology and Allied Sciences, New Vallabh Vidyanagar, Anand, Gujarat India
[2]Department of Biotechnology, G. B. Pant Engineering College, Pauri Garhwal, Uttarakhand, India
[3] Institute of Genomics and Integrative Biology, Sukhdev Vihar, Mathura Road, Delhi- 110025 India
**Corresponding author*

**Keywords**

Glucose oxidase (GOD), Cholesterol oxidase (COD), Polyvinyl resin (PVR)

**A B S T R A C T**

The present study demonstrates the functional stability enhancement of glucose oxidase (GOD) and cholesterol oxidase (COD) through co-immobilization on to a new immobilization support. Polyvinyl resin was used to prepare the membrane after dissolving in different organic solvents and the enzymes were co-immobilized onto the membrane by entrapment method. Enzyme activity was measured by Amperometric method. The membrane was subjected to various stress conditions of temperature and pH to check the stability and activities of both GOD and COD. Interestingly, the co-immobilized enzymes displayed enhanced stability (at room temperature) over a long period of time in comparison to native enzymes. Native GOD and COD enzymes showed a drastic decline in activity within 5 and 25 days respectively, whereas immobilized GOD and COD showed detectable activity till 65 days of storage and can be stored at room temperature for 20 days without any loss in activity. Immobilized enzymes maintained significant activity, even at 50ºC in contrast to the native enzymes. Also the immobilized GOD could withstand 70ºC showing detectable activity. pH 7 was optimized for both native and immobilized enzymes. Nearly 2-fold increase in the immobilized enzyme activity was observed when compared with native enzymes in highly acidic conditions.

## Introduction

Glucose and cholesterol are biomolecules of prime importance to human life as they are associated with a number of diseases such as diabetes, cardiac diseases, kidney failure, etc. Quantitative determination of these biomolecules is very important in biochemistry, clinical chemistry and food biology. Numerous methods such as spectrometry, amperometry, HPLC, polarometry, and capillary electrophoresis

for analysis have been reported. However, most of the current adopted methods are time consuming or costly. Biosensors containing enzymes have been widely applied in chemistry and biology due to their high sensitivity and potential selectivity, in addition to the low cost and the possibility of miniaturization or automation. Glucose oxidase (GOD) and cholesterol oxidase (COD) are widely used for the determination of glucose and cholesterol in body fluids and removing residual glucose, cholesterol and oxygen from beverages and foodstuffs (Yildiz et al., 2005).

Instability and high cost are restriction for the use of enzymes in the analytical methods. These problems can be partly overcome by immobilizing of these enzymes on suitable matrices. Immobilized enzymes provides several operational advantages over free enzyme. It is possible to use a single batch of enzymes repetitively and stop the reaction by physical removal of immobilized enzyme from the solution. Also, high enzymatic activity in a small volume, long life, predictable decay rate and elimination of reagent preparation are further advantages of enzyme immobilization. Although enzyme immobilization technology is in use since 1916 (Nelson and Griffin, 1916), yet the extensive development of this noble technology is still in progress (Trevan, 1980; Juta et al., 2006). The enzyme immobilization technology, which is an integral component of the biosensor design provides highly selective, stable and rapid analytical method for the determination of small molecules in food as well as blood samples.

A number of methods are available for immobilization and co-immobilization of enzymes on different inorganic and organic supports (Braco et al., 1992; Kumar et al., 1999; Clark, 2004), as no single method is perfect for all enzymes. However, applications of enzyme immobilization have been limited due to the short life of the enzymes and possibility of severe leaching during long term operation. There are different possible ways to overcome such problems. Firstly, to solubilize and modify the enzyme in such a manner that it retains its maximum activity and entrap these modified enzymes in a suitable carrier. Secondly, to search for the new carrier materials for better enzyme activity after immobilization.

The immobilization of enzymes on polymers serves as an effective choice to retain their maximum activities. This may be attributed to the fact that when an enzyme is immobilized onto a polymer (either in solution or in a hydrogel), drastic environmental changes occur in the polymer conformation which significantly affects the enzyme activity and substrate (Fernandez-Romero et al., 1987; de Velde et al., 2000; Gautam and Kumar, 2008; Chauhan and Pundir, 2011).

Immobilization of GOD and COD separately has been variously reported (Yildiz et al., 2005, Kumar et al., 1999, Gautam and Kumar, 2008). Also the coimmobilization of COD with enzymes other than GOD such as cholesterol esterase, peroxidase and other enzymes have been documented (Torabi et al., 2007; Tank et al., 2005; Chauhan and Pundir, 2011), but hardly any attempt has been made to coimmobilize COD and GOD. Thus, the present investigation was designed to develop a single step process for the co-immobilization of GOD and cholesterol COD enzymes onto polyvinyl resin in the presence of organic solvents. It is hypothesized that the developed enzyme membrane will possess high selectivity and sensitivity to estimate glucose and

cholesterol simultaneously in the serum and other food stuffs.

## Materials and Methods

The enzymes (COD, E.C. 1.1.3.6; GOD, E.C. 1.1.3.4; horseradish peroxidase (POD), E.C. 1.11.1.7) and Poly- vinyl resin (Commercial name Formvar) were purchased from M/S Sigma Chemicals (St. louis Mo, USA). The source of GOD was *Aspergillus niger*, whereas COD enzyme was extracted from *Rhodococcus* sp. All other reagents were of analytical grade and were purchased locally. Milli-Q Water was used for preparing reagents and buffers (Millipore. Inc. Bedford, MA).

### Coimmobilization of COD and GOD Enzymes onto Poly-vinyl Resin Membrane

The membrane was prepared from 2 ml of 4% (w/v) poly-vinyl resin and chloroform and ethylene dichloride (1:1 ratio, v/v). The enzymes Glucose oxidase (GOD) and cholesterol oxidase (COD) (5 units/ml each) were added to the membrane mixture and stirred for two minutes at room temperature (at 25$\pm$2$^{\circ}$C) to achieve a homogenous distribution of enzymes within the polymer. The polymerization was carried out at room temperature resulting the formation of a thin membrane with entrapped enzymes. This immobilized enzyme membrane was allowed to air dry for 4-6 hours. Any unbound enzymes (if present) were removed by washing the membrane with distilled water. The enzyme membrane was stored at room temperature for further studies. Extending the studies for further improving the enzyme efficiency, this membrane was incubated with Glutaraldehyde (a cross linking agent) at different concentrations ranging from 0.5 % to 2.5% for 4-5 hours at room temperature as well as at 4$^{\circ}$C.

### Assay of Membrane Bound GOD and COD Enzymes

Glucose oxidase activity was measured on the basis of two step enzymatic assays. Glucose is oxidized by glucose oxidase with the formation of $H_2O_2$ that is subsequently used by POD to oxidize o-dianisidine. The reaction mixture contained 2.5 ml of dye buffer (1% dye in 0.1M phosphate buffer, pH 6.0), 54 mg D-glucose, 10 units of horse-radish peroxidase in total volume of 3.0 ml. The reaction was started by adding 100 µl (10 units) of the enzyme solution. The light brown color of oxidized o-dianisidine was determined spectrophotometrically at 460 nm. One unit of glucose oxidase was defined as the amount of enzyme producing one µ mole of $H_2O_2$ per minute at 25$^{\circ}$C.

Cholesterol oxidase activity was measured by the method based on the conversion of cholesterol to 4 cholesten-3-one according the method described by Allain et al.,1974. The assay buffer mixture contained 400mM $KH_2PO_4$, 360mM KOH, 2.7mM EDTA di-sodium salt, 24mM 4-hydroxybenzoic acid, 40.7mM -amino anti pyrine, 2g/l Triton X-100, 5ml/l methanol and 20 units/100ml peroxidase. The substrate (cholesterol) was prepared in isopropanol and TritonX-100. The reaction mixture was prepared by adding 2.5 ml of assay buffer and 0.125 ml of substrate solution. The reaction was started by adding 50 µl COD enzyme solution. The red color of 4-cholestene-3-one was measured spectrophotometrically at 500 nm for 5 min. One unit of cholesterol oxidase is defined as the amount of enzyme that converts 1 µmol of cholesterol/min at 37$^{\circ}$C. Immobilized COD and GOD were also assayed for their activities in the similar manner as that of the free enzymes and 1X1 $cm^2$ enzyme membrane was used in place of free enzymes.

**Enzyme Membrane Characterization Studies**

For the characterization studies, both native and immobilized enzyme membranes (1X1 $cm^2$) were subjected to various environmental conditions such as extremes of temperature (10-80°C for 2 hours) and pH (pH 3.0-10.0). Characterization at preliminary level was also done to check the shelf-life of the enzyme membrane at room temperature in terms of enzyme activity. To supplement the study, the response of enzyme membrane on Oxygen electrode was also assessed by varying the GOD and COD concentrations (100 mg/dl to 800 mg/dl).

**Application Based Assays**

**Determination of Serum Glucose and Cholesterol with 'Enzyme Membrane'**

**Assay of Serum Glucose**

The glucose concentration was measured based on colorimetric assay by Loft and Turner (1975). In this method glucose is oxidized in the presence of GOD enzyme into gluconic acid and liberates hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine in presence of phenol and peroxidase enzyme and form quinoneimnine, a pink colored compound which has absorption maxima at 505 nm. The reaction mixture consisted of 0.1M phosphate buffer, 0.4 mM 4-aminoantipyrene, 40mM phenol, 10 units of peroxidase, glucose oxidase in phosphate buffer (membrane in the case of immobilized enzyme 1x1 $cm^2$) and serum 100 µl in place of glucose. The reaction mixture was incubated at 37 °C for 30 min and enzyme activity was measured at 505 nm.

**Determination of Serum Cholesterol**

The total cholesterol in serum was measured using enzyme membrane (1X1 $cm^2$) instead of free enzymes. The serum was pretreated with Triton X-100, isopropanol and sodium phosphate buffer and used as a substrate. Serum (100 µl) was used in place of cholesterol.

The same batch of the serum was further analyzed by the commercially available Enzo kit. (data not shown)

## Results and Discussion

The methods of enzyme immobilization on polymers have been well documented (Chang, 1977; Karube et al., 1982; Juta et al., 2006, Sumritta et.al.2013). Such type of enzyme membranes has been proposed for the estimation of cholesterol, glucose, urea, uric acid etc. in blood and urine samples. These enzyme membranes are also useful in long term operation in bioconversions. However, suitable methods for effective co-immobilization of COD and GOD are not available. To explore the possibility of a suitable method, it is necessary that a random screening of matrices and optimization of the enzyme immobilization conditions should be carried out. Our present study was based on the strategy that enzymes behave differently in organic medium and once they are immobilized they remain active and stable up to several years (Igor and Matiasson, 1999). Therefore, we selected polyvinyl resin for the immobilization of GOD and COD in presence of organic solvents. After several washings of the immobilized enzyme membrane, 80% enzyme retention was observed. No leaching of immobilized GOD and COD from the membrane was observed during the enzyme assay experiments. The individual enzyme activities were increased several folds when they were co-immobilized. Lower and upper detection limits of both enzymes in the samples were

50-140 mg/dl for GOD and 50-450 mg/dl for COD.

## Characterization of Enzyme Membrane

### Effect of Glutaraldehyde on Enzyme Membrane

On the basis of earlier findings (Clair and Navia, 1992), it was suggested that interaction of cross linking agent with enzyme may alter its activity. It may either enhance the activity of the enzymes or may inhibit the activity due to its toxic nature. We used different concentrations of glutaraldehyde ranging from 0.25 to 2.5 % for the evaluation of enzyme membrane. Our results suggested that glutaraldehyde did not show any enhancement of enzyme activity and also did not inhibit the enzyme activities at lower concentrations (up to 0.25%). However, the higher concentrations (more than 0.25 %) showed an adverse effect on enzyme activity (Fig. 1).

### Stability of Enzyme Membrane

The stability of enzyme membrane was investigated by keeping it at room temperature (25°C) for 60 days. The stability of this membrane was determined in terms of the retention of the enzyme activities. It was found that the GOD and COD activity decreased after 20 days and retained 50% activities after 60 days at room temperature (Fig.2).

### Effect of Temperature on Enzyme Membrane

The influence of temperature on the enzyme membrane was investigated by keeping the membrane at different temperatures ranging from 10-80°C for two hours. The enzymes entrapped in membrane were found to be more stable than native enzymes (Fig. 3). Native COD activity declined sharply as the temperature was raised above 10°C, whereas immobilization of this enzyme enhanced the stability upto 25°C. Similarly, stability in GOD activity was also enhanced from 25°C to 35°C after the immobilization process. Significant activity was maintained by immobilized enzymes even at 50°C in contrast to the native enzymes. Also, the immobilized GOD could withstand 70°C showing detectable activity.

### Influence of pH on the Enzyme activity

To investigate the efficiency of enzyme membrane at different pH levels, the enzyme membrane was subjected to different pH buffers ranging from pH 3 to pH 10 as shown in Fig. 4. Surprisingly, the results suggested that immobilized enzymes retained good activity in both acidic and alkaline (pH 5.0 to pH 8.0) conditions when compared to native enzymes. Maximum activity of the enzymes in both native and immobilized state was found at pH 7. In highly acidic conditions (pH 3) the immobilized enzymes showed nearly 2-folds increase in the enzyme activity when compared with native enzymes.

### Response of Enzyme Membrane on Oxygen Electrode

The co-immobilized enzyme membrane was attached to the oxygen electrode. When different concentrations of glucose and cholesterol were used the current starts decreasing and as a result of oxygen consumption and finally achieved the stationary phase. The result suggested that enzyme electrode responded differently on glucose and cholesterol standard solutions. The standard curve showed linearity up to 700 mg/dl in case of glucose and cholesterol (Fig. 5). This membrane was used to determine cholesterol and glucose

concentration in various samples and the results were compared with different available methods (Fig-6).

The two enzymes GOD and COD were successfully immobilized on polyvinyl resin (Commercial name Formvar). The enzyme membrane was very thin and retained maximum enzymatic activity without leaching. The stability of enzyme membrane (after immobilization) at different pH and temperature, improved to a great extent as compared to native enzymes. The enzyme membrane was successfully used in the determination of glucose and cholesterol concentrations in different pathological and food samples. This enzyme membrane has a longer shelf - life which may be attributed to its hydrophobic nature. Therefore, the use of this co-immobilized enzyme membrane in the estimation of glucose and cholesterol in different pathological and food samples is recommended. Further, the membrane also retains its potential to be used in biosensors and bioreactors.

**Figure 1: Effect of glutaraldehyde on co-immobilized membrane**

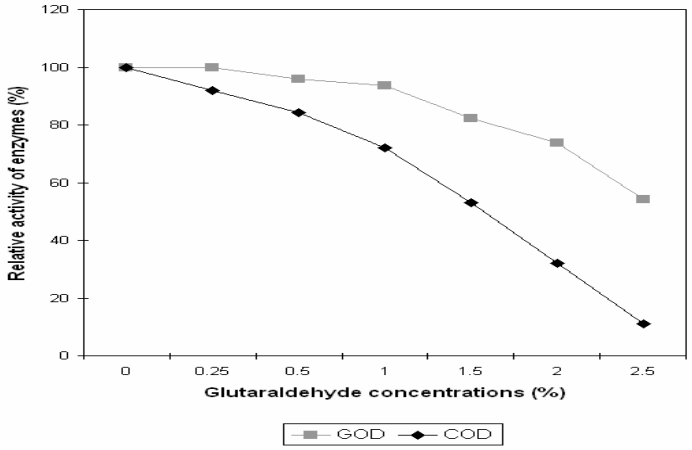



**Figure 2: Stability of co-immobilized enzymes**

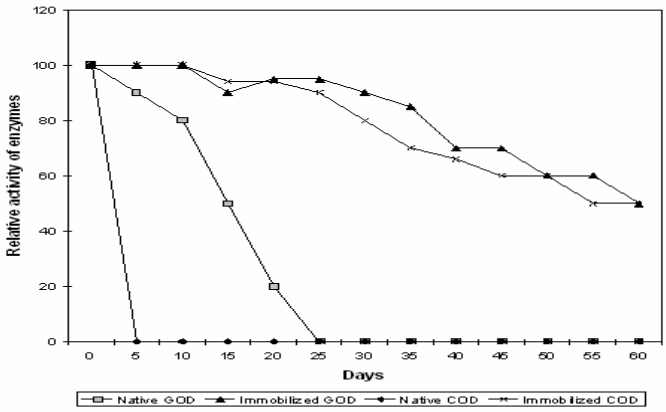


**Figure 3: Effect of temperature on the co-immobilized enzymes**

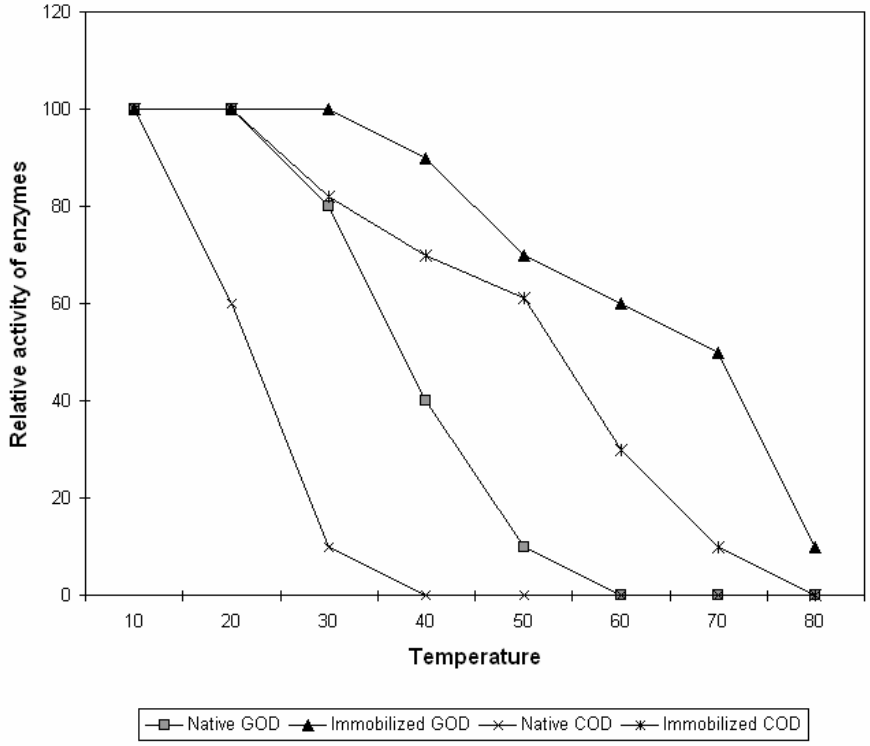



**Figure 4: Effect of pH on enzyme membrane**

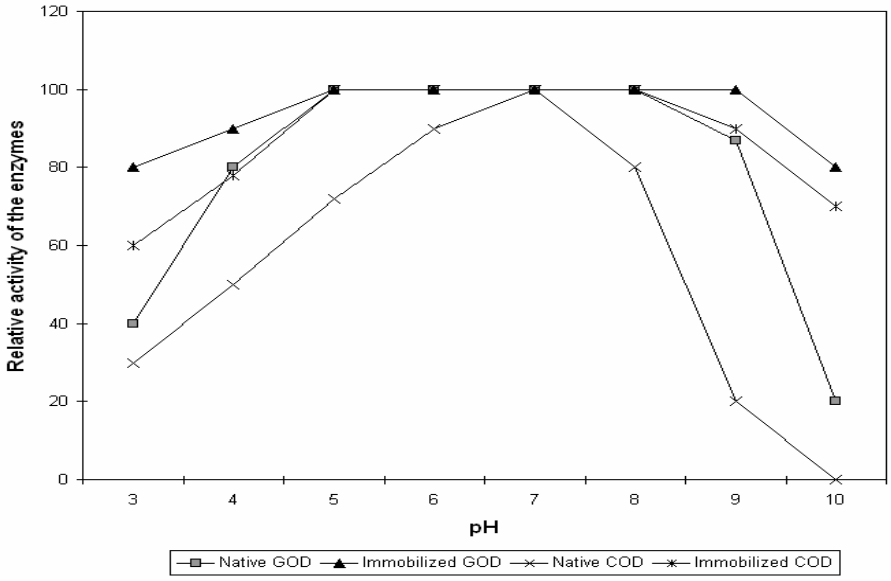


Figure 5: Response of oxygen electrode on enzyme membrane

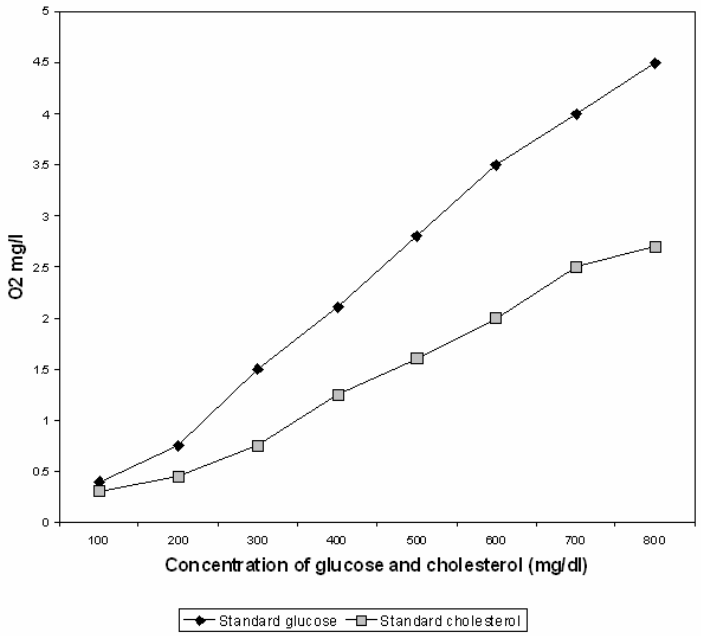
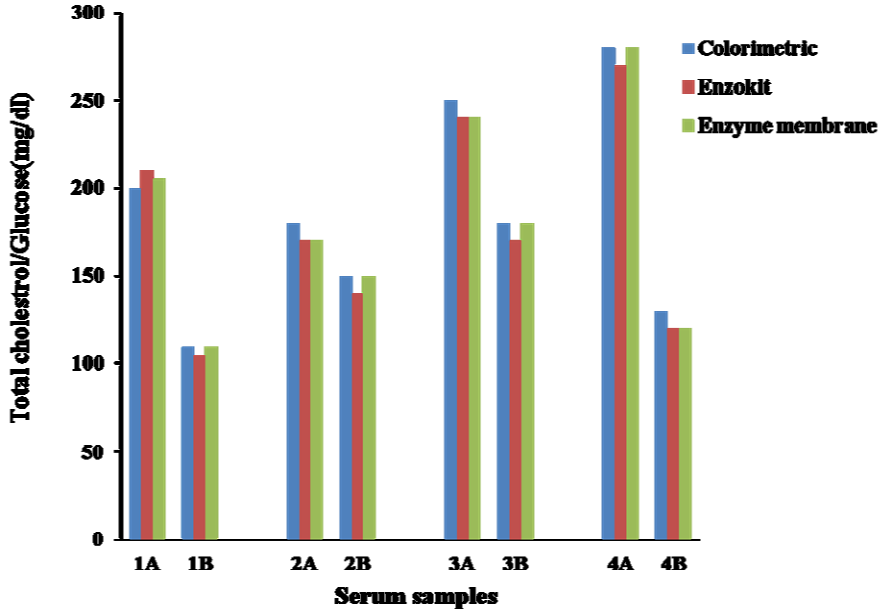



**Figure 6: Estimation of cholestrol and glucose by different methods**



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