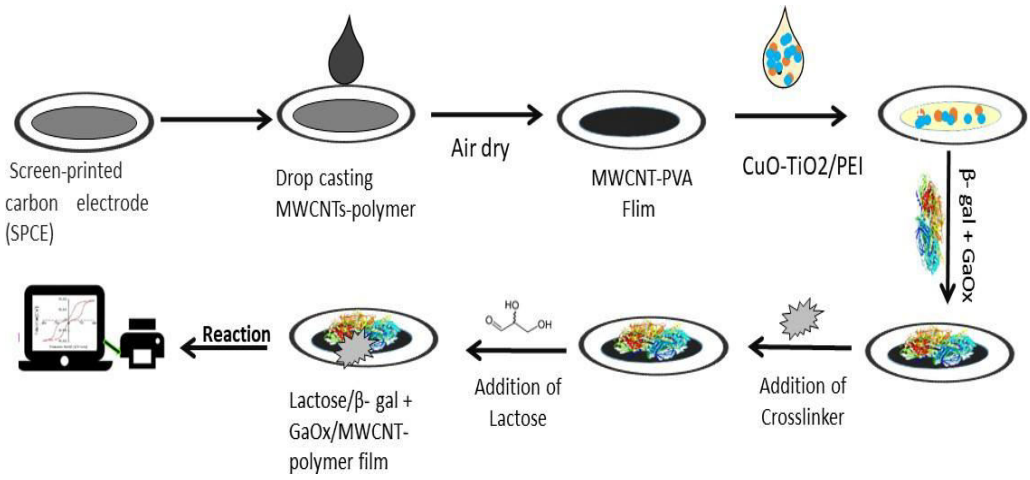
CHAPTER 3

DEVELOPMENT OF LACTOSE BIOSENSOR USING IMMOBILIZED β-GALACTOSIDASE AND GALACTOSE OXIDASE WITH MULTI-WALLED CARBON NANOTUBES AND $CuO\text{-}TIO_2$

Research Highlights:

- Novel fabrication chemistry to design lactose biosensor.
- Functionalized MWCNTs and metal oxide nanoparticles (MNOPs) were incorporated into screen printed carbon electrode.
- The electrode showed linear range 0.05 to 1.5 mM for lactose detection and limit of detection 0.005 to 4 mM.

Graphical abstract:



3.1 Introduction:

Milk is an important source of human diet that supplies the disaccharide Lactose (β-D-galactopyranosyl (1 → 4) α-D-glucopyranose), calcium and dairy products. Lactase is a hydrolase that breaks down lactose into its monomers, glucose and galactose. This enzyme is found in the human digestive system, and most children start to produce less of it between the ages of 2-5 years. Majority of people only retain 10% of the lactase activity found in infants (Brito et al., 2021).

Lactose intolerance refers to the symptoms reported by individuals who experience difficulty digesting lactose after consuming milk and dairy products. When ingested, lactose reaches the small intestine where it remains incompletely hydrolysed, triggering symptoms of differing intensities such as nausea, abdominal pain, diarrhoea, flatulence, and others. These symptoms typically manifest around 30 min. to 2 h. following the consumption of lactose-containing foods (Luthy et al., 2017, Troise et al. 2016). In this scenario, individuals with lactose intolerance commonly decrease or eliminate their consumption of milk and milk products. This cautious approach is adopted due to the possibility that ingesting lactose, depending on the dosage, could lead to gastrointestinal discomfort in a short timeframe (K. Pawłowska et al., 2016).

As a result, dairy industries have recognized this situation and are directing resources towards the production of milk and other related products with reduced lactose content or zero lactose options as special purpose foods. (Monti L et al., 2017; Brito et al., 2019). Therefore, it is crucial to identify the amount of lactose in dairy products.

There are several methods available for lactose detection, including spectrophotometry, polarimetry, infrared spectroscopy, titration, and chromatography (Claudia et al., 1998; Conzuelo et al., 2010). The challenges allied with these techniques, such as the lack of easy and continuous monitoring, dependence on expensive equipment, need for skilled professionals, use large amounts of reagents and elaborate sample preparation.

Therefore, electrochemical methods emerge as an efficient solution, offering results that are according to traditional analysis techniques in terms of precision, selectivity and specificity

(Handan et al., 2002; Thanh et al., 2013). Among the electrochemical methods, the application of electrochemical biosensors becomes an effective alternative. It is a device capable of providing real-time information about the system studied. They are very sensitive, economic, providing the possibility of miniaturization and automation (Thakur and Ragavan 2013; Mortari and Lorenzelli 2014).

Enzyme immobilization is a fundamental step for the development of amperometric biosensors since it can enhance enzyme stabilization facilitate continuous operation ((Brito et al., 2021; Brito et al., 2019). Recently, carbon nanotubes have been applied as matrices for enzyme immobilization augmenting the performance of the electrodes, due to their excellent properties of high surface area, electrical conductivity, chemical stability, and electrochemical properties (Gupta et al., 2016).

Various methods have been developed to detect lactose. Marrakchi et al investigated the development of a biosensor that integrates two distinct enzymatic activities, β-galactosidase (β-gal) and glucose oxidase, to quantitatively detect lactose in milk. The enzymes were immobilized in films using glutaraldehyde. Tasca et al. developed a third-generation biosensor utilizing single-walled carbon nanotubes in the electrode modified with cellobiose dehydrogenase and aryl diazonium for lactose determination.

In this work, functionalised multiwalled carbon nanotubes were used as a matrix, for the immobilization of the β-gal and galactose oxidase (GaOx) enzymes by adsorption, subsequently metal oxide nanoparticles (MONP) were used to facilitate the reaction. Thus, the objective of this work is to develop an efficient electrochemical biosensor based on MWCNTs/MONPs to detect lactose.

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-gal}} \text{D-Glucose} + \text{D-Galactose} \quad (3.1)$$

$$\text{Galactose} + O_2 \xrightarrow{\text{GaOx}} \text{d- Galacto- hexodialdose} + H_2O_2 \quad (3.2)$$

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \quad (3.3)$$

As shown in the chemical equations, in the enzymatic cascade involving beta-galactosidase and galactose oxidase, lactose undergoes hydrolysis catalyzed by beta-

galactosidase, yielding D-glucose and D-galactose in the presence of water (Equation 3.1), breaks down lactose into its constituent monosaccharides. Subsequently, D-galactose produced from lactose **Equation 3.1** reacts with molecular oxygen under the catalysis of galactose oxidase to form D-galacto-hexodialdose and hydrogen peroxide (H2O2), as showed in **Equation 3.2.** Lastly, the generated hydrogen peroxide participates in a redox reaction involving molecular oxygen, protons, and electrons, as shown in **Equation 3.3**. Electrones produced during this reaction are measured by the electrode.

3.2. Materials and Methods:

3.2.1. Reagents and Apparatus

The enzymes β-Galactosidase (β-Gal) from *Aspergillus oryzae* (EC 3.2.1.23, ≥8.0 units/mg solid, G5160) and Galactose oxidase (GaOx) from *Dactylium dendroides* (EC 1.1.3.9, ≥3,000 units/g solid, G7400) and PEI (Polyethylene imine) solution 50 % (w/v) were purchased from Sigma Aldrich, India. MWCNTs were purchased from Ad nanotechnologies, India. Glutaraldehyde (GA - 03965), polyvinyl alcohol (PVA - 0531500500) and uric acid (LOT – 17606) were obtained from LOBA Chemie Pvt. Ltd., India. Na2HPO4 (sodium phosphate dibasic anhydrous - 1944143), NaH2PO4 (sodium phosphate monobasic anhydrous - 59443), BSA (85171), KCl (84984) and Potassium ferricyanide (K3 [Fe (CN6)]- 59558), nano-TiO2 suspension (94632-15 nm) and Copper (II) oxide (CuO – 544868 - < 50 nm), lactose (124936), dextrose (51758) and D (-) fructose were purchased from SRL Pvt. Ltd., India. Screen-printed carbon electrodes were purchased from PalmSens BV, Netherlands.

All reagents, except for multi-walled carbon nanotubes (MWCNTs), were of extra pure grade and used without further purification. Autoclaved Milli-Q water was employed throughout the experimental procedures.

3.2.2 Apparatus:

Electrochemical measurements were performed on EmStat3+ electrochemical workstation (PalmSens BV, Netherlands) to carry out CV measurements. Cyclic voltammetry was carried out from -0.3 to + 0.6 V at 0.1 V/s scan rate on screen printed carbon electrode in 50 mM $K_3[Fe(CN_6)]$ with 0.1 M KCl in 50 mM phosphate buffer, pH 7.0. Ultrasonicator bath was used to sonicate MWCNTs in PVA polymer to prepare a suspension.

3.2.3. Fabrication of electrochemical biosensor

In the present study, novel fabrication chemistry to coat the electrode combined the use of carboxylic acid modified MWCNTs and CuO and nano-TiO_2 dispersed into PEI. Screen printed carbon electrode was used to develop the electrochemical sensor, which contains the working, reference and counter electrodes on the same chip. In this study, a suspension of Multi-Walled Carbon Nanotubes (MWCNTs) was prepared by dispersing 1 mg of MWCNTs into 0.5 ml of 1 mg/ml polyvinyl Alcohol (PVA) solution. The suspension sonicated for a duration of 3 h. in an ultrasonicator bath. 0.03 % (w/v) of polyethyleneimine (PEI) stock solution was prepared to disperse a total 2.5 mg of titanium dioxide (TiO_2) and copper (II) oxide (CuO) nanoparticles in the ratio of 2:1. To develop electrode, 4 µl of the MWCNT in PVA solution was deposited onto a Screen-Printed Electrode (SPE) and subsequently air dried at room temperature. After that 4 µl of TiO_2-CuO/PEI solution was drop cast and air dried at room temperature. Then, 20 µL of 50 mM phosphate buffer, pH 7.0 solution containing β- Galactosidase (10 mg/ml) and galactose oxidase (10 mg/ml) in the ratio of 1:1, was layered on the electrode. The solution was allowed to air dry at RT. After that, 2 µL of 2.5% glutaraldehyde was added on to the electrode surface to crosslink the enzymes to PVA. The solution was allowed to air dry at room temperature. Then, the electrode was washed three times with phosphate buffer to remove excessive glutaraldehyde and again allowed it to air dry. The procedure was a modification of the previous work by the laboratory (Gupta et. al., 2018; Thakkar et. al., 2023). The electrode was subjected to pre-treatment by applying 10 µl of 1% bovine serum albumin (BSA) for 10 min. This procedure was applied to block nonspecific binding (Ishiwaka et. al., 2009). The electrode was washed with Milli Q water 3-4 times. The electrolyte solution in a 50 µL system of 50 mM K_3 [Fe $(CN)_6$] and 0.1 M KCl in

50 mM phosphate buffer (pH 7.0) was tested for change in the current at a scan rate of 100 mV/s. Then the electrode was washed with 50 mM phosphate buffer, pH 7.0. The electrode was air dried and stored at 4°C. The electrodes with and without enzyme immobilization were compared by running cyclic voltammetry from -0.3 to 0.6 V in 50 µl of electrolyte solution which contains 0.1 M KCl and 50 mM K_3 [Fe $(CN)_6$] prepared in 50 mM phosphate buffer, pH 7.0.

3.3. Results and Discussion:

3.3.1 Electrochemical behaviour of lactose electrode:

Electrochemical properties of the bare SPE, SPE/MWCNTs/PEI-TiO_2-CuO, SPE/MWCNTs/PEI-TiO_2-CuO/GA and SPE/MWCNTs/PEI-TiO_2-CuO/GA/β-gal-GaOx were studied by running cyclic voltammetry in 50 mM phosphate buffer, pH 7.0 (PB) containing 50mM K_3[Fe $(CN)_6$] and 0.1 M KCl at 100 mV/s scan rate. The current increased after layering MWCNTs. Due to nanoparticles on the electrode, the surface area was increased, resulting in enhanced electron transport between the electrode and electrolyte solution. Following it, PEI-TiO_2-CuO was layered on the electrode and was cross-linked with glutaraldehyde (GA). The current was increased after coating with PEI-TiO_2-CuO/GA layer compared to SPE/MWCNTs. The increase in the magnitude of current due to TiO_2 provides large surface area for CuO, which increases the electrochemical activity. After that, when enzymes were immobilized onto the surface of electrode using GA, the current decreased. Thus, β-gal and GaOx hinder the electron transfer involved in the redox process **(Fig. 3.1a)**.

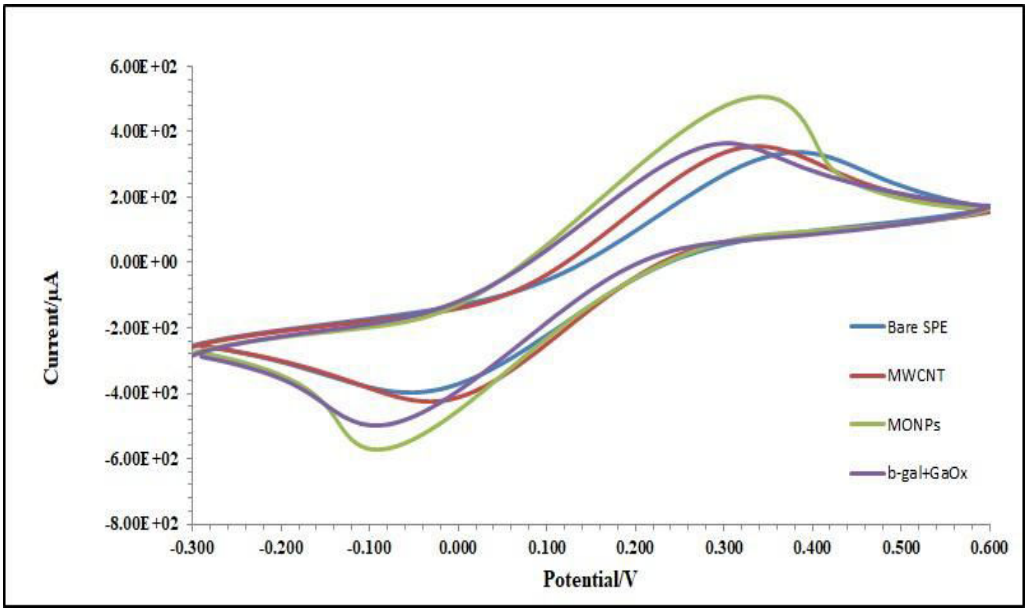


Fig. 3.1 (a). Cyclic voltammetry scans of bare SPE, SPE/MWCNTs, SPE/MWCNTs/CuO-TiO_2 and SPE/MWCNTs/CuO-TiO_2/GA/β-gal-GaOx electrode in 50 mM phosphate buffer, pH 7 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

The coated electrode was then tested for different scan rates from 25-250 mV/s in 50 mM phosphate buffer (50mM $K_3[Fe(CN)_6]$, 0.1M KCl, pH 7.0). From the observations in **Fig. 3.1(b).** it is evident that the height of redox peak current varies depending on the scan rate. **Fig.3.1(c)** showing the plot for the scan rate versus magnitude of anodic and cathodic peak heights, the R^2 values for anodic and cathodic peak heights were calculated and were found to be 0.9723 and 0.9927 respectively. Thus, at the cathodic peak 100 mVs^{-1} with a value was considered for further experiments to estimate lactose concentration. Further, the ratio of cathodic and anodic peak current (Ipc/Ipa) was found to be near to 1. The results agreed that the electron transfer largely occurs through surface-controlled mechanisms.

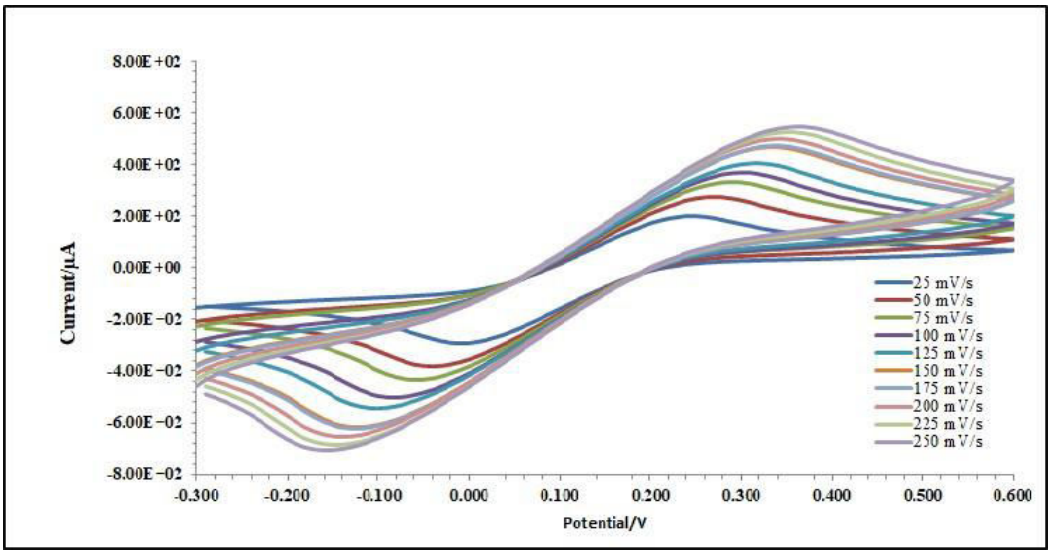


Fig. 3.1 (b). Cyclic voltammetry scans of electrode (SPE/MWCNTs/CuO-TiO_2/GA/β-gal-GaOx) at different scan rate ranging from 25 mV/s to 250 mV/s in 50 mM phosphate buffer, pH 7 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

The electrode was fabricated in triplicates to check the replicability of the sensor. The current response of each electrode was observed in 50 mM phosphate buffer (50 mM $K_3[Fe(CN)_6]$, 0.1 M KCl, pH 7.0) at a 100 mV/s scan rate. The sensor can be replicated as shown in **Fig. 3.2.**

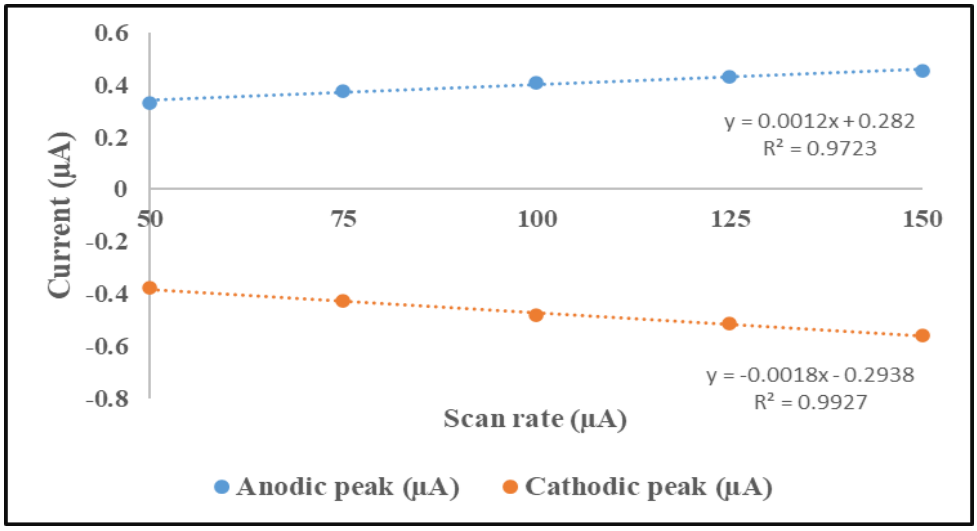


Fig. 3.1 (c). Plots of corresponding anodic and cathodic peak of current vs scan rate.

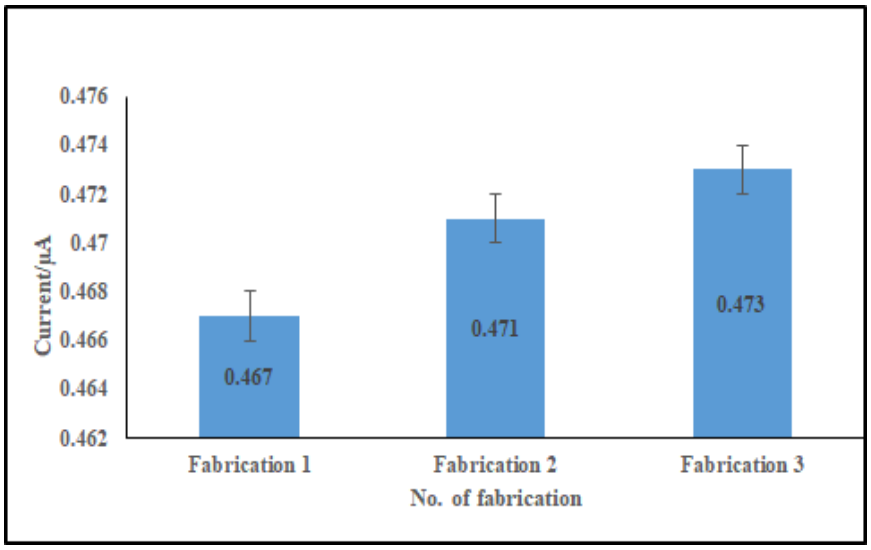


Fig. 3.2. Cathodic peak currents of laccase chip in 50 mM phosphate buffer, pH 7 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

3.3.2 Effect of pH on electrode:

Cyclic voltammetry was carried out at a scan rate of 100 mV/s to examine the effect of pH on the enzymes immobilised SPE in a solution of 50 mM $K_3[Fe\ (CN)_6]$ and 0.1 M KCl in 50 mM phosphate buffer solution in the pH range of 5.5 to 8.0 **(Fig. 3.3 a)**. The concentration of lactose

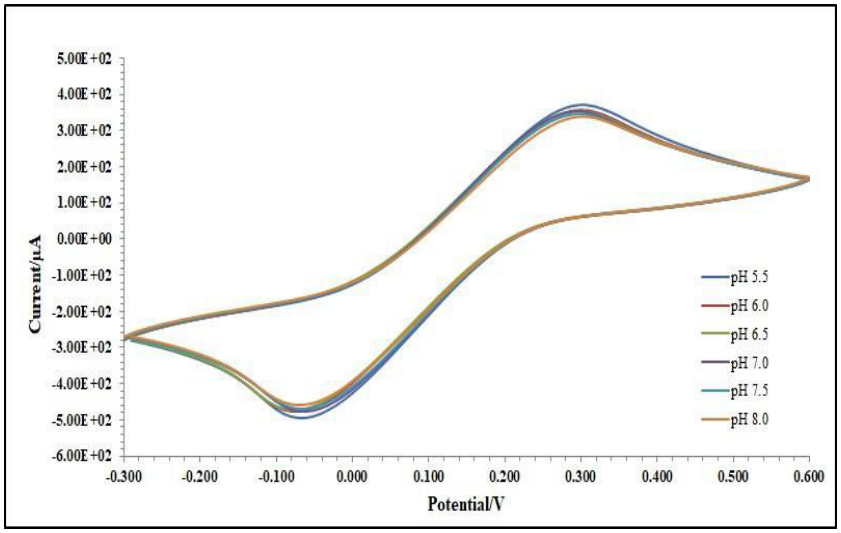


Fig. 3.3 (a). Cyclic voltammetry scans of lactose chip function of pH in 50 mM phosphate buffer with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

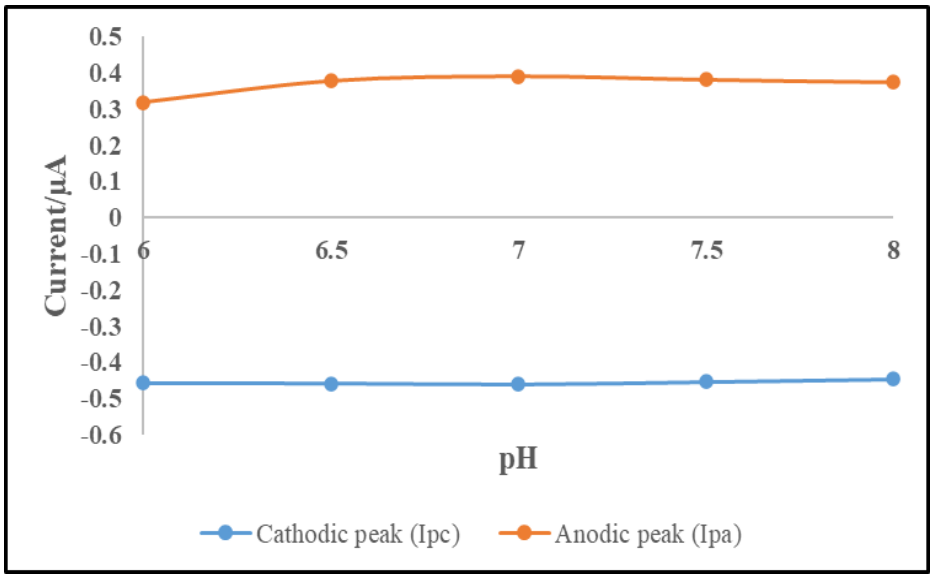


Fig. 3.3 (b). Effect of pH on the current density of anodic and cathodic peaks of lactose chip in 50 mM phosphate buffer with 50mM $K_3[Fe(CN)_6]$, 0.1 M KCl and 0.5 mM lactose at 100 mV/s scan rate.

0.5 mM in the experimental solution. The anodic and cathodic peak currents reduced when the pH of the solution rose from 5.5 to 8.0, which can be explained by a decrease in the number of positively charged moieties in the electrolyte. Also, it is evident from the graph **Fig. 3.3(b)** that pH 7.0 gives higher current at cathodic peak so cathodic peak was considered as the measure of magnitude of the current at pH 7.0 and was considered as optimal value for all further experiments.

3.3.3 Calibration curve for lactose using cyclic voltammetry:

Cyclic voltammetric response for different lactose concentrations was recorded in 50mM $K_3[Fe(CN)_6]$, 0.1 M KCl in 50 mM phosphate buffer (pH 7.0) at the scan rate of 100 mV/s. As the concentration of the analyte increases, cathodic peak current increases. This increase in magnitude of the current can be explained by the pH sensitive behaviour of the biosensor. Protons were released during the enzymatic reaction, which in turn altered the pH of the reaction system. This change in pH led to change in magnitude of the current. As seen in **Fig. 3.4**, change in the cathodic peak current magnitude with respect to the lactose

concentration followed linear regression model with the R^2 value of 0.9871. The lower and higher detection limits were found to be 5 µM and 4.0 mM, respectively.

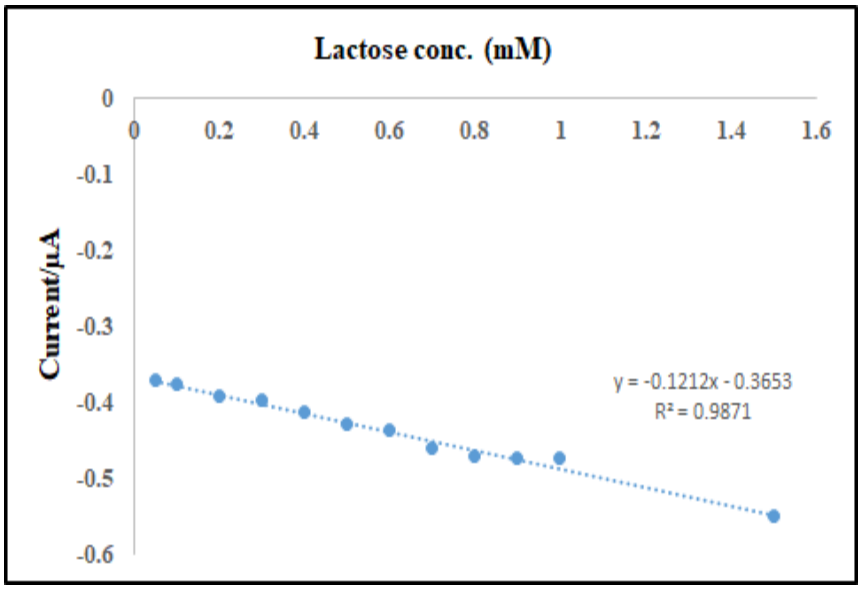


Fig. 3.4 Calibration curve of cathodic current vs the different concentrations of lactose in 50 mM phosphate buffer, pH 7 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

3.3.4 Selectivity study:

To estimate the selectivity of the biosensor, the impact of possible interfering substances was examined under ideal conditions. Current response of the biosensor was examined in a solution with the substrate and the interferents in the ratio of 1:1. Herein, the substances checked for interference was dextrose **Fig. 3.5**. The current decreases at cathodic peak form IPc -0.461 (Lactose) to -0.449 (Lactose/Dextrose) with the addition of the interferent used in the study.

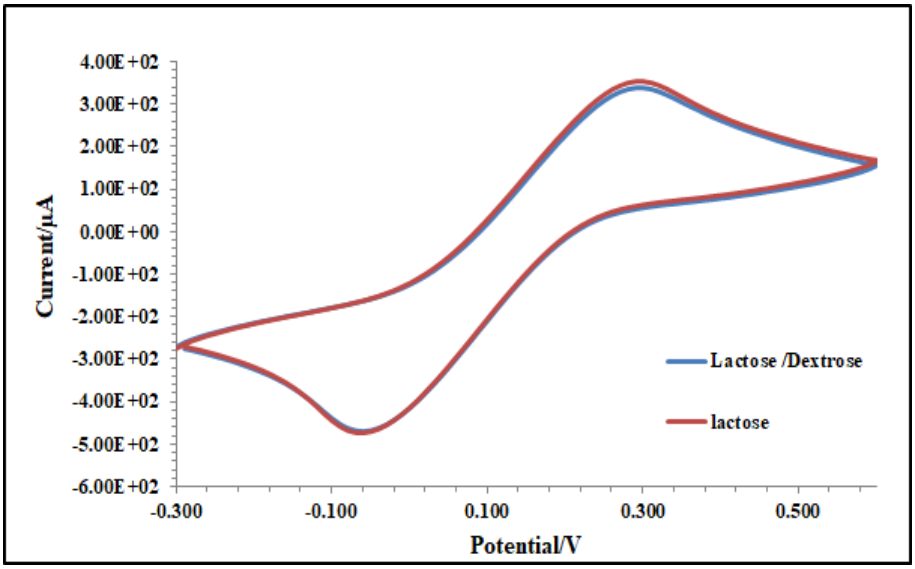


Fig. 3.5 Comparison of lactose chip for selectivity study using 1:1 concentration of substrates in 50 mM phosphate buffer, pH 7 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

3.3.5 Real sample detection:

The applicability of the biosensor with the target analyte was checked in the presence of milk. Amul taaza Milk was used as a real sample. Milk was diluted without any pre-treatment for real sample analysis. The results showed reliable current change at cathodic sweep segment of cyclic voltammogram. **Table**. **3.1** shows detection lactose of milk real samples using lactose biosensor.

Sample	Pre-treatment	Dilutions	Conc. (mM)	RSD %
Milk	None	1:10	0.675±0.00473	1.061
		1:100	0.575±0.003	0.69
		1:1000	0.44±0.00265	0.633

Table 3.1 Determination of lactose in milk samples using biosensor.

3.3.6 Conclusion:

In brief, a lactose biosensor was successfully designed using an innovative fabrication chemistry, establishing an effective connection between the enzyme's active site and the electrode surface. Enzymes were attached to the electrode via glutaraldehyde covalent crosslinking. The electrode exhibited linear response to lactose with the range of 0.05 to 1.5 mM, with a detection limit of 0.005 mM to 4 mM. Its specificity was assessed against interfering substances, indicating good specificity for lactose. Real sample testing further proven its performance. The biosensor's reproducibility was confirmed, suggesting its capability as a reliable tool for lactose detection.