International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Development and Characterization of a Visual Eco-Friendly, Cost Effective Glucose Biosensor Using Immobilized Glucose Oxidase on Natural Fibers

Dolly Sharma[1], Madhuri Das[2]

Gauhati University

Abstract: ***Enzymes must be immobilized into a matrix in order to construct a biosensor. The matrix that immobilizes enzyme permits exchange of substrate-containing media. For immobilization of enzymes number of matrices are available. Glucose oxidase, used in the food industry to measure glucose in processed foods, is used in our effort to fabricate and characterize a glucose biosensor. Synthetic materials are generally used to immobilize enzyme but are not biodegradable and produces large amount of waste. We have utilized natural materials as matrices for immobilization of enzyme, including pomelo shell, coconut fiber, onion inner membrane, eggshell inner-membrane, sugarcane fiber, and Luffa cylindrica. Nafion is utilized to attach the enzyme by cross linking to the matrix. Immobilized enzymes activity is monitored by employing substrates such as starch, glucose, honey, and sugarcane juice. The sugarcane juice atpH7.5-8.5, elicits the maximum activity, whereas glucose atpH6- 8, elicits the lowest activity. Matrices exhibit their maximum stability at higher pH values and lower substrate concentrations. With glucose substrate, the stability of the matrices was consistent for six hours. Additionally, we have tried to develop a color-changing optical glucose biosensor using the immobilized GOx enzyme, transforms from blue to colourless with glucose.***

Keywords: Glucose oxidase, enzyme immobilization ecofriendly matrix, visual biosensor

1. Introduction

The current demands of the world's biotechnological industries are enhancement in enzyme productivity and development of novel techniques for increasing their shelf life. One of such technique is enzyme immobilization technique. Enzyme immobilization provides an excellent base for increasing availability of enzyme to the substrate with greater turnover over a considerable period of time. Immobilization of enzyme is defined as the imprisonment of enzyme in a distinct support or matrix. The support or matrix on which the enzymes are immobilized allows the exchange of medium containing substrate or inhibitor molecules. Glucose oxidase (GOx) was used to develop a glucose biosensor, which is the most widely studied biosensor due to the importance in the monitor of blood glucose for treatment and control of diabetes. GOx (β-d-glucose: oxygen 1-oxidoreductase) catalyzes the oxidation of β-d-glucose to gluconic acid, by utilizing molecular oxygen as an electron acceptor with simultaneous production of hydrogen peroxide. Among the various classes of biosensors the amperometric transducer-based biosensors are widely acclaimed not only for their inherent potential to exhibit the improved functional properties such as fast, label free and sensitive detection of analytes but also for bearing the scope of scaling down their size with tailored low production cost, easy fabrication, and simple operation with low or no sample loss. The desired biological material (usually a specific enzyme) is immobilized by conventional methods (physical or membrane entrapment, non- covalent or covalent binding). There are several methods of monitoring of glucose such as chemical, chromatographic, spectroscopic etc. Although a few methods are reliable, they are complex, time consuming, expensive instrumentation and trained operator [Eun-Hyung Y and Soo-Youn L. 2010]. Such disadvantages can be overcome by using biosensor devices incorporating with enzyme as bio-recognition element.. GOx was used to develop a glucose biosensor, which is the most widely studied biosensor due to the importance in the monitor of blood glucose for treatment and control of diabetes [Eun-Hyung Y and Soo-Youn L. 2010]. GOx is an oxidoreductase which catalyzes the oxidation of glucose to gluconolactone. It is highly specific for β-D-glucose and does not act on α-D-glucose. This enzyme associated with FAD as cofactor which avidly bound to the protein matrix. GOx is widely used for the determination of free glucose in body fluids. In this study about GOx, immobilizing it in different matrices and using different types of substrates of GOx and designing of glucose visual biosensor. The matrices used are pomelo, coconut fiber, *Luffa cylindrica*, sugarcane fiber, inner membrane of onion, inner membrane of egg shell. I have used these matrices because these are naturally available, biodegradable, low cost, easily available and these matrices do not give out any toxic product after the enzyme-substrate reaction. During my study about immobilization of GOx into various matrices I have used the cross linking method for the immobilization to take place. For cross linking nafion is used. Nafion helps in cross linking of the matrix with the enzyme and do not let the enzyme to come out of the matrix. The cross linking method that is used for enzyme immobilization is an irreversible method of enzyme immobilization that does not require a support to prevent enzyme loss.

1.1 Materials Used for Preperation of Matrix

a) *Chemicals Used Sodium hydroxide* (2% w/v), sodium chloride (Nacl), potassium chloride(Kcl), disodium hydrogen phosphate(Na_2HPO_4), potassium dihydrogen phosphate(KH_2PO_4) , distilled water, PBS buffer, NaOH.
b) *Glasswares and Plastic Wares* Used Beaker, Measuring cylinder, conical flask, petri plate, glass rod, Spatula
c) *Instruments Used* Weighing balance, shaker, digital hot plate, hot air oven, Spectrophotometer, Vortex, weighing balance
d) *Matrices Used for Enzyme Immobilization*. These includes:

- Coconut fiber, Pomelo, Inner membrane of egg shell, Inner membrane of onion, *Luffa cylindrica*, Sugarcane fiber

e) IMMOBILIZATION OF GLUCOSE OXYDASE

- Materials Used for Immobilization, Chemicals Used: GOx, nafion, polyethylenimine, sodium Chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, PBS buffer (p^H ranges from 6,6.5,7,7.5,8,8.5).
- GLASSWARES AND PLASTICWARES USED: Beaker, micro pipette and tips, cuvate, ependroff tubes, petri plates, measuring cylinder.

1.2 Method of Preperation of Enzyme Immobilization Matrix

Coconut fiber was used for preparation of enzyme immobilization. Two methods were used for the preparation of matrix The two methods are described briefly below

1) Boiling: The coconut fiber was at first washed with water , then dipped in water and boiled for 2 hours. After boiling coconut fiber is dried in hot air oven for 1 hour and stored at room temperature.
2) Shaking by dipped in NaOH: coconut fiber is washed with water and then dipped in 2% NaOH and placed in shaker and shacked for 6 hours (Thais Milona de Souza Bezerra et al,2015). After shaking coconut fiber is again washed with distilled water and stored at room temperature by dipping into PBS buffer.

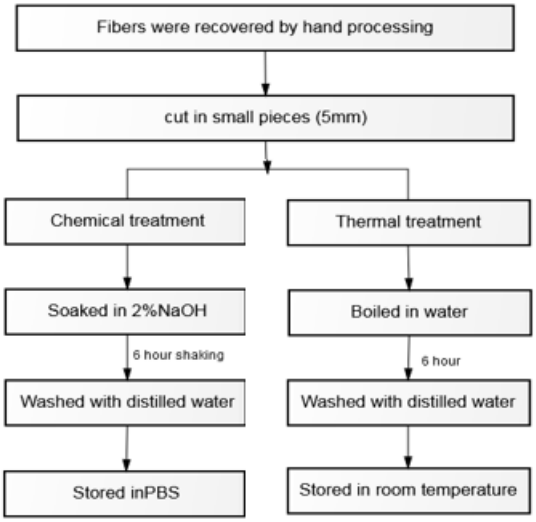

Figure 2: Flowchart showing matrix preparation from coconut fiber

Different parts of **pomelo** was used for the preparation of enzyme immobilization matrix, which are the whole shield of the pomelo, outer part of the shield and the spongy part of the shield. All the three matrices are prepared by using two methods. The two methods are described below briefly

1) Boiling: All the three parts of pomelo were at first washed with water, then dipped in water and boiled for 2 hours . After boiling all the parts are dried in hot air oven for 1 hour and stored at room temperature.
2) Shaking by dipped in NaOH: All the three parts of pomelo were washed with water and then dipped in 2% NaOH and placed in shaker and shacked for 6 hours (Non Fazelin Matzin et al,2014). After shaking all the parts are again washed with distilled water and stored at room temperature by dipping into PBS buffer.

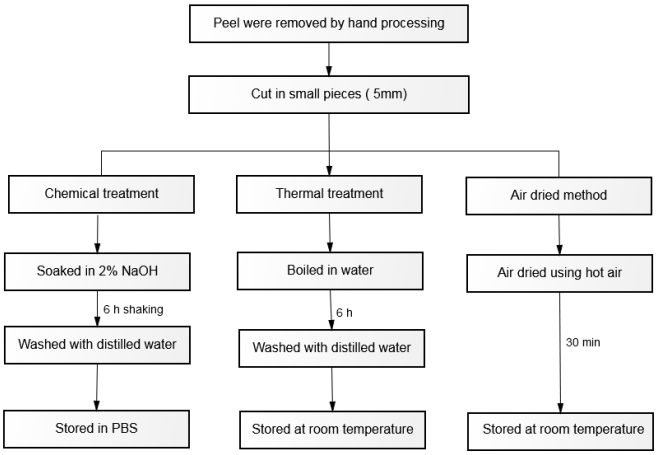

Figure 3: Flowchart showing matrix preparation from pomelo

The inner membrane of onion has been used for preparation of enzyme immobilization matrix. One method is used for the preparation of the matrix. The process is described below

1) Shaking by dipped in PBS buffer: The inner membrane of egg shell are washed with distilled water and then dipped in PBS buffer and allowed to shake foe 6 hours. After shaking is over, the membranes are washed with distilled water and stored at room temperature by dipping into PBS buffer(Matej Balaz,2015).

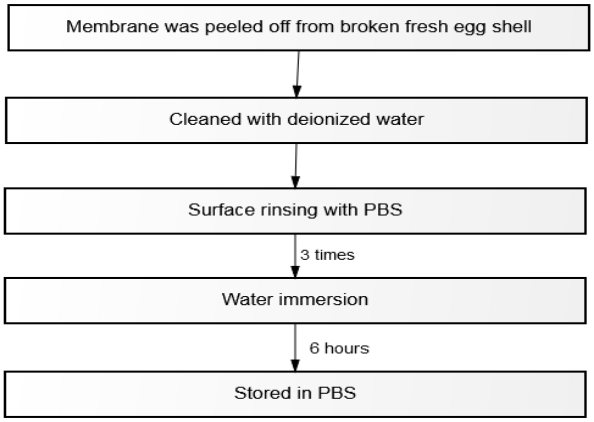

Figure 4: Flowchart showing matrix preperation from egg shell inner membrane

The inner membrane of onion has been used for preparation of enzyme immobilization matrix. One method is used for the preparation of the matrix. The process is described below

1) Shaking by dipped in PBS buffer: The peeled inner membrane of onion are washed with distilled water and then dipped in PBS buffer and allowed to shake foe 6 hours. After shaking is over, the membranes are washed with distilled water and stored at room temperature by dipping into PBS buffer.

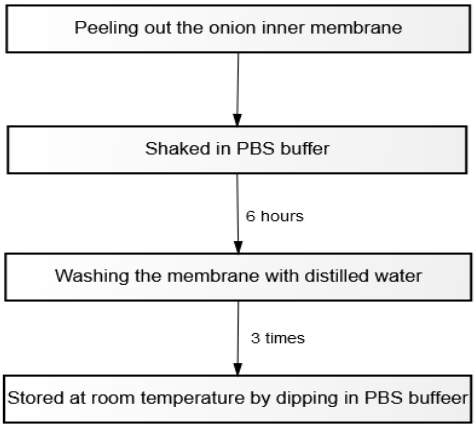

Figure 5: Flowchart showing matrix preparation from inner membrane of onion

The fibers of ***Luffa cylindrica*** has been used for preparation of enzyme immobilization matrix. One method is used for the preparation of the matrix,the process is described below:

1) Washing the *Luffa cylindrica* fruit: The fruits were decorticated and seeds were removed. Then the fruits were socked in tap water until the water becomes clear. Then these are dried and stored in polypropylene bag at room temperature.

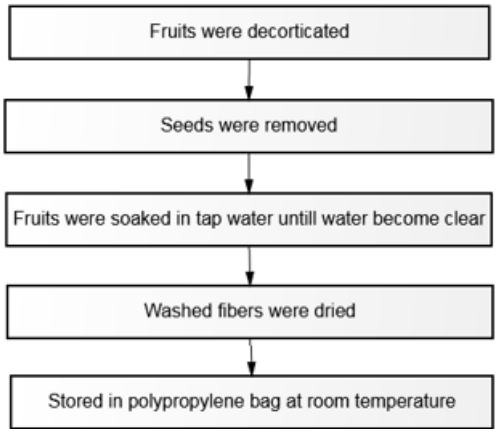

Figure 6: Flowchart showing matrix preparation from inner membrane of *Luffa cylindrica*

The fibers of Sugarcane have been used for preparation of enzyme immobilization matrix. One method is used for the preparation of the matrix.The process is described below:

1) Washing the sugarcane baggage with distilled water: The baggages were grounded. Then the baggages were socked in tap water until the water becomes clear. Then these are dried and washed with distilled water and stored at room temperature by dipping in PBS buffer.

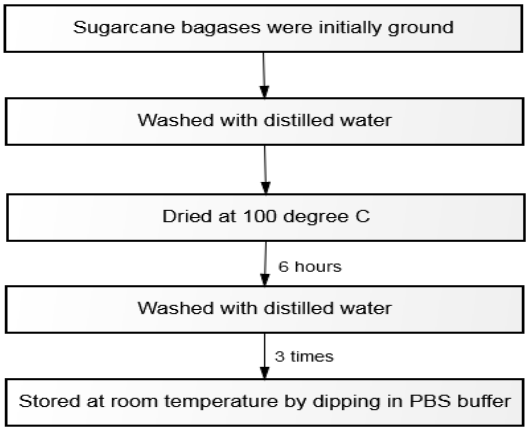

Figure 7: Flowchart showing matrix preparation from Sugarcane baggage

1.3 Method of Preperation of Chemicals for Immobilization of Glucose Oxydase

- Preparation of GOx 1mg of GOx is dissolved in 1ml of PBS buffer solution at -20^{o}C and stored at the same temperature.
- Preparation of Nafion 5% Nafion is dissolved in isopropanol and stored at 4^{o}C.
- Preparation of Polyethylinimine 50% polyehylinimine dissolved in distilled water and stored at room temperature.
- Preparation of Substrate Glucose has been used as substrate for the immobilization of GOx. For the preparation of substrate 10mg of glucose has been dissolved in 50ml of distilled water.

1.3.1 Method of Immobilization of Glucose Oxydase

The immobilization of GOx involves the following steps:

- Cut the matrices into small pieces and give a layer of nafion over it. Let it dry for 20-30 minutes at 4^{o}C.
- After 20-30 minutes, the enzyme solution given upon the matrix and let it dry for 30 minutes at 4^{o}C.
- After 30 minutes polyethylinimine solution is given over the matrix containing the enzyme and let it dry for 4-5 hours at 4^{o}C.
- After immobilization of enzyme, substrate is added to the enzyme and enzyme and substrate is submerged in PBS buffer of p^{H} ranging from 6, 6.5, 7, 7.5, 8, 8.5.
- Let the enzyme to react with the substrate for 30 minutes and then the absorbance are taken at a wavelength of 540nm.
- The p^{H} of the enzyme substrate complex are taken.

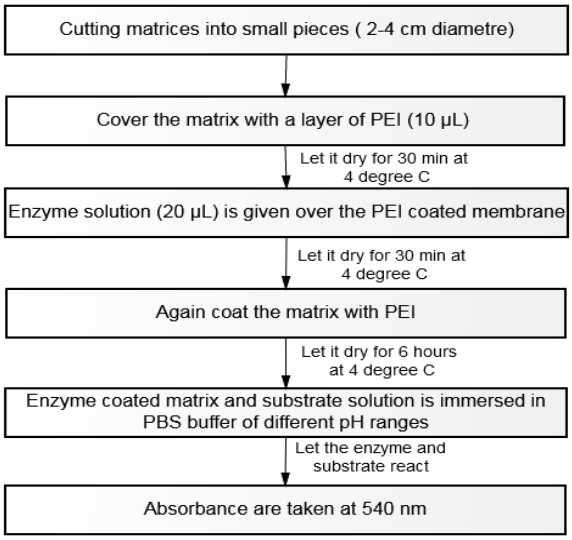

Figure 8: Flowchart showing method of immobilization of Gox

1.4 Detection of Glucose using Glucose Oxidase

When potassium per magnate is added to glucose in acidic environment in presence of GOx enzyme; hydrogen peroxidase gas releases and the color of potassium permanganate disappears which ends up with formation of potassium and manganese salt.

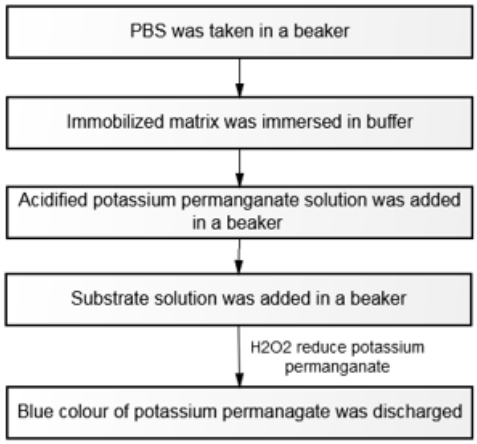

Figure 9: Flowchart showing the method of detection of glucose in acidic environment using GOx enzyme.

1.5 Detection of Glucose by Making A Visual Biosensor

For detecting glucose by glucose visual biosensor, at first the enzyme immobilized matrix is placed upon the support strip. Then acidified potassium permanganate is introduced into the immobilized enzyme and dried. When substrate solution is added to it, the blue color of potassium permanganate reduces and the support material becomes colorless due to release of hydrogen peroxidase and formation of manganese and sulfate salt as end product.

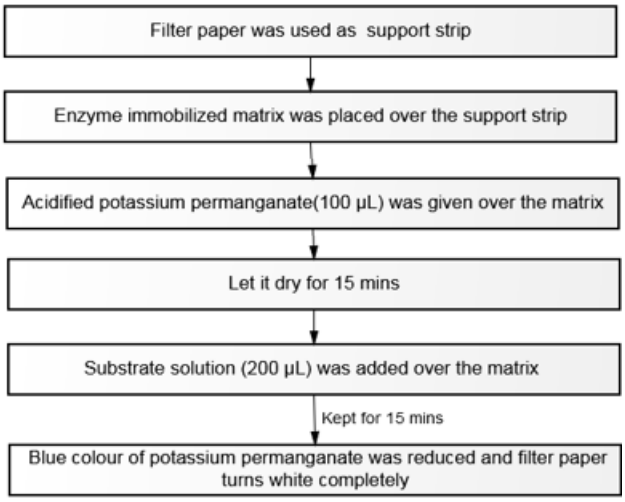

Figure 10: Detection of presence of glucose-by-glucose visual biosensor

1.6 Total No of Matrix Used for Immobilization of Glucose Oxydase

During the study period, a total of 4 enzyme immobilization matrices have been studied. All of the matrices are isolated from natural sources. The 6 matrices used for enzyme immobilization studies are pomelo, coconut fiber, inner membrane of onion, inner membrane of egg shall, sugarcane fiber and *Luffa cylindrica*.

1.7 Stability Period of the Matrices

As the first step of study, I have observed the stability of each of the matrix. The stability of the matrices are given below

Table 1: Stability period of each matrix

Matrix	Stability period (in months)
Coconut Fiber	10
Pomelo	7
Egg shell inner membrane	6
Onion inner membrane	1
Lufa Cylindrica	10
Sugarcane fiber	6

1.8 Absorbance of the Product of the Enzyme-Substrate Reaction after Enzyme Immobilization

After immobilization of enzyme, the it is added to the substrate and allowed the reaction to take place in PBS buffer of different p^H. The p^H of the buffer taken for the enzyme substrate reaction to take place are 6, 6.5, 7, 7.5, 8 and 8.5. I have used 4 substrates of glucose oxydase, which are glucose, honey, sugarcane juice and starch.

Table 2: Absorbance of the Product of Enzyme-Substrate Reaction at Different P^h of PBS Buffer on Pomelo Matrix at a Wevelength 540nm on all the Four Substrates

p^H	Absorbance			
	Glucose	Starch	Honey	Sugarcane
6	0.041	0.041	0.080	0.366
6.5	0.034	0.052	0.055	0.366
7	0.035	0.043	0.070	0.196
7.5	0.143	0.047	0.056	0.221
8	0.037	0.049	0.057	0.354
8.5	0.038	0.051	0.053	0.224

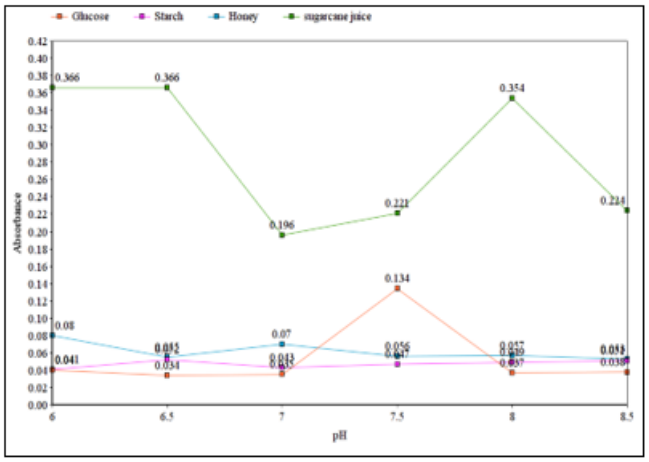

Figure 7: Effect of p^H graph

Table 3: Absorbance of the Product of Enzyme-Substrate Reaction at Different P^h of PBS Buffer on Coconut Fiber Matrix at a Wevelength 540nm on all the Four Substrates

p^H	Absorbance			
	Glucose	Starch	Honey	Sugarcane
6	0.051	0.061	0.065	0.158
6.5	0.049	0.057	0.063	0.165
7	0.055	0.061	0.067	0.248
7.5	0.049	0.063	0.067	0.419
8	0.061	0.057	0.057	0.320
8.5	0.039	0.050	0.063	0.315

Figure 8: Effect of p^H graph

Table 4: Absorbance of the Product of Enzyme-Substrate Reaction at Different P^h of PBS Buffer on EGG Shell Inner Membrane Matrix at a Wevelength 540nm on all the Four Substrates

p^H	Absorbance			
6	0.055	0.061	0.065	0.258
6.5	0.053	0.057	0.063	0.185
7	0.051	0.053	0.057	0.298
7.5	0.063	0.058	0.058	0.319
8	0.049	0.045	0.053	0.0315
8.5	0.045	0.055	0.091	0.0351

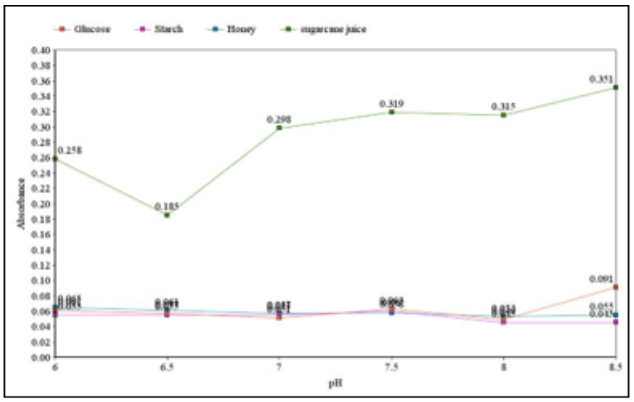

Figure 9: Effect of p^H graph

Table 5: Absorbance of the Product of Enzyme-Substrate Reaction at Different P^h of PBS Buffer on Onion Inner Membrane Matrix at a Wavelength 540nm on all the Four Substrates

p^H	Absorbance			
	Glucose	Starch	Honey	Sugarcane juice
6	0.041	0.060	0.068	0.158
6.5	0.072	0.055	0.055	0.125
7	0.052	0.059	0.050	0.198
7.5	0.043	0.053	0.057	0.219
8	0.055	0.048	0.055	0.115
8.5	0.048	0.048	0.059	0.151

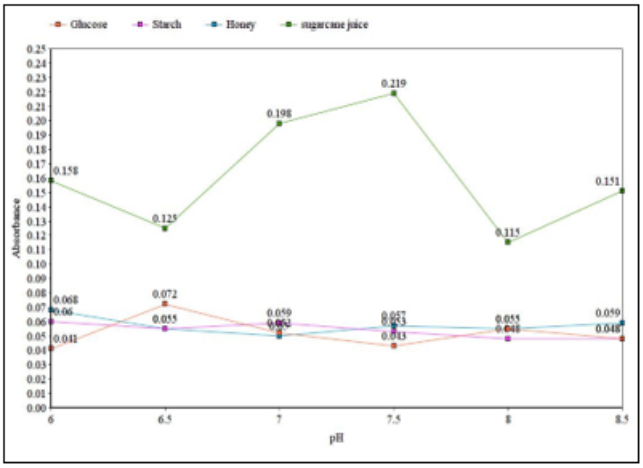

Figure 10: Effect of p^H graph

Table 6: Absorbance of the Product of Enzyme-Substrate Reaction at Different P^h of PBS Buffer on LUFA Cylindrica Matrix at a Wevelength 540nm on all the Four Substrates

p^H	Absorbance			
	Glucose	Starch	Honey	Sugarcane juice
6	0.275	0.08	0.091	0.091
6.5	0.048	0.048	0.048	0.958
7	0.049	0.049	0.049	1.082
7.5	0.051	0.051	0.051	0.719
8	0.046	0.046	0.046	0.809
8.5	0.48	0.048	0.048	1.315

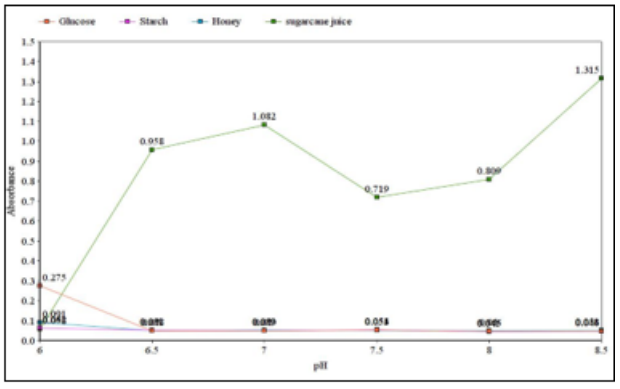

Figure 11: Effect of p^H graph

Table 7: Absorbance of the Product of Enzyme-Substrate Reaction at Different P^h of PBS Buffer on Sugarcane Fiber Matrix at a Wevelength 540nm on all the four substrates

p^H	Absorbance			
	Glucose	Starch	Honey	Sugarcane juice
6	0.057	0.062	0.067	0.103
6.5	0.057	0.052	0.052	0.093
7	0.046	0.046	0.067	0.374
7.5	0.045	0.045	0.061	0.349
8	0.044	0.046	0.069	0.300
8.5	0.046	0.046	0.064	0.423

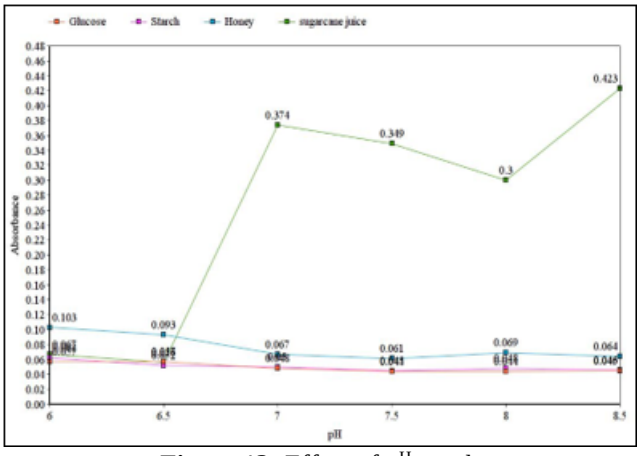

Figure 12: Effect of p^H graph

1.9 Detection of Glucose using GOx

It is a colorimetric method. In this reaction GOx in the presence of acidified potassium permanganate react with alcohol and release H_2O_2 which reduce $KMnO_4$, as a result the blue color of potassium permanganate solution is discharged and potassium sulfate and manganese sulfate are produced as end product.

$$2KMnO_4 + 2H_2SO_4 + 5H_2O_2 \longrightarrow K_2SO_4 + 2MnSO_4 + 8H_2O + 5O_2$$

Figure 13: Visual detection method of glucose using glucose oxidase

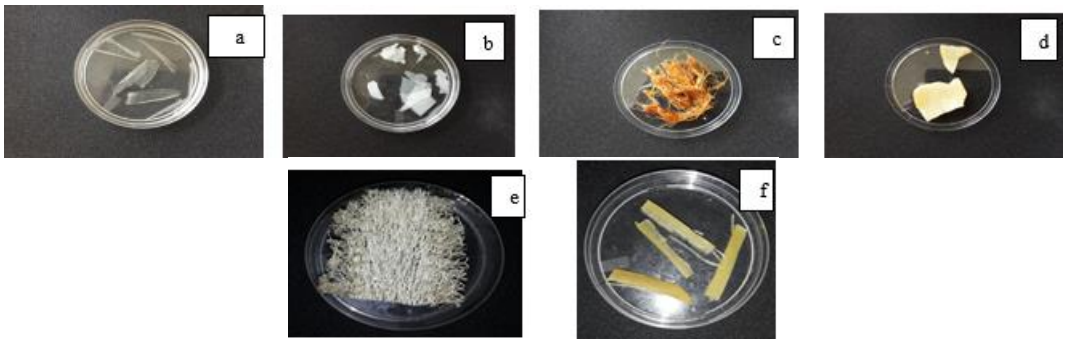

Figure 23: (a) Onion inner membrane matrix (b) Egg shell inner membrane matrix (c) Coconut Fiber matrix (d) Pomelo matrix, (e) *Luffa Cylindrica* (f) Sugar cane fiber

Figure: Glucose visual biosensor test on whatman filter paper

2. Results and Discussion

In our study, we prepared different natural matrices and each matrix showed stability for a different period of time. In which coconut fiber and *Luffa cylindrica* matrix has showed highest stability and onion inner membrane matrix has showed lowest stability. Immobilizing GOx enzyme on the pomelo, coconut fiber, onion inner membrane egg shell inner membrane, sugarcane fiber and *Luffa cylindrica* matrices, we found that *Luffa cylindica* showed highest activity with sugarcane juice substrate at a p^H of 8.5 and lowest activity with glucose substrate at a p^H of 6 , coconut fiber showed highest activity on sugarcane juice substrate at a p^H of 7.5 and lowest activity with glucose substrate at a p^H of 6, onion inner layer showed highest activity on sugarcane juice substrate at a p^H of 7.5 and lowest activity with glucose substrate at a p^H of 6, egg shell inner membrane showed highest activity with sugarcane juice substrate at a p^H of 8.5 and lowest activity with glucose substrate at p^H 6, pomelo showed highest activity with sugarcane juice at a p^H of 6 and 6.5 and lowest activity with glucose at a p^H of 6.5 and sugarcane fiber matrix showed highest activity with sugarcane juice at a p^H of 8.5 and lowest activity with glucose at a p^H of 8 . From the study we can conclude that matrices show highest stability at higher p^H and lower substrate concentration. Also, we have tried to prepare a colour changing visual glucose biosensor with the immobilized glucose oxidase enzyme, which changes its colour from blue to colourless in presence of glucose. This colour changing indicates the presence of glucose in a sample.

3. Conclusion

In our study, easily available, low cost, nontoxic, natural matrix which are coconut fiber, pomelo, onion inner membrane, egg shell inner membrane, *Luffa cylindrica* and sugarcane fiber are used for the immobilization of enzyme GOx. Among all these matrices, coconut fiber and *Luffa cylindrica* showed 83.33% stability, pomelo matrix showed 58.33% stability, egg shell inner membrane and sugarcane fiber matrix showed 100% stability and onion inner membrane showed 8.33% stability. The values on operational and storage stability were also found to be comparable to many reports on GOx. Thus, we can conclude that above mentioned matrices can be used as a potential immobilization support for enzyme. Thus, we conclude that these costeffective easily available materials can be a choice of immobilized glucose oxidase for large-scale application. For constructing visual biosensor, easily available filter paper was used as test strip and potassium permanganate as indicator and it show good result.

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