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Development and Characterization of a Visual Biodegradable Alcohol Biosensor Using Immobilized Alcohol Oxidase on Natural Fibers

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Abstract: ***The study focuses on the development of a cost effective and portable alcohol biosensor using alcohol oxidase AOx as a biocatalyst. AOx, isolated from yeast strains, efficiently catalyzes the oxidation of alcohol to aldehyde and hydrogen peroxide, which forms the basis of detection. Various natural materials such as coconut fiber, egg shell membrane, pomelo peel, onion inner layer, sugarcane bagasse, and luffa cylindrica were used as enzyme immobilization matrices. The immobilized enzymes exhibited high bioactivity at room temperature and physiological pH levels. The biosensor responds to ethanol with a visible color change, offering a simple, rapid, and equipment free method for alcohol detection. This low- cost biosensor is suitable for applications in clinical, forensic, and food analysis.***

Keywords: alcohol biosensor, alcohol oxidase, enzyme immobilization, ethanol detection, natural matrix

1. Introduction

The quantitative detection of alcohol with high sensitivity, selectivity and accuracy are required in clinical and forensic analysis, food, beverage industries etc. Many analytical methods based on chemical, chromatographic, and spectroscopic principles have been developed for the determination of alcohol. Although, a few of these methods are reliable, they are complex, time consuming and require prior separation processes, expensive instrumentation and trained operators. Such disadvantages may be overcome by using enzyme-based biosensors.

In an alcohol biosensor, two enzymes have been extensively used in the determination of alcohols, namely alcohol dehydrogenase (ADH) and alcohol oxidase (AOx). Alcohol dehydrogenase (ADH) has been widely studied to develop alcohol biosensors. Alcohol oxidase (AOx; Alcohol: O_2 oxidoreductase, EC 1.1.3.13) is an oligomeric enzyme. AOx consisting of eight identical sub-units arranged in a quasi-cubic arrangement and each subunit containing a strongly bound cofactor, flavin adenine dinucleotide (FAD) molecule. Alcohol oxidase has been isolated from several strains of yeast, such as Candida, Pichia, and Hansenula. AOx utilize methanol as a sole carbon and energy source. AOx catalyzes the oxidation of an alcohol to an aldehyde and hydrogen peroxide. Alcohol oxidase catalyzes the oxidation of short-chain, primary, aliphatic alcohols to their respective aldehydes (Das and Goswami 2013). It has the highest affinity for methanol. AOx is widely used for the determination of ethanol in many fields ranging from clinical analysis (e.g. blood, serum, saliva, urine, breath and sweat) to food and alcoholic beverages (wine, beer and spirits). Alcohol can be quantified by monitoring of O_2 consumption or H_2O_2 formation. Here, we try to develop a miniature and cost effective visual biosensor device using AOx as a biocatalyst. As miniature biosensor device require small sample volume and make the system cost effective and easy for transportation which are some advantages of that device.

One of the main steps for constructing novel, low cost, easily available biosensor is, to use of easily accessible, inexpensive, stable, suitable for regeneration and biodegradable, easily transportable matrix to immobilization of enzyme Visual detection method is a means of quantitative analysis by the naked eye through the comparison of color intensity or type of colour change. Owing to its simplicity, low-cost, rapid operation, and equipment-free, visual detection was widely used in the detection of numerous targets. (Xio- Ming Ma et.al 2018). A simple visual biosensor responds to ethanol via a colour change from green to blue, due to the enzymatic reaction of ethanol that produces acetaldehyde and hydrogen peroxide, when the latter oxidizes the PANI film. (B. Kuswandi et.al. 2014)

Natural fibers are now emerging as viable alternatives to synthetic fibers either alone or combined in composite materials for various applications. In addition to their low density, availability and low cost, natural fibers are recyclable and biodegradable. Besides, natural fibers being eco-friendly are expected to give less health problems for the people producing the composites. In the case of natural fibers, presence of hydroxyl groups on the surface promotes anchoring of enzymes which can be enhanced by surface treatment of natural fibers in order to promote interfacial adhesion and improve the water resistance. Considering the above facts, this work focus attention on the studies of various natural materials as a matrix. Biosensors based on AOx are easy prepared since AOx uses only molecular oxygen (O_2) as the cofactor. O_2 is involved in the reaction of oxidation of ethanol to acetaldehyde and hydrogen peroxide. Therefore, the catalytic reaction can be easily followed amperometrically. (K. Sevinc , and L. Toppare ,2015)

$$RCH_2OH + O_2 + AOx \rightarrow RCHO + H_2O_2$$

2. Materials and Methods

Enzymes are immobilized onto a matrix with the use of some type of polymer matrix. This polymer scaffold should keep the enzymes stable and allow for the facile diffusion of molecules and ions in and out of the matrix. Most polymers

used for this type of immobilization are nafion and Polyethyleneimine (PEI).

Nafion: It is a sulfonated tetrafluroethylene based fluoropolymer discovered in the late 1960s by Walther Grot of Dupont. Nafion is widely used as supporting matrix to confine biomolecules at the electrode surface in the construction of various biosensors (Fan and Harrison, 1992). It is an extremely suitable material for solubilizing MWCNT. (P.Vatsyayan et. al. 2010).

Polyethyleneimine (PEI):It is a poly-cationic polymer (R. Rochefort et al., 2008). PEI is use to activate supports and immobilize enzymes *via* ion exchange, as well as to improve immobilized enzymes by coating with PEI. PEI is a polymer containing primary, secondary and tertiary amino groups, having a strong anion exchange capacity under a broad range of conditions, and the capability to chemically react with different moieties on either an enzyme or a support. This polymer (in combination with other anionic ones) permits the generation of "saline" environments around enzyme molecules, improving enzyme stability in the presence of hydrophobic compounds. The use of PEI as a physical glue useful to crosslink enzyme subunits in multimeric enzymes, monomeric enzymes immobilized *via* physical interactions or production of enzyme multilayers will be specially emphasized as new open avenues for enzyme coimmobilization. (J. Ortize et.al. 2017)

2.1 Experimental Approach

Chemicals, reagent and apparatus used in matrix preparation

Distilled water, Sodium hydroxide, PBS buffer (p^H ranges from 6, 6.5, 7, 7.5,8, 8.5)**,** Alcohol oxidase from *Pichia pastories*, (Nf), Polyethylenimine (PEI) were bought from Sigma Aldrich, micro pipette, Glass rod, Beakers Petri dishes, cuvate, ependroff tubes, measuring cylinder, Analytical balance, Hot air oven, P^H meter, Hot plate, Shaker, Vortex, weighing balance, spectrophotometer, multimode reader.

2.2 Matrix Preparation

2.2.1 Methods

Matrix preparation

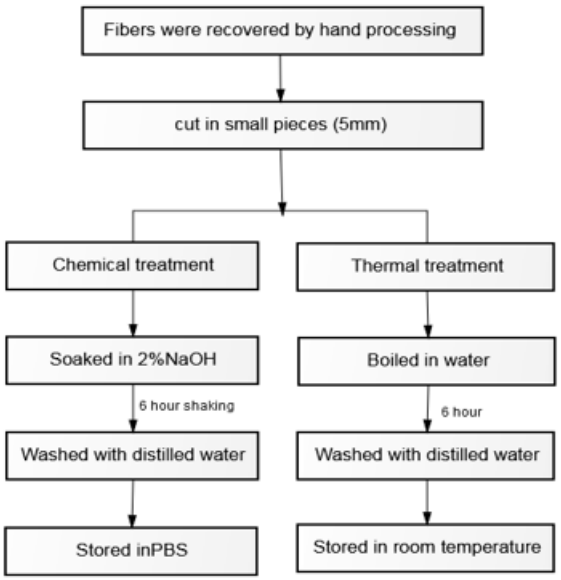
In my study I have taken three materials for matrix preparation. Those ares-

Coconut fiber, Egg shell membrane, Onion inner layer, Sugarcane fiber, *Luffa cylindrical*, Pomelo fruit peel

1) Coconut fiber preparation:

Coconut coir is one of the most versatile materials man has ever extracted from Nature. Coco fiber is 100% environmentally friendly. It is a renewable resource that is consistent in quality. Coco fiber has the best physical and chemical properties. For the preparation of matrix the fibers were recovered by hand-processing. After that step the fibers were treated by two different processes.

a) The first process is chemical treatment. In this process the fibers were soaked with NaOH 2% (w/v) for about 6h with shaking (Thais Milona de Souza Bezerra et.al. 2015). Residual solids were washed with water and stored in PBS at room temperature.
b) The second process is thermal treatment. In this process the fibers were boiled in water for 6h and dried in oven and stored in room temperature.



Flowchart 1: Coconut fiber matrix preparation method

2) Egg shell membrane preparation:

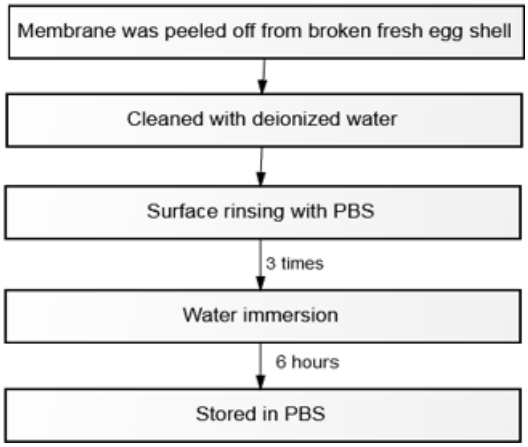
Eggshell membrane (ESM) is a unique biomaterial, which is generally considered as waste (M. Balaz, 2015). The egg-shell membrane is made with cross-linked protein fiber and exhibits exceptional gas and water permeability. The egg-shell membrane also possesses flexibility with aqueous solutions and provides a stable and biocompatible environment hence the immobilized enzymes can retain their shape and activity. An eggshell membrane was manually stripped from a broken fresh eggshell after removing albumen and yolk from the egg. The separated membrane was washed several times with DW .The cleaning procedure sequence were-

Surface rinsing with PBS $\xrightarrow{1min}$ Water immersing $\xrightarrow{2h}$

Surface rinsing with PBS $\xrightarrow{1min}$ Water immersing $\xrightarrow{2h}$

Surface rinsing with PBS $\xrightarrow{1min}$ Water immersing $\xrightarrow{2h}$

After completing the cleaning procedure the egg shell membrane were kept in phosphate buffer (pH -6.9) at room temperature until further use.

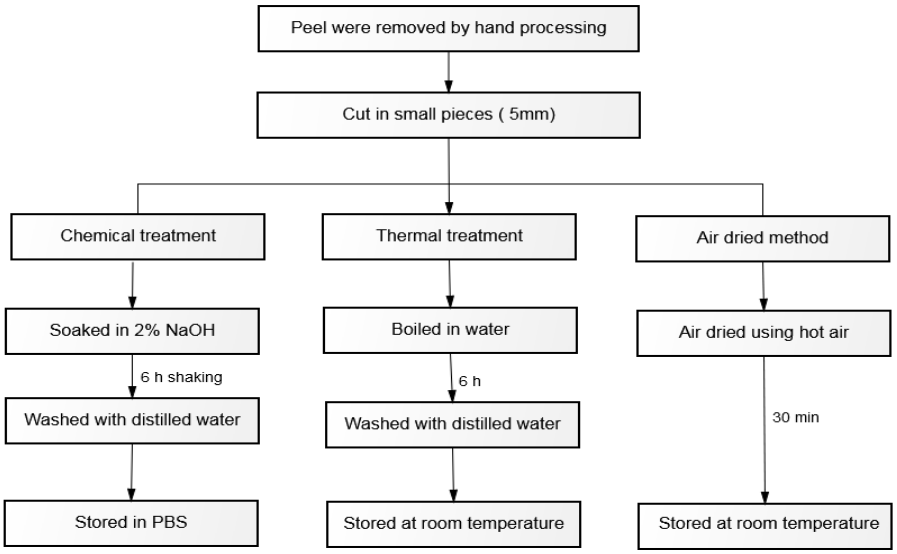


Flowchart 2: Egg shell membrane matrix preparation method

3) Pomelo peel matrix preparation:

The pomelo *(Citrus maxima)* is a natural (non-hybrid) citrus fruit. Pomelo has thick enveloping membranous material around the segments which is considered inedible, and thus is usually discarded. Pomelo peel is one of the under-utilized waste materials that have potential in the production of functional ingredients, due to its high fiber content (F. Matzain et.al, 2014). For the preparation of matrix the peels were removed by hand processing. I had taken three different layer of peel, those are - the outer thin layer, spongy layer and the whole peel. The peels were treated by three different methods.

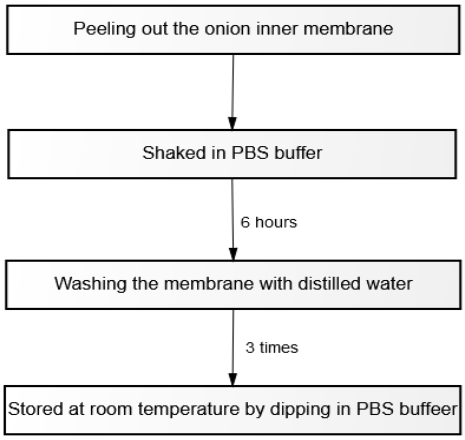
a) In the first process the layers were soaked in 2% NaOH for 6h with shaking. Residual solids were washed with water and stored in PBS at room temperature.
b) In the second method the layers were boiled with water for about 6h. Residual solids were washed with water, dried and stored at room temperature.
c) The third one was air dried method. In this process the layers were dried using hot air oven and stored.



Flowchart 3: Pomelo peel layers matrix preparation method

4) Onion membrane matrix preparation:

The inner layers of onion provide a protective layer against viruses and fungi that may harm the sensitive tissues. The clear epidermal cells exist in a single layer and do not contain chloroplasts. For the preparation of matrix the fresh inner layers of onion are peeling out by hand processing. The separated membranes were washed several times with DW. After that inner membrane were soaked in PBS buffer for 6 hours. Residue were washed with distilled water and stored at room temperature.

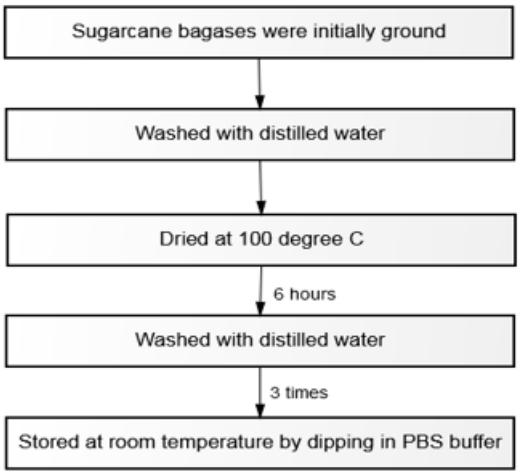


Flowchart 4: Onion inner layer matrix preparation method

5) Sugarcane matrix preparation:

Sugarcane bagasse, a lignocellulosic material, as a low-cost support. Lignocellulosic materials have been gaining importance in the industry, mainly as support for immobilization of enzymes and cells, considering their high

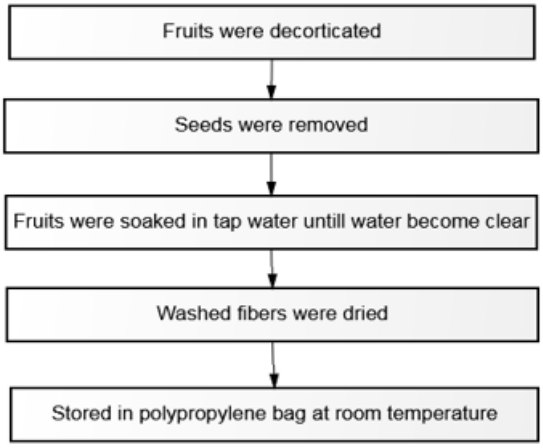
availability as an agricultural byproduct. (Nathalia L.F et.al. 2016).For the preparation of matrix sugarcane bagasse matrix first sugarcane bagasse were initially ground and washed with distilled water. After that bagasse were dried at 100^0C and stored at room temperature.



Flowchart 5: Sugarcane bagasse matrix preparation method

6) *Luffa cylindrica* matrix preparation:

Luffa cylindrica is a lignocellulosic material composed mainly of cellulose, hemicelluloses and lignin. Luffa sponge is very suitable for enzyme immobilization for industrial purposes (D. A.R. Mahmoud, 2011). For the preparation of matrix luffa sponge was soaked in tap water until water become clear and washed fiber were dried and stored in polypropylene bags.



Flowchart 6: *Luffa cylindrica* matrix preparation method

2.3 Immobilization of Alcohol Oxidase on Prepared Matrix

2.3.1 Methods of Preparation of Chemicals for Immobilization Of AOx

Preparation of AOx

2µl of AOx is dissolved in 1ml of PBS buffer (1M, p^H 7) at -20^0C and stored in the same temperature.

Preparation of Polyethylenimine (PEI)

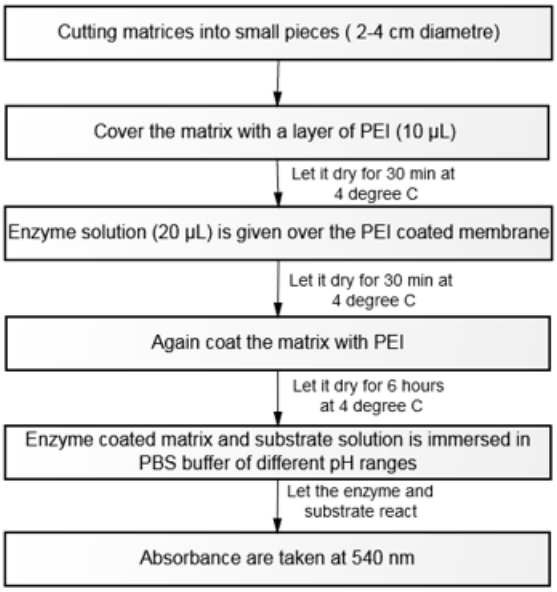
50% of PEI is dissolved in distilled water and stored in room temperature.

2.3.2 Method of Immobilization of Enzyme

Immobilization of AOx on different matrices

The immobilization of enzymes involves the following steps:

- Cut the matrices into small pieces and aliquot of 10 µl of PEI was dropped on each piece. Let it dry for 20-30 minutes at 4^0C.
- After 20-30 minutes, an aliquot of 20 µl of enzyme were dropped on semidried matrix and let it dry for 30 min in 4^0C.
- After 30 minutes PEI solution is given over the enzyme-PEI conjugated matrix and let it dry for 6 hours at 4^0C.
- After immobilization of enzyme, substrate is added in the beaker and enzyme immobilized matrix and substrate is submersed in PBS buffer of p^H ranges from 6, 6.5, 7, 7.5, 8, 8.5.
- Let the enzyme to react with the substrate for 30 min and then absorbance are taken at a wavelength 540 nm.

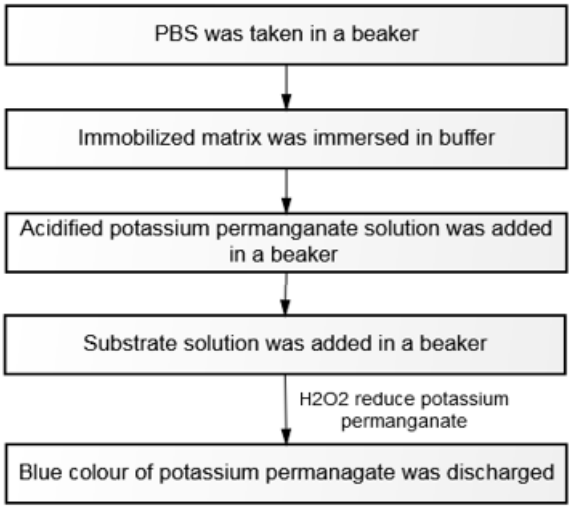


Flowchart 7: Enzyme immobilization method

2.4 Detection of Alcohol Using Alcohol Oxidase

2.4.1 Visual Detection of Alcohol

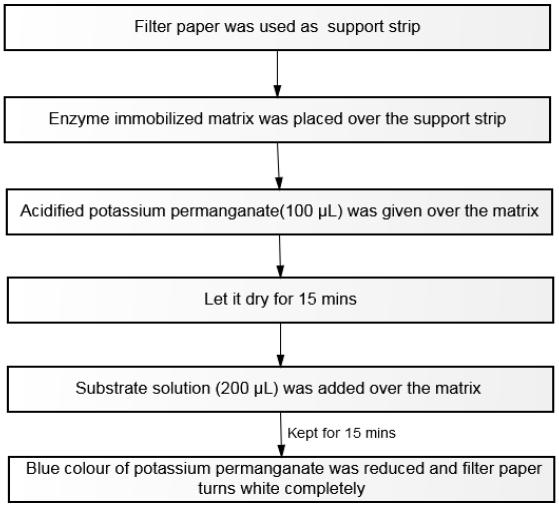
Visual detection method is means of quantitative analysis by the naked eye through the comparison of color intensity or type of colour change. In the colour changing reaction AOx in the presence of acidified potassium permanganate react with alcohol and release H_2O_2 which reduce $KMnO_4$ as a result the blue colour of potassium permanganate solution is discharged and produce potassium sulfate and manganese sulfate.



Flowchart 8: Colorimetric method for detection of alcohol

2.4.2 Visual Biosensor Method for Detection of Alcohol

For constructing visual biosensor, filter paper is use as test strip because of its inertness property. Test strips include an indicator which changing colour in response of a particular substance being tasted. Visual biosensor can be constructing in various steps, those are-



Flowchart 9: Visual biosensor preparation method

3. Results and Discussion

3.1 Total no. of matrix used for immobilization of Alcohol oxidase

During the study period, a total of 6 enzyme immobilization matrices have been studied. The 6 matrices used for enzyme immobilization studies are coconut fiber, egg shell membrane, Pomelo peel, onion inner layer, sugarcane bagasse, and *luffa cylindrica*.

3.2 Stability period of the matrices

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

Table 2: Stability period of the matrices

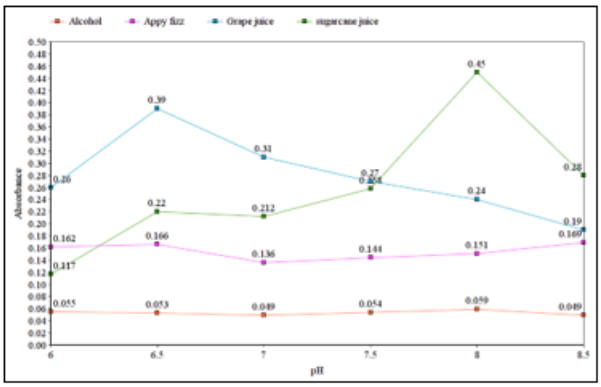
Matrix	Stability period (in months)
Coconut fiber	6
Egg shell membrane	5
Onion inner layer	4
Pomelo peel	3
Sugarcane bagasse	5
Luffa cylindrica	12

3.3 Absorbance of the product of the enzyme- substrate reaction after enzyme immobilization

After immobilization of enzyme, substrate is added and allowed the reaction to take place in PBS buffer of different p^H. The p^H of the buffers is 6, 6.5, 7, 7.5, 8 and 8.5. I have used Alcohol, Grape juice; Appy fizz soft drink (commercial apple juice) and Sugar cane juice as substrate for AOx.

Table 3: Absorbance of the product of enzyme- substrate reaction at different p^H of PBS buffers using Alcohol, Grape juice; Appy fizz soft drink (commercial apple juice) and Sugar cane juice as a substrate on coconut fiber matrix at a wavelength 540 nm

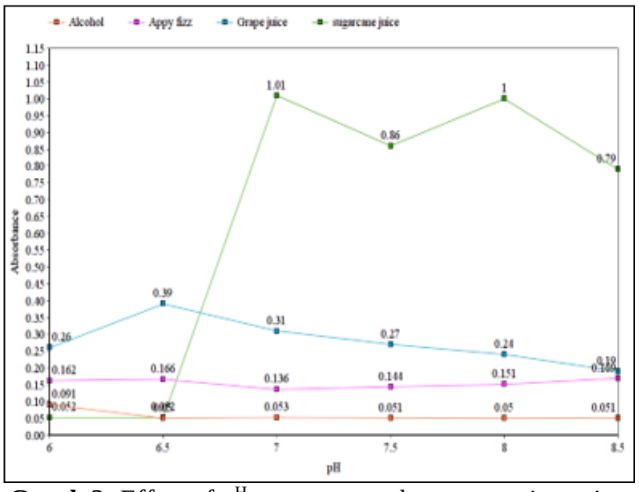
p^H	Absorbance			
	Alcohol	Grape juice	Appy fizz	Sugarcane juice
6	0.055	0.1620	0.26	0.117
6.5	0.053	0.166	0.39	0.22
7	0.049	0.136	0.31	0.212
7.5	0.054	0.144	0.270	0.258
8	0.059	0.151	0.24	0.485
8.5	0.049	0.169	0.19	0.28



Graph 1: Effect of enzyme-substrate reaction using coconut fiber

Table 3: Absorbance of the product of enzyme-substrate reaction at different p^H of PBS buffers using Alcohol, Grape juice; Appy fizz soft drink and Sugar cane juice as substrate on egg shell matrix at a wavelength 540 nm

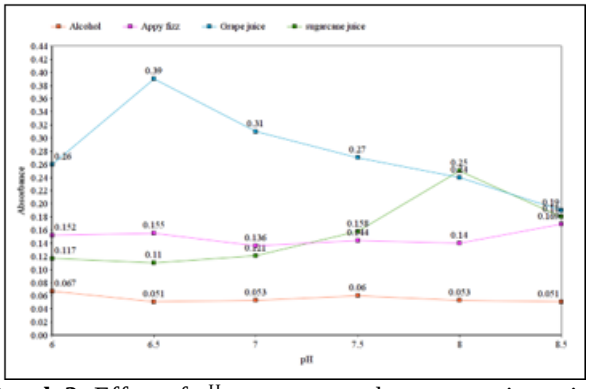
p^H	Absorbance			
	Alcohol	Grape juice	Appy fizz	Sugarcane juice
6	0.091	0.162	0.260	0.052
6.5	0.053	0.166	0.390	0.052
7	0.053	0.136	0.311	1.01
7.5	0.051	0.144	0.270	0.860
8	0.050	0.151	0.241	1.000
8.5	0.051	0.169	0.192	0.790



Graph 2: Effect of p^H on enzyme substrate reaction using eggshell membrane

Table 4: Absorbance of the product of enzyme- substrate reaction at different p^H of PBS buffers using Alcohol, Grape juice; Appy fizz soft drink and Sugar cane juice as substrate on Pomelo peel matrix at a wavelength 540 nm.

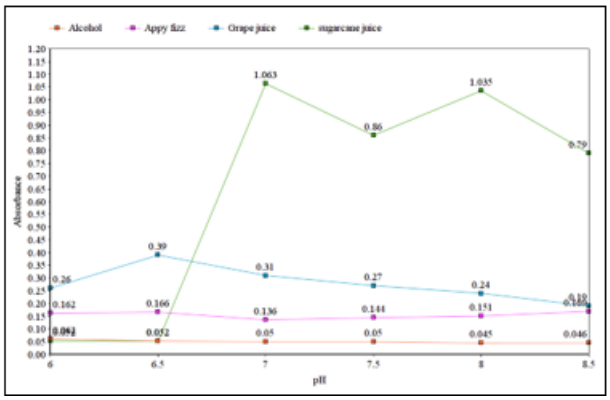
p^H	Absorbance			
	Alcohol	Grape juice	Appy fizz	Sugarcane juice
6	0.091	0.162	0.260	0.052
6.5	0.053	0.166	0.390	0.052
7	0.053	0.136	0.311	1.01
7.5	0.051	0.144	0.270	0.860
8	0.050	0.151	0.241	1.000
8.5	0.051	0.169	0.192	0.790



Graph 3: Effect of p^H on enzyme-substrate reaction using Pomelo peel

Table 5: Absorbance of the product of enzyme- substrate reaction at different p^H of PBS buffers using Alcohol, Grape juice; Appy fizz soft drink and Sugar cane juice as substrate on onion inner membrane matrix at a wavelength 540 nm

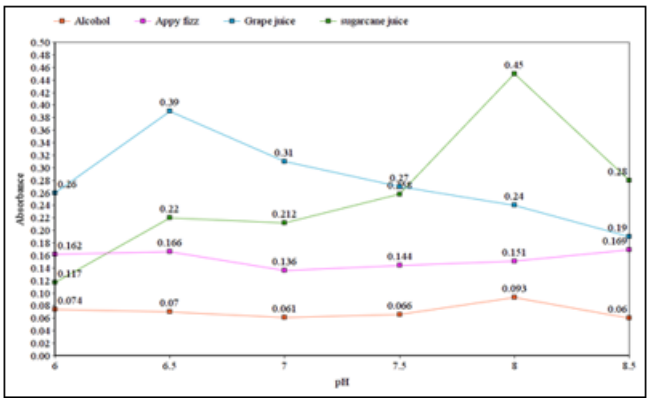
p^H	Absorbance			
	Alcohol	Grape juice	Appy fizz	Sugarcane juice
6	0.062	0.162	0.260	0.062
6.5	0.052	0.166	0.390	0.052
7	0.053	0.136	0.311	1.069
7.5	0.051	0.144	0.270	0.860
8	0.045	0.151	0.241	1.035
8.5	0.046	0.169	0.192	0.790



Graph 4: Effect of p^H on enzyme-substrate reaction using onion membrane

Table 6: Absorbance of the product of enzyme- substrate reaction at different p^H of PBS buffers using Alcohol, Grape juice; Appy fizz soft drink and Sugar cane juice as substrate on sugarcane bagasse matrix at a wavelength 540 nm.

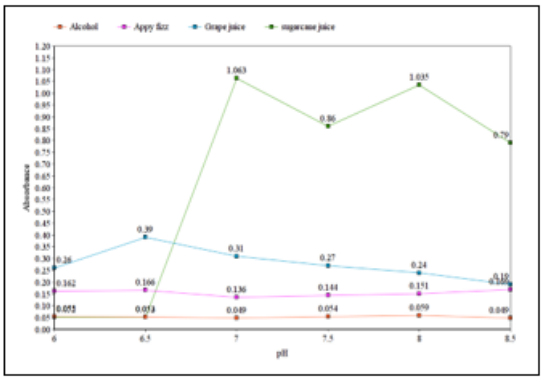
p^H	Absorbance			
	Alcohol	Grape juice	Appy fizz	Sugarcane juice
6	0.074	0.162	0.260	0.117
6.5	0.070	0.166	0.390	0.220
7	0.061	0.136	0.311	0.212
7.5	0.066	0.144	0.270	0.258
8	0.093	0.151	0.450	0.45
8.5	0.060	0.169	0.28	0.28



Graph 5: Effect of p^H on enzyme substrate reaction using sugarcane bagasse

Table 7: Absorbance of the product of enzyme- substrate reaction at different p^H of PBS buffers using Alcohol, Grape juice; Appy fizz soft drink and Sugar cane juice as substrate on *Luffa matrix* at a wavelength 540 nm

p^H	Absorbance			
	Alcohol	Grape juice	Appy fizz	Sugarcane juice
6	0.052	0.26	0.162	0.052
6.5	0.053	0.39	0.166	0.053
7	0.049	0.31	0.136	1.063
7.5	0.054	0.27	0.144	0.86
8	0.059	0.24	0.151	1.035
8.5	0.049	0.19	0.169	0.79



Graph 6: Effect of p^H on enzyme substrate reaction using *Luffa cylindrica*

3.4 Detection of alcohol using AOx

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

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

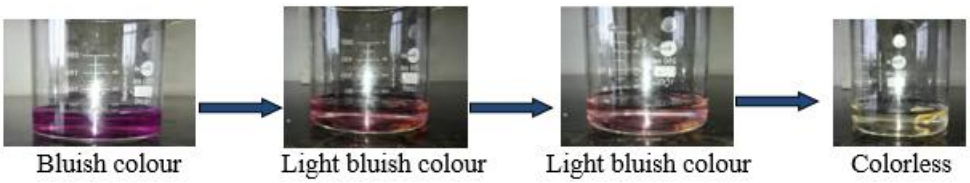


Figure 1: Visual detection method of alcohol using AOx

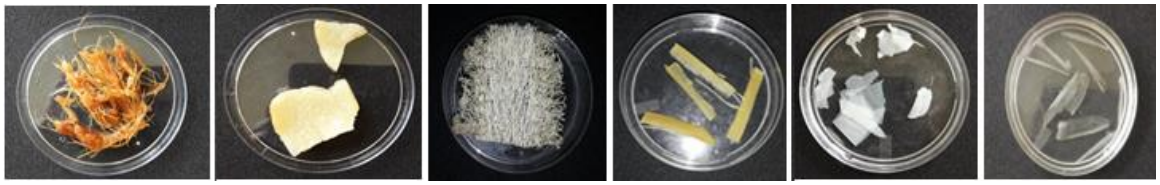
3.5 Detection of alcohol using visual biosensor

It is a method in which a disposable test strip is use for detecting ethanol in aqueous solution. The test strip includes an inert support pad, enzyme alcohol oxidase, and potassium permanganate that react with hydrogen peroxide to give a compound of changed color.

Figure 2: Visual AOx biosensor

In this study, we had prepared different natural matrices and its matrix shows stability for a different period. *Luffa cylindrica* show higher stability and Pomelo peel shows lower stability. After immobilization of AOx enzyme on the coconut fiber, egg membrane, Pomelo matrices, onion membrane, sugarcane bagasse and *luffa cylindrica*, I found that on coconut fiber matrix, for alcohol, appy fizz, grape juice and sugarcane juice enzyme shows highest activity at p^H range of 8, 8.5, 8, 6.5 respectively and shows lowest enzyme activity at p^H range of 8.5, 7, 6, 6 respectively. In Egg membrane, for alcohol, appy fizz, grape juice and sugarcane juice enzyme shows highest activity at p^H range of 6, 8.5, 6.5, 7 respectively and shows lowest enzyme activity at p^H range of 8, 7, 8.5, 6 respectively. In Pomelo peel, for alcohol, appy fizz, grape juice and sugarcane juice enzyme shows highest activity at p^H range of 6, 8, 8, 6.5 respectively and shows lowest enzyme activity at p^H range of 8.5, 7.5, 6, 8.5 respectively. In onion matrix, for alcohol, appy fizz, grape juice and sugarcane juice enzyme shows highest activity at p^H range of 6.5, 6.5, 6.5, 7 respectively and shows lowest enzyme activity at p^H range of 8, 8.5, 8.5 and 6 respectively. In sugarcane bagasse for alcohol, appy fizz, grape juice and sugarcane juice enzyme shows highest activity at p^H range of 6.5, 6.5, 8, and 6.5 respectively and shows lowest enzyme activity at p^H range of 8.5, 8, 6, 8.5 respectively. In luffa cylindrica, for alcohol, appy fizz, grape juice and sugarcane juice enzyme shows highest activity at p^H range of 6.5, 6, 6.5, 7 respectively and shows lowest enzyme activity at p^H range of 8.5, 7, 8.5, 8.5 respectively.

Thus, from the investigation we can confer that lower p^H increases the enzyme activity and higher p^H decreases the enzyme activity. For the detection of alcohol I have use colorimetric method in which potassium permanganate is use as an indicator.



(a) (b) (c) (d) (e) (f)

Figure 3: (A) Coconut fiber matrix, (B) Pomelo peel matrix (C) Egg shell matrix (D) Onion membrane matrix (E) *Luffa cylindrica* matrix, (F) Sugarcane matrix

4. Conclusion

An easily available and inexpensive matrix, coconut fiber, pomelo peel, egg membrane, onion membrane, sugarcane bagasse and *luffa cylindrica* were used for cross linking of alcohol oxidase. The immobilized enzymes possess good bioactivity and response at room temperature and physiological p^H. The values on operational and storage stability were also found to be comparable to many reports

on alcohol oxidase. Thus, we conclude that these nonexpensive, easily available materials can be a good choice of immobilized alcohol 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.

References

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