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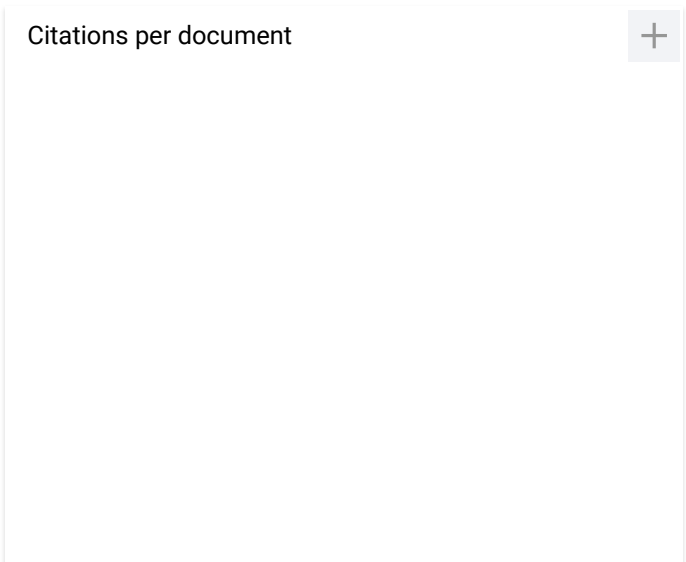
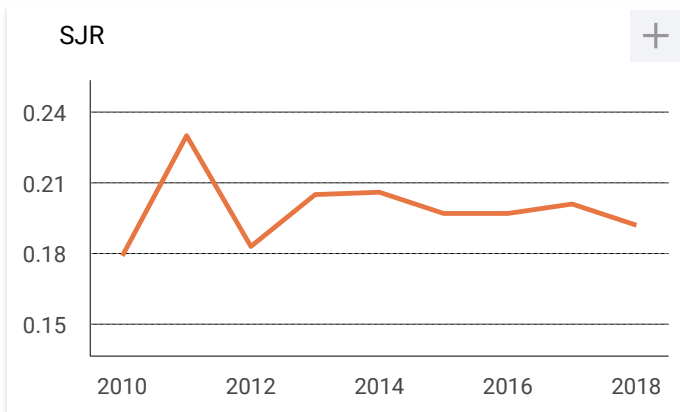


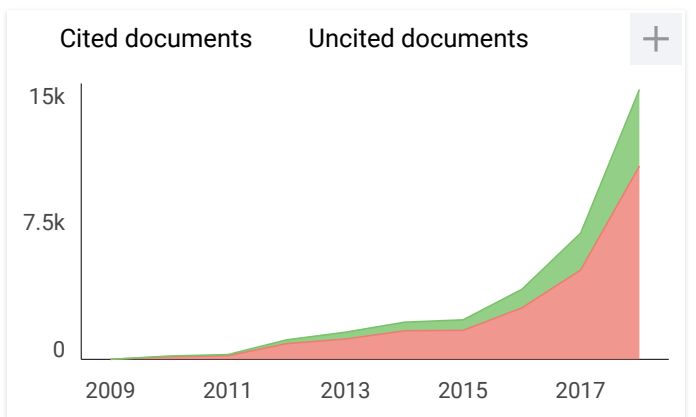
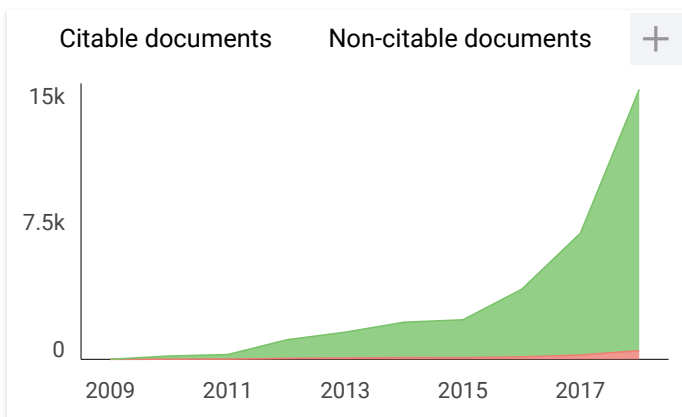
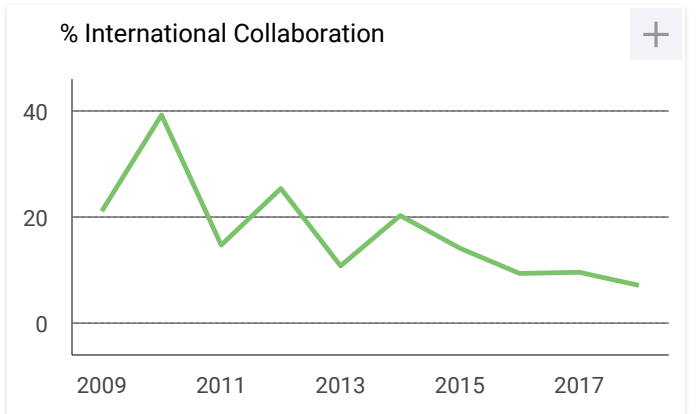
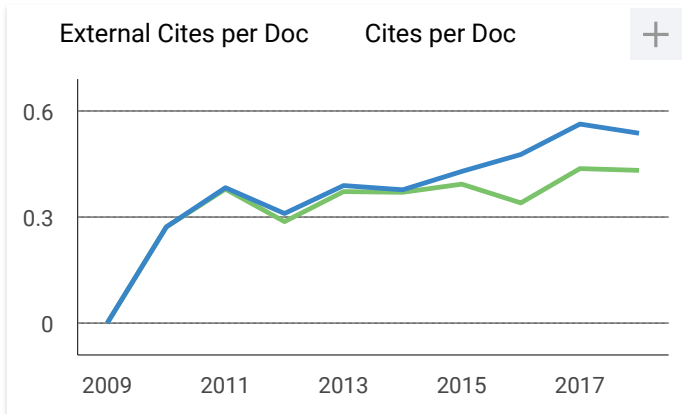
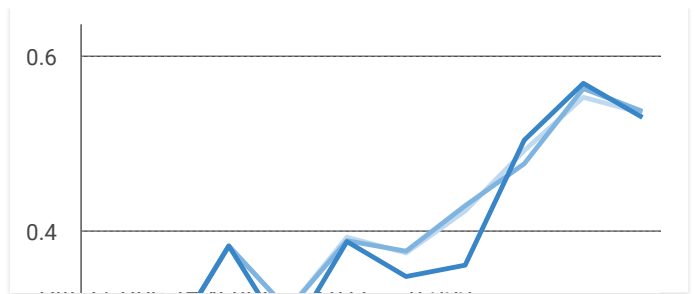
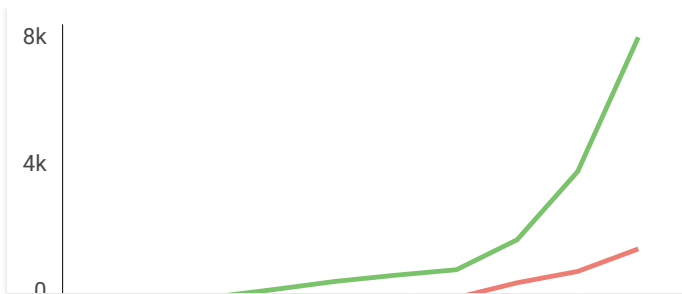
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Abstract. Wafer glucose oxidase (GOx) enzymes was produced by addition of PAH (Poly-Allyamine Hydrochloride) polymer into immobilized GOx enzyme on modified-Tetramethylammonium Hydroxide (TMAH) 5%-calsium-bentonite. The use of surfactant molcul (TMAH) is to modify the surface properties and pore size distribution of the Ca-bentonite. These properties are very important to ensure GOx molecules can be bound on the Ca-bentonit surface to be immobilized. The addition of the polymer (PAH) is expected to lead the substrates to be adsorbed onto the enzyme. In this study, wafer enzymes were made in various concentration ratio (Ca-bentonite : PAH) which are 1:0, 1:1, 1:2 and 1:3. The effect of PAH (Poly-Allyamine Hydrochloride) polymer added with various ratios of concentrations can be shown from the capacitance value on LCR meter and enzyme activity using DNS method. The addition of the polymer (PAH) showed effect on the activity of GOx, it can be shown from the decreasing of capacitance value by increasing of PAH concentration.

1. Introduction

Biocatalysis of redox enzymes has fascinated escalating attention for development of electronic biomaterials and devices for industrial, clinical and environmental applications [1]. One of the enzymes which are being extensively used is glucose oxidase (GOx). The GOx is widely used as a reagent in medical diagnostics [2,3], especially to determination of glucose in blood. However a major problem with the enzymatic methods is the high cost of the enzyme itself. Immobilized enzyme technology has been used to solve the problem [4]. Tests involving immobilized GOx have been used quite frequently in recent year, and one of the tests is immobilized GOx using surfactant-modified bentonite [5].

The method of sol-gel entrapment of biomolecules is a very promising technique of immobilization for biosensors construction, because of its simplicity, low temperature of the process, large amount and low leakage of entrapped material. When the sol-gel technique is used to construct the optical biosensors, the conditions of the process must allow obtaining transparent gels. A simple enzyme electrode can be obtained by immobilization of oxidase on the surface of oxygen electrode. The oxygen concentration depletion is proportional to the concentration of oxidase substrate. Local changes of oxygen concentration caused by enzymatic reaction are measured by oxygen electrode.



Enzyme immobilization matrix using polymer for the development of biosensors has been investigated by many researchers [6-8]. Conducting polymers are capable of incorporating different functionalities in their matrix during or after polymerization. The properties of synthesized polymer films are affected by electropolymerization condition. Many results of the synthesis of polymer films which can be used as a polymer matrix for immobilization of biocomponents have been reported [9,10]. Various conducting polymers have been considered for immobilization of enzymes [11,12]. Amperometric enzymatic electrodes based on GOx, which generates hydrogen peroxide in the presence of oxygen and glucose, are the most widely used for the measurement of blood glucose concentration. Several biosensors with the enzymatic method have been reported [13-17].

However, still it is essential to continue the research in this field with new material and approach, so that the sensitivity and stability of the sensor can be improved. In the present study, we used Ca-bentonite modified by Tetramethylammonium Hydroxide (TMAOH) as an immobilizer. We also used Poly-Allyamine Hydrochloride (PAH) as a polymer GOx matrix. The use of surfactant molecule (TMAH) is to modify the surface properties and pore size distribution of the Ca-bentonite. These properties are very important to ensure GOx molecules can be bound on the Ca-bentonite surface to be immobilized. The addition of the polymer (PAH) is expected to lead the substrates to be adsorbed onto the enzyme.

2. Experimental

Reagents: The enzyme glucose oxidase (GOD) from *Aspergillus niger* (273 U/mg) was obtained from Nacalai Tesque, Ca-bentonite from Pacitan, Indonesia, TMAOH 25%wt (E. Merck), Dinitrosalicylic Acid (Sigma-Aldrich), Sodium sulfite (E. Merck), D-Glucose p.a. (Nacalai Tesque), cellulose acetate (Sigma-Aldrich), PAH (Poly-Allyamine Hydrochloride MW 15.000) (Sigma-Aldrich), reagent Folin-Ciocalteu (E. Merck).

Apparatus: Amperometric (capacitance) measurement was carried out with LCR meter (DEKKO). Preparation of Immobilized GOx Wafer Enzyme: The preparation was divided into two steps; *first step* was preparation of immobilized GOx wafer enzyme in vary of Ca-bentonite:PAH (1:0, 1:1, 1:2, 1:3). The immobilization of GOx was followed the procedure which was described more detailed before [5]. To obtain Tetramethylammonium Hydroxide (TMAH) modified bentonite, 250 mL of several concentrations of TMAH solution (in range 0-5%) were heated until 75 °C and 5 g of bentonite was gradually added to it. Mixtures of heated TMAH solution and bentonite suspension were refluxed for 5 h. The solid phase was separated by filtration and washed with distilled water until the pH become 7.0 to remove the unabsorbed TMAH. The TMAH modified bentonite was dried overnight at a temperature of 100 °C. The modified bentonite powder was sieved with a 140 mesh sieve and the

resulting filtrate was used in further experiments. Immobilization of GOx was carried out by dissolving 0.2 g of TMAH-modified bentonite powder and 1 mL of GOx solution (100 IU) in 4 mL 0.1 M phosphate buffer pH 7.0. After being incubated at 20 °C and shaken with rotary shaker overnight, the bentonite enzyme dispersion was centrifuged at 4,000 rpm at 4 °C for 10 min. The supernatant was tested for Hartree Lowry protein assay to determine the amount of un-immobilized enzyme. The pellet was washed several times with phosphate buffer pH 7.0 until no protein was detected in the supernatant. The proteins detected in the supernatants from washing steps were also considered as un-immobilized enzyme.

Immobilization percentage was calculated using the equation below:

$$\% \text{ immobilization} = (\text{total amount of immobilized enzyme}) / (\text{total amount of initial enzyme}) \times 100\%$$

The total amount of immobilized enzyme was defined as total amount of protein in supernatant before immobilization minus the total amount of protein after immobilization. The total amount of initial enzyme defined as total amount of protein in supernatant before immobilization. The amounts of protein in the supernatant before and after immobilization were determined using Hartree Lowry's method using GOx as the standard. The quantity of protein immobilized on the support was calculated by subtracting the protein recovered from the combined washings of the modified and unmodified bentonite-enzyme complexes from the total amount of added protein.

The preparation of enzyme wafer was carried out by mixing the suspension of immobilized GOx with PAH solution. The mixture was then centrifuged for 7 h at 4°C. The pellet obtained was swollen in 0.1M phosphate buffer solution (pH 7). The wafer enzyme activity test was measured by DNS method and capacitance values. 500 µL of 1000 ppm of glucose substrate was mixed into 100 µL wafer enzyme, and then incubated for 30 min. After it was centrifuged, the supernatant was measured by DNS method. The second step was pre-design of wafer enzyme device and its performance test by capacitance measurement. The capacitance was measured by adding 100 µL of 1000 ppm of glucose substrate into 100 µL wafer enzyme in the chamber which was connected with LCR meter in room temperature.

3. Results and Discussion

3.1. Activity test of immobilized and free GOx

The use of Ca-bentonite as a support material for adsorption, catalytic and immobilization of enzyme has attracted interest in recent year. Modification of Ca-bentonite is required to increase its capability as a support material. In this study, modification of Ca-bentonite was carried out by intercalation of Ca-bentonite using 5% TMAH. Phenomenon of pores structure formation, very

important characteristic of Ca-bentonite as a support material, was characterized using FTIR and XRD which has been reported more detail before [18]. Nanostructuring is verified by microscopic inspection allowing studying locally the relevance of the intercalating agents for improvement of Ca-bentonite structure using SEM techniques. The SEM images of TMAH-modified Ca-bentonite in Figure 1 show that the porous structure of bentonite is improved by intercalation process using TMAH. SEM images of Ca-natural bentonite as shown in this figure reveal non-porous structure. On the other hand SEM images of TMAH-modified-Ca-bentonite reveal porous structure. It indicates that TMAH molecules may introduce inside the bentonite layer to improve the pore size distribution of the Ca-bentonite.

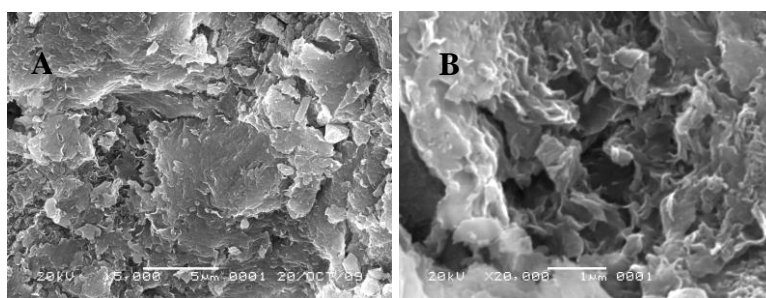


Figure 1. SEM images of Ca-bentonite (A), and intercalated bentonite using TMAH (B)

The modified Ca-bentonite was then used as immobilization material for GOx. The result of immobilized GOx is described in Table 1.

Table 1. Percentage of Immobilized GOx on 5% TMAH-Modified Bentonite

Replication	Initial GOx concentration (IU/ml)	Immobilized GOx (IU/ml)	% Immobilization
I	54.3	41.9	77.2
II	56.9	42.8	75.2
III	54.9	42.2	76.9

This result indicates that the immobilization GOx using 5% TMAH give stable and consistent result. The addition of TMAH into bentonite leads increasing the distance of bentonite layer. This is due to ammonium cation from TMAH exchange with potassium and magnesium cation in the bentonite. As a consequence, modified bentonite has higher affinity to organic molecules. It causes the GOx molecules can recline easily into the bentonite pores, and then they form interaction to shape immobilized GOx structure [5].

The free and immobilized GOx enzyme activities can be seen in Figure 2. The data shows that the activity of immobilized GOx enzyme is lower than that of free GOx enzyme. The decreasing of the activity is about 32.5%. The immobilization process may leads to the conformational changes of the GOx, and moreover it also causes some GOx molecules covered by surfactant molecules in the layer

of bentonite, which may decrease its affinity and accessibility of substrate molecules thus cause decreasing of enzyme activity [5].

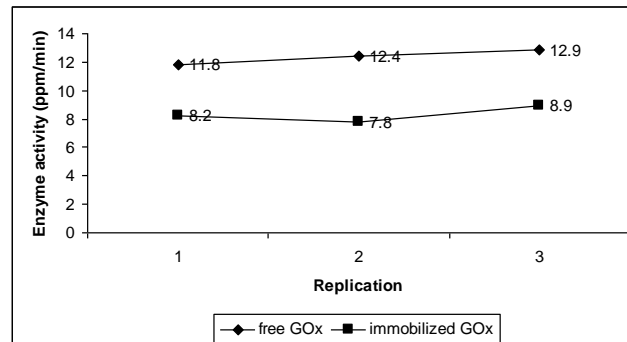


Figure 2. The Activity of Free and Immobilized GOx Enzyme

3.2. Preparation of Immobilized GOx Wafer Enzyme

The main composition of pre-designed device is a wafer. This wafer consists of membranes, immobilized GOx and PAH. This section describes the effect of molar ratio of immobilized GOx:PAH toward the enzyme activity (Figure 3) and its capacitance values (Figure 4).

The addition of the polymer (PAH) plays a role in increasing of the stability of immobilized GOx enzyme. The data shows that by increasing of molar ratio of PAH, the GOx enzyme activity is decreased (Figure 4). This probably increasing of the number of PAH molecules lead to increase the steric hindrance around the sites active of the enzyme which may hinder the substrate molecule interact with the enzyme. As a consequence, the glucose molecule which may oxidize is decreased.

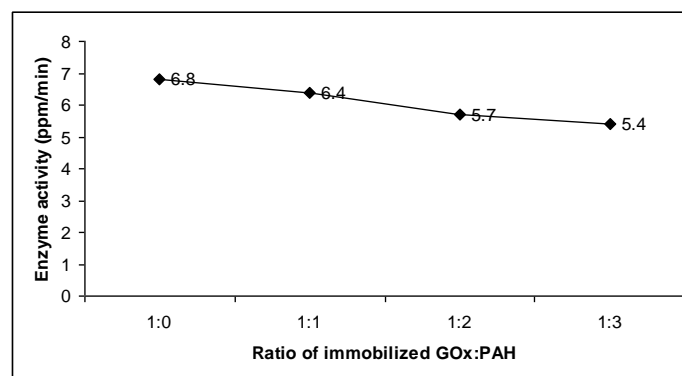


Figure 3. Effect of Ratio of Immobilized GOx and PAH Toward Enzyme Activity

The addition of the polymer (PAH) also plays a role in decreasing of the capacitance of GOx enzyme during the process the substrates oxidize to form gluconic acid and peroxide, and moreover there are some electrons involve in this reaction. The transfer of the electrons can be detected by LCR meter to give its capacitance. The electrons from the reaction is cached by the PAH and then pass through into

dielectric solution which leads to increase the conductivity. The conductivity value is contrary with capacitance value.

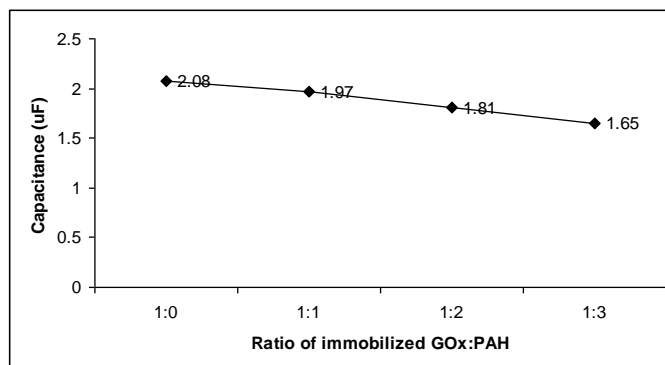


Figure 4. Effect of Ratio of Immobilized Gox and PAH Toward Wafer Capacitance

Thus, the more substrate oxidizes, the more electrons involved the lower capacitance is. It is very clearly described in Figure 4. It can be concluded that the addition of the polymer lead the substrates to be adsorbed onto the enzyme and to be oxidized more easily.

4. Conclusion

In this paper, immobilized glucose oxidase wafer enzyme on Ca-bentonite modified by TMAH has been presented. GOx can be immobilized successfully and still showed very good activity. The wafers consist of immobilized GOx and PAH have also been successfully prepared and show its activity. Increasing of molar ratio of PAH, the GOx enzyme activity is decreased. The present work demonstrates the existence promising applications of immobilized glucose oxidase wafer enzyme on Ca-bentonite modified by TMAH as a health device.

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