University POLITEHNICA of Bucharest

SCIENTIFIC BULLETIN

Series B: Chemistry and Materials Science

Volume 73 Issue 1 2011

ISSN 1454-2331

Series B - Chemistry and Materials Science

UNIVERSITY POLITEHNICA of BUCHAREST
SCIENTIFIC BULLETIN

Revista online

Series B - Number 3

Contents

IMMOBILIZATION OF GLUCOSE OXIDASE ON ACID ACTIVATED-BENTONITE AND ITS PERFORMANCE EXAMINATION

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Glucose oxidase (GOx) was immobilized on acid activated-bentonite. The activation of bentonite was carried out by adding 1-3 M of hydrochloric acid, and the immobilized GOx enzymes were examined for the oxidation of glucose solution under certain conditions. Besides, the effect of pH and working temperature were also investigated. The result showed that the immobilized GOx increased the rate of catalytic reaction. The optimal catalytic activity of immobilized GOx was at 30^{o}C and pH 7. The values achieved for the Michaelis-Menten constant (Km) and and maximum reaction rate (Vmax) were 48.01 mM and 5.41 x 10^{-3} mM.min^{-1}, respectively. The result also showed that effectiveness factor was 0.68. Immobilization of GOx on acid activated-bentonite also provides a stable matrix shown by the ability to reuse it up to seven times before its activity decreases to a level of 68%.

Keywords: bentonite, glucose oxidase, immobilized enzymes, oxidation, glucose.

1. Introduction

Enzymes are protein molecules that reduce the activation energy to accelerate chemical reactions in living cells. However, some of these uses require immobilization of molecules. The enzyme molecule is often used without losing its activity and might be used repeatedly and continuously [1, 2]. The immobilization development has increased where they are used as biocatalysts in textile industries and other applications [3-6]. The use of immobilized enzymes is preferred because of their repeated use, the ease of harvesting products, and their greater stability. Glucose oxidase enzyme (GOx) is used as a reagent in the glucose determination in blood, though it is always costly [7, 8]. The development of immobilized enzyme technology has always received attention [8]. For instance, explorations relating to immobilized GOx have been carried out many times in recent years. The process of immobilizing biomolecules using insoluble support materials is critical in the fabrication of various functional materials [9].

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Therefore, some materials are extensively tested as support, such as nanomaterial [10] including magnetite [11, 12], two-dimensional layered clay minerals [13, 14], gold nanoparticles (AuNPs) [15], magnetite nanoparticles [16], polymethylmethacrylate (PMMA) [17, 18], and graphene oxide [19,20].

A previous study reported the utilization of clay as support for immobilized of various enzymes [3,21,22], for instance clay for immobilized biocatalyst and biosensor [4], bentonite for immobilized of various types of enzymes, such as for lipase [23], for phosphatase [24], for amylase [25], and for catalase [26]. Bentonite is a popular type of smectite abundant in Indonesia. It has properties that might be explicitly set to suit needs, which is quite advantageous. This arrangement can be conducted by a number of simple techniques, such as activation using acids, intercalation, and pillarization by metals or organic molecules. The properties can be adjusted through the use of acid to increase the site, which functions as a binding center through the enzyme -NH_2 group during adsorption. This bond is more durable than physisorption because it is ionic [3]. In the previous work, immobilization of GOx using surfactant-modified bentonite was studied. Its ability, kinetics, and reusability were examined [27, 28].

This study examines the immobilization of glucose oxidase (GOx) on the acid activated-bentonite. XRD and FT-IR techniques were used to characterize the matrices. Analysis of the effect of working temperature, pH, and reusability was carried out by measuring of immobilized and free GOx enzymatic activity. Additionally, the kinetic of the matrices was also investigated.

2. Experimental Section

2.1. Materials

Glucose oxidase (GOx) from Aspergillus niger (257 IU/mg) and peroxidase from horseradish (250-300 IU/mg) were obtained from MP Biomedical and Sigma, hydrogen peroxide 30% (UP. Kobika Puslit LIPI), glycerol (Nacalai Tesque), D-glucose (Nacalai Tesque), o-Dianisidine (Sigma), Aquadest, Folin-Ciocalteau (Merck).

2.2. Instrumentation

Spectrophotometer UV-vis Genesys 10 was used for the detection of the free enzyme by the Hartree Lowry method, while X-Ray diffractometer Shimadzu XRD 1000 was used for characterization. The characterization of the surface functional groups was based on FTIR (Bruker Tensor 27) spectrometer.

2.3. Procedure

2.3.1. Immobilization of GOx enzyme

Natural bentonite mined from Pacitan, Indonesia was used as starting clay, while modification involved adding 100 mL of hydrochloric acid 1 - 3 M to 5 g of bentonite. The mixture was heated at 90^{o}C for 1 hour and agitated. The solid phase was then rinsed with condensed water and dried at 100^{o}C for 24 hours. Additionally, the modified bentonites were characterized by an XRD diffractometer.

The GOx enzyme solution (5 mL, 10.14, 14.81, 15.33 IU/mL in pH 7 of phosphate buffer) was added to 5 g of acid activated-bentonite. The mixture was stirred using a rotary shaker at 20^{o}C for 24 hours and centrifuged at 4000 rpm for 10 minutes. To determine the amount of free GOx enzymes, the supernatant was analyzed using Hartree Lowry. The obtained pellet was treated with phosphate buffer to form a suspension. Afterwards, the suspension was rinsed with an identical buffer solution to eliminate free GOx enzymes. It was then centrifuged at 11,000 rpm for 15 minutes to obtain the immobilized. Immobilization fraction was determined using the equation below

$$\%\ \text{immobilized GOx} = \frac{\text{total amount of immobilized GOx}}{\text{total amount of initial GOx}}\ x\ 100 \tag{1}$$

2.3.2. Measurement of enzyme activities

The immobilized GOx activity was determined spectrophotometrically at 525 nm with 2% of glucose solution as the substrate at 30^{o}C and pH 7 based on Whittington's method [28,29]. Also, 10 µl of immobilized GOx was added with 500 µl of glucose solution, 50 µl of horseradish peroxidase solution, 400 µl of glycerol, and 50 µl of o-dianisidine. The mixture was incubated at pH 7 for 60 minutes and then centrifuged at 11,000 rpm for 10 minutes to stop the reaction. The supernatant was measured by determining the difference between the absorbance of the initial and final solutions. The presence of oxidase o-dianisidine in the supernatant was quantified based on its absorbance detection using a spectrophotometer UV-Vis at 525 nm. The sucrose concentrations were varied at 1 - 15% to study the influence of substrate concentration on the reaction rate. Lineweaver-Burk plot was used to determine the Michaelis constant Km. To compare the effectiveness of free and immobilized enzyme activities, the effectiveness factor (EF) was established.

$$EF = \frac{V_{\max}\left(\text{immobilized GOx}\right)}{V_{\max}\left(\text{free GOx}\right)} \tag{2}$$

Studies of immobilized GOx reusability were carried out by centrifuging the product mixture after each reaction. The isolated, immobilized enzyme was

collected and used for further activity test. To determine the thermal stability of the immobilized GOx, the temperatures used were 30, 40, 50, and 60^{o}C.

3. Result and discussion

3.1. Immobilization of GOx enzyme

Table 1 shows the effectiveness of the acid-adding process into bentonite for the immobilization of GOx. The percentage of immobilized GOx in acid modified-bentonite was much higher compared to natural bentonite. There is a higher possibility of an acid-adding process forming hydrogen ion layers on the bentonite surface. These ions might have formed ionic bonding with an amino acid in the GOx enzyme, increasing the effectiveness of enzyme immobilization. The concentration of acid into bentonite affected the immobilization of GOx.

Table 1

GOx immobilization in acid modified-bentonite

Materials		Enzyme concentration		Immobilized GO_X (%)
		Initial (IU/mL)	Immobilized (IU/mL)	
Natural bentonite		10,14	2,05	20.22
Acid modified - bentonite	(HCl 1 M)	15.33	14.1	92.16
	(HCl 1.5 M)	15.33	14.1	92.16
	(HCl 2 M)	15.33	15.33	100
	(HCl 2.5 M)	14.81	9.93	67.05
	(HCl 3 M)	14.81	10.3	69.55

The acid activation of bentonite possibly released some impurities, such as Al, Fe, Mg cations, leading to an exchange of cations between Ca and hydrogen. At the end of the process it was expected that the surface area of bentonite was expected to expand. The adsorption of hydrogen cation on the bentonite surface forms ionic bonding with the negative part of amino acid in the GOx molecule. Therefore, the immobilization of GOx into bentonite might have occurred. From table 1, the most effective concentration of acid-adding into bentonite for immobilization of GOx was 2 M. Increasing acid concentration leads to immobilization of GOx into bentonite, making it less effective. Generally, increasing acid concentration destroys the bentonite structure by releasing of Al-O alluminosilicate.

3.2. Characterization of matrix

The X-Ray Diffraction spectrums for immobilized glucose oxidase are presented in Fig. 1. The peak at $2\theta = 5.92^{o}$ corresponding to a d-spacing of 8.54Å is considered for natural (fresh) bentonite. This peak was the d001 plane equivalent to the interlayer spacing. Its shifting part was lower 2θ (5.58^{o}) with a

distanced 15.81Å after the addition of 2M HCl to bentonite, indicating the expansion of the layer. Therefore, activation by adding HCl occurred in the bentonite layer. The new peak intensity was quite high, meaning that almost all the layers of bentonite are intercalated with O-Si-O and H+ ion [30]. The GOx immobilization on the activated bentonite cause this peak to shifts slightly higher 2θ (5.78^{o}) with a d-spacing of 15.26Å representing a contraction of layers. However, the distance of the layers was still wider than that of the fresh bentonite, indicating not the whole GOx molecules intercalated between the layers vertically, others were horizontal. This caused the layers covered by the GOx molecules and their distance to be constricted.

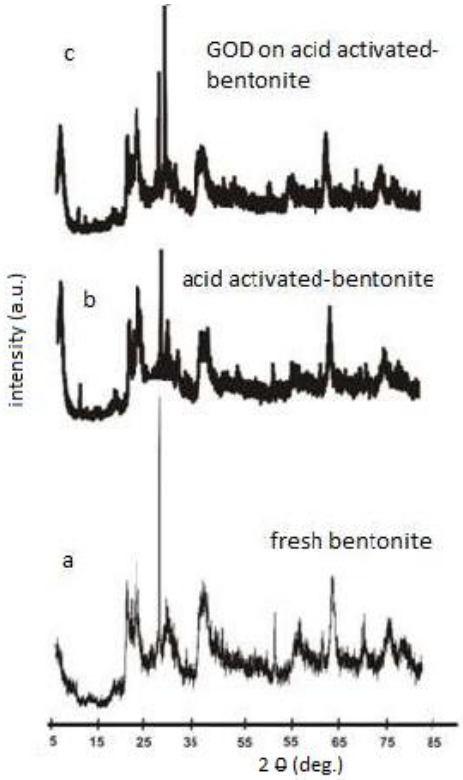

Fig. 1. XRD patterns of support and immobilized glucose oxidase. (a)fresh bentonite, (b) activated bentonite using HCl 2M, (c) immobilized GOx on acid-activated bentonite

The characterization of immobilized glucose oxidase was also studied by Fourier Transform Infrared spectroscopy (FTIR) (Fig. 2). The acid-activated bentonite indicates pores structural transformation, unlike the fresh bentonite. This is shown by the wider and higher intensity of O-Si-O peak at 1000-1100 cm^{-1}. The absorption was also supported by the change of peak absorption at 800 -

900 cm^{-1} due to O-Si-O interaction with H^+ from the acid on the acid-activated bentonite configurations. This was strengthened by the presence of the band at 400-550 cm^{-1} and reinforced by a weak peak at about 3000 cm^{-1} due to amide A and amide B of GOx absorption and by specific absorption of –OH group at about 3500 cm^{-1}. The band transformation designates the pores creation within bentonite sheets.

The appearance of the band at 800-900 cm^{-1} was due to Si-O-Si asymmetry vibration between two tetrahedral SiO4 in the bentonite. The interaction between GOx and activated bentonite was indicated by the changes in band character at 800-900 cm^{-1} between acid-activated and GOx-acid-activated bentonites. The absorption peak at 1650 cm^{-1} corresponds to the symmetry stretching vibration of Si-O---H^+ bond from acid-activated bentonite as well as GOx. The increase in the amount of interaction between Si-O from bentonite and H from GOx is indicated by the presence of a wider and higher absorption intensity.

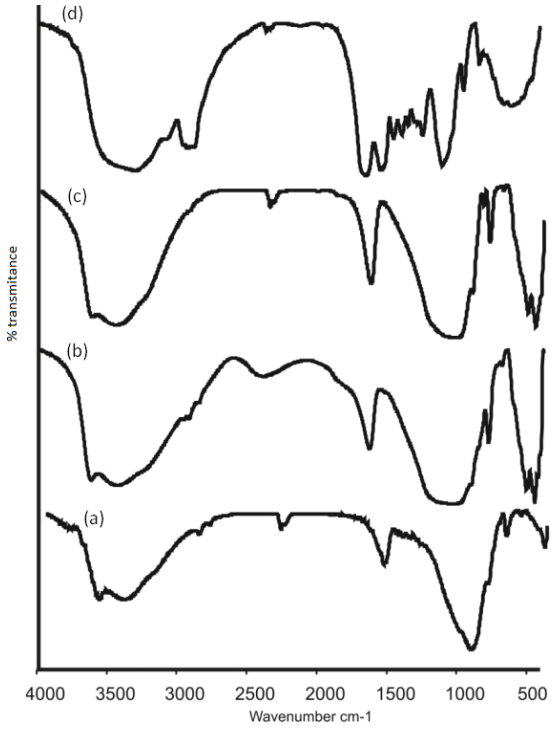

Fig. 2. FTIR spectra of (a) fresh bentonite, (b) activated bentonite using HCl 2M, (c) immobilized GOx on acid-activated bentonite, (d) GOx enzyme

3.3. Enzyme activities studies

The effects of pH and working temperature on the activity of free and immobilized GOx are given in Fig. 3, and Fig. 4. The free GOx activity was higher than immobilized GOx in almost all pH ranges studied. This indicates the immobilization process leads to the structural change of GOx, decreasing its affinity and accessibility for glucose molecules to be oxidized. Figure 3 shows that even though the immobilized and free GOx has a wide range of pH, 5.5-7.5, the optimum is 7 because this is where the highest free GOx activity occurs. The immobilized GOx shows constant activity from pH 5.5 to 7 but declining from pH 7 to 7.5. The exceptional result is demonstrated by data at pH 7.5, where the free GOx activity is lower than that of the immobilized. This indicates that immobilized process makes the GOx structure more stable at higher pH.

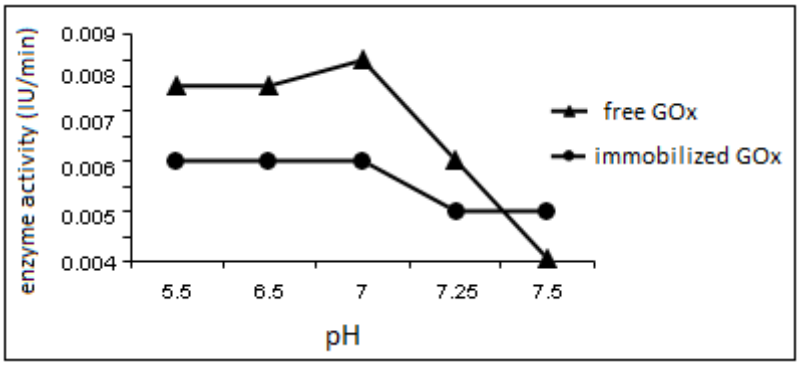

Fig. 3. The effect of pH toward the activity of free and immobilized GOx enzyme

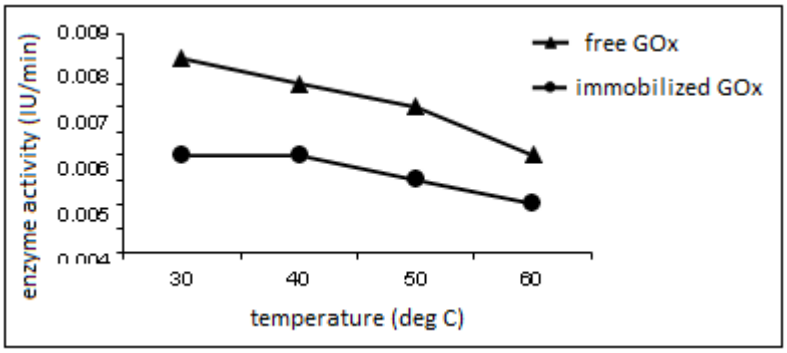

Fig. 4. The effect of working temperature toward the activity of free and immobilized GOx enzyme

Working temperature affects the specific activity of the free and immobilized enzyme. To examine its influence, the activity of immobilized and free GOx was examined at temperatures between 30 and 60°C. The working temperature with the maximum activity for both immobilized and free GOx matrices was 30°C (Fig. 4). The activity of immobilized GOx declined slowly after 30°C. Possibly, at a temperature above 30°C, the enzyme starts to denature. However, the decreasing activity of free GOx was faster than that of immobilized GOx. The immobilization of the GOx enzyme does not change the working temperature, though, it affects its activity. This is because the immobilized activity is lower than free GOx. The immobilization material covers the enzyme active site in acid-bentonite. Therefore, fewer substrate molecules lie on the active site of the enzyme.

3.4. Kinetic study of free and immobilized GOx

The investigation of enzyme kinetics was carried out by measuring the Km and Vmax values from Lineweaver-Burk plots of free and immobilized GOx activity. This was conducted at a temperature of 40°C and pH 7 in various concentrations of glucose substrate (Fig. 5). The plot is linear for both the free and immobilized invertase indicating that the kinetics of the enzyme comply with the Michaelis–Menten equation.

Table 2

Kinetic values for free and immobilized GOx

Kinetic parameter	Free GOx	Immobilized GOx
K_m (mM)	35.8	48.01
V_{max} (x 10^{-3} mM.min^{-1})	8	5.41
Effectiveness Factor (EF)	1	0.68

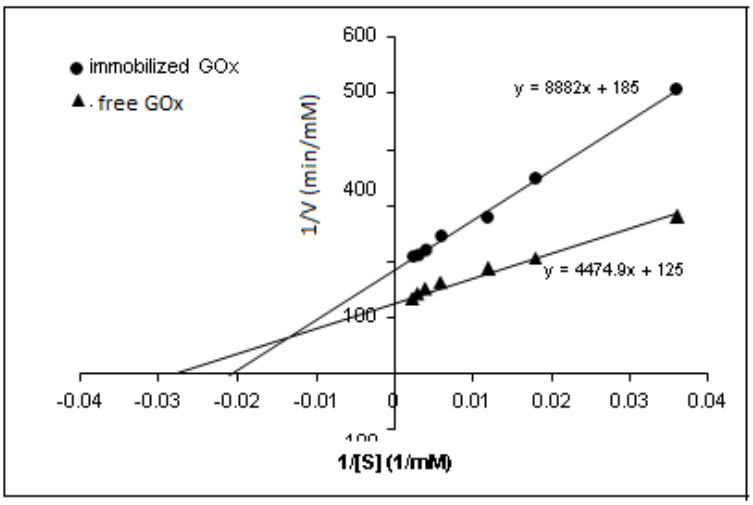

Fig. 5. Lineweaver-Burk plot of the free and immobilized GOx

Table 2 presents Km and Vmax values for free and immobilized GOx. The apparent Michaelis constant, Km exhibited intensification on immobilization. This is due because immobilization causes a decline in substrate affinity due to changes in enzyme conformation. Additionally, the maximum rate Vmax of immobilized enzymes is lower than that of the free enzyme. Therefore, the decrease in activity was probably due to the limited access of substrate molecules to active sites of enzymes not bound to the supporting surface. Also, it may have been caused by reduced substrate mobility to reach the active centre due to immobilization [31]. This result is in line with the outcome of the previously reported work [27]. It is supported with the efficiency of immobilization which is about 68%, possibly due to diffusional limitations.

3.5. Reusability of immobilized GOx

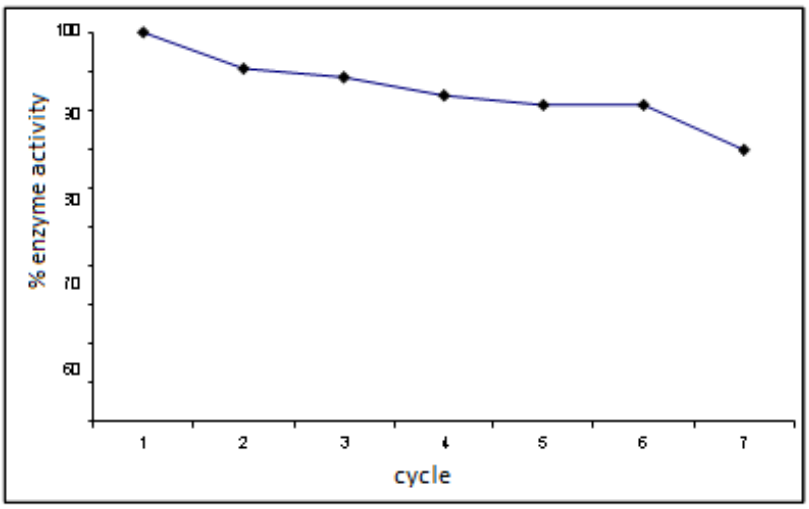

Fig. 6. Enzyme activity levels in each cycle of reusability assay

Utilization of enzyme immobilization ease separation and reuse. The reusability test of the immobilized GOx enzymes using acid-activated bentonite at room temperature showed different levels of activity in each incubation cycle presented in Figure 6. Immobilized GOx activity decreased to a level of 69.8% after being used seven times. After the 6th use, the enzyme was reasonably stable on the bentonite surface and may be entrapped in the pores, causing little leaching.

4. Conclusion

Study of GOx immobilization on acid-bentonite was successfully carried out. The results show that immobilization of GOx enzyme does not change the working temperature of GOx, though it decreases its activity. The immobilized

glucose oxidase was stable and could be re-used 7 times before its activity declined to level 69.8%.

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