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Development of Lactose Biosensor Based on β-Galactosidase and Glucose Oxidase Immobilized into Gelatin

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In this article, we describe the preparation of a new lactose biosensor based on electrode coating with β-galactosidase and glucose oxidase immobilized gelatin. For this purpose, β-galactosidase and glucose oxidase enzymes were immobilized onto gelatin by crosslinking with glutaraldehyde. Properties of the immobilized β-galactosidase and glucose oxidase enzymes electrode have been studied. The effects of glutaraldehyde concentration, temperature and pH variations and reusability were among the subjects analyzed. Lactose biosensors were subjected to continuous repeated use in order to observe reusability and shelf life; where standard lactose and milk samples were used as substrate solutions. Continuous reuse experiments showed that most of the lactose biosensors activities were retained even after the 10th use in a period of 30 days.

Keywords lactose determination, amperometric biosensor, β-galactosidase, glucose oxidase, crosslinking, glutaraldehyde, co-immobilization, gelatin

Introduction

A biosensor is a sensing device that transforms chemical information (concentration of a specific compound) to an analytically useful signal. To accomplish this transformation efficiently, a reaction fast enough to obtain a high concentration of product near the electrode surface is required. The sensing phase (measurements) should be quick and repeatable. In addition to these properties, biosensors should be cheap, their shelf life should be long enough and recalibration should be easy to be used commercially. The most efficient way of obtaining a high product concentration near the electrode is coating the electrode surface with a catalyst. The support system used in coating, should be hydrophilic, and have good mechanical properties and stable over the temperature and pH ranges used in measurements. Finally, the catalyst immobilized on the support system should retain its properties for a long time to enable reusability.

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Among the biosensors, the importance of amperometric ones has been increased due to the combined advantages offered by the selectivity of the biological recognition elements and the electrochemical transduction process. Numerous clinical, food, and wastewater applications of amperometric biosensors have been reported (1). The key element for the design of suitable sensor architecture is the immobilization of the specific biological recognition element, particularly an enzyme, at the transducer surface, which (in the case of an amperometric biosensor) is usually a noble metal or carbon surface.

Milk contains lactose and other carbohydrates in small quantities (2). The quantitative determination of lactose, an essential carbohydrate in milk and milk products, is important and routinely carried out in the dairy industry to ensure effective process and product control. Low levels of milk are measured for cows suffering from mastitis.

Congenital lactose intolerance was first described by Durand (3). Severe congenital lactose intolerance was viewed by some as a transient form of congenital lactase deficiency. However, the disease is now known to have distinct features. It is a more serious disorder with vomiting, failure to thrive, dehydration, and disaccharidurie including lactosuria, renal tubular acidosis, and aminoaciduria. Liver damage is also observed. Abnormal absorption of lactose and other disaccharides was suggested by the work of Berg, Dahlqvist, Lindberg and Studnitz (4). Lactose, not normally found in the blood, may have toxic effects, as does fructose-1-phosphate in fructose intolerance and galactose-1-phosphate in galactosemia. Russo, Mollica, Mazzone and Santonocito (5), documented that lactosuria is due to gastric absorption because it disappeared when lactose was given intraduodenally.

Although, various methods, such as spectrophotometry, polarimetry, titrimetry, chromatography are available for lactose detection, all the methods are complex, expensive, time consuming and difficult.

Biosensors are versatile analytical devices with a high degree of selectivity, which have found application in the fields of food, biotechnology, medicine, environmental and agriculture (6). Biosensors are analytical systems that combine the specific properties of biologic and physicochemical compounds. Biosensor systems produced by using immobilized β-galactosidase (β-GAL) and glucose oxidase (GOD) as biological active materials are called lactose biosensors.

β-Galactosidase (EC 3.2.1.23) enzyme is widely used for hydrolysis of lactose in milk, milk products, producing glucose and galactose.

$$\text{Lactose} \xrightarrow{\beta\text{-Galactosidase}} \text{glucose} + \text{galactose}$$

Glucose oxidase enzyme (E.C.1.1.3.4) catalyzes the conversion of β-D-glucose to hydrogen peroxide and δ-D-glucanolactone in the presence of molecular oxygen.

$$\text{Glucose} + \text{GOD (FAD)} \longrightarrow \text{Gluconolactone} + \text{GOD (FADH}_2\text{)}$$
$$\text{GOD (FADH}_2\text{)} + \text{O}_2 \longrightarrow \text{GOD (FAD)} + \text{H}_2\text{O}_2$$

FAD and $FADH_2$ are the oxidized and reduced forms of flavine adenine dinucleotide (the active center of GOD, respectively). The H_2O_2 can be detected at the electrode surface in accordance with the equations:

$$\text{H}_2\text{O}_2 \longrightarrow 2\text{H} + + \text{O}_2 + 2\text{e}^-$$

The oxidation current of H_2O_2 is normally proportional to the concentration of glucose in the solution. Classical devices for glucose determination were based on monitoring either the consumption of oxygen or the production of hydrogen peroxide. In recent years, the development of cheap and reliable enzyme electrodes for industrial and medical applications has become a major research area. Among these electrodes most widely investigated, one is the glucose biosensors.

The performance of the biosensors does not only depend on the biological material and the transducer but also on the immobilization method. So, developments in enzyme immobilization techniques are very important for enzyme-based sensors. Some examples of the recent works on this subject are; co-immobilization of β-galactosidase and glucose oxidase in a redox polymer, polyvinylferrocenium perchlorate by Gülce, Gülce and Yıldız (7), β-galactosidase and glucose oxidase immobilized in protein membrane (pigs small intestine) by Adanyi, Szabo, and Varadi (8).

Hydrogels have been extensively used to immobilize enzymes for the construction of aqueous-and organic-phase biosensors. A large amount of hydroxyl groups in the hydro gel provide a biocompatible microenvironment for the enzyme to maintain its natural configuration. Due to this structure, high sensitivity has been expected from the biosensors based on the hydro gels, but the swelling property of the hydro gel may limit the practical application of these biosensors in some cases (9). Gelatin (G) is a water-soluble protein resulting from the partial hydrolysis of collagen. The most characteristic physical property of gelatin and the basis for many of its uses is the ability of gelatin to form reversible elastic gels when its aqueous solutions are chilled. In the cooling process, the viscosity of gelatin solutions increases sharply but there does not seem to be any particular point at which gelation occurs. Reactive groups present in gelatin are primarily hydroxyl, carboxyl, and amino functions. Their abundance is approximately 100, 75, and 50 miliequivalents (per 100 g high quality gelatin), respectively. Inorganic, as well as organic, compounds can harden gelatin. Among the former, which react in general with the carboxyl groups of the gelatin through their divalent metallic ions, the most often encountered are alum and chromium salts which cross-link to the ionized carboxyl groups. Among the organic hardeners, formaldehyde and glutaraldehyde are the most commonly used compounds. Hardening with aldehydes occurs by the formation of cross-links between the amino groups of gelatin. In our study, gelatin was used as a carrier system and glutaraldehyde (GA) [$OHC(CH_2)_3CHO$] has been used as the hardener (10–12).

In this work, we aimed to develop a lactose-biosensor to be used in food industry and medical applications by coating its platinum electrode with β-GAL and GOD immobilized gelatin. Gelatin was used as carrier and β-galactosidase and glucose oxidase enzymes were immobilized into gelatin with chemical crosslinking by glutaraldehyde (13). The properties of immobilized β-galactosidase and glucose oxidase enzyme electrode was studied. Affect of the crosslinker concentration on the response of the biosensor, temperature, pH dependencies and reusability were also analyzed. Standard lactose and milk samples were used as substrate solutions.

Experimental

Reagents

β-Lactose, glucose oxidase enzyme, β-galactosidase enzyme and glutaraldehyde were purchased from Sigma (USA).

Granular photographic gelatin was obtained from Croda Gelatin Co. (England).

Other chemicals were purchased from Merck (Germany) and were analytical grade.

All reagents were prepared with chemicals of analytical reagent grade and double distilled water.

Electrode Preparation

Gelatin (0.075 g) was added to 0.1 M phosphate buffer (pH 6.0) to obtain a final volume of 1 ml. The mixture was allowed to rest for 30 min, at 25°C and then heated to 50°C in a water bath and cooled to 32°C after the gelatin had been dissolved. β-galactosidase (100 U) and glucose oxidase (50 U) were added to the immobilization solutions at 32°C. The solutions were stirred vigorously for 1 min, then glutaraldehyde with concentrations varying from 0.004 to 0.015 M was added. Afterwards, the crosslinker addition solutions were stirred again for 1 min to accelerate the reaction. Platinum plates (1.5 cm^2) were drop-coated with 0.2 ml gel using 0.1 ml for each surface. The prepared electrodes were allowed to rest for 48 h at 25°C. The electrodes were washed 3 times with phosphate buffer to release the unbounded enzyme. Washing operation was performed three times and each lasted for 1 h. The gelatin-β-GAL-GOD electrodes were stored in 0.1 M phosphate buffer (pH 7.0) at 4°C between measurements. Blank electrodes were not contained β-galactosidase and glucose oxidase.

Electrochemical Measurement

Electrode response experiments were carried out in a 60 ml cell. Determination of activity by the amperometric method was accomplished by using a three-electrode system, consisting of the working enzyme electrode, a platinum counter electrode and an Ag/AgCl reference electrode.

The current generated at the working electrode was held at 0.7 V. All measurements were made at 25°C in a cell containing a phosphate buffer (0.1 M pH 7.0) by using Potentio-Galvanoscan Wenking PGS 95 model (Germany) Potentio-Galvonoscan analyzer equipped with x-y-t recorder. The developed system was based on the measurement of H_2O_2 produced by the reaction.

In response time measurements, lactose was added to the cell after obtaining a stable background current.

Results and Discussion

Enzyme Immobilization

To investigate the effect of cross-linker concentration on the activity of the immobilized enzymes, 7.5% gelatin gel containing β-galactosidase and glucose oxidase was mixed with different concentrations of glutaraldehyde (0.004–0.015 M). Working electrodes were prepared with all of the crosslinker concentrations tested. The highest activity was obtained with a glutaraldehyde concentration 0.012 M by using amperometric analysis. Results are given in Figure 1.

As seen from the figure, there was a slight increase up to 0.012 M (about 17%) and a considerable decrease (about 29%) above that concentration was observed. According to

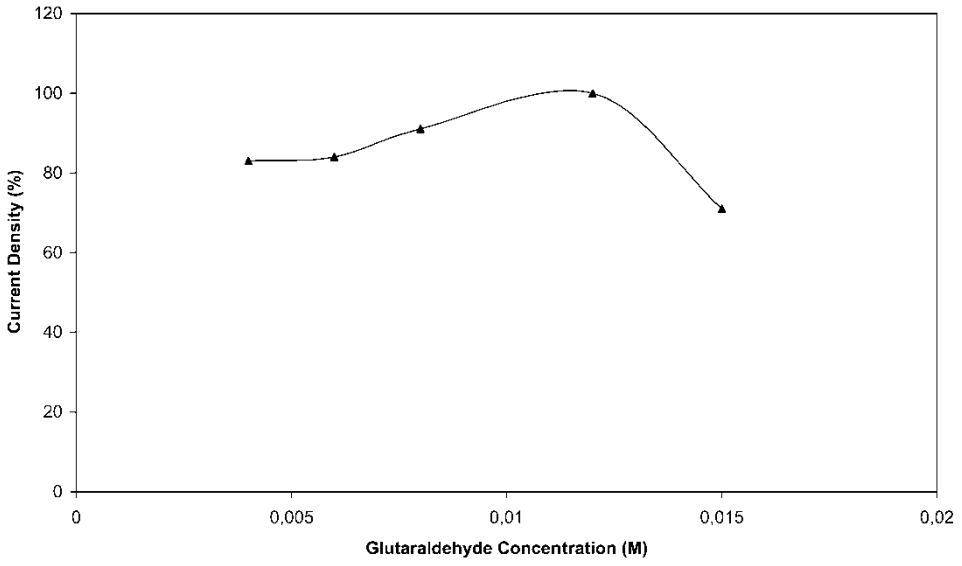

Figure 1. Effect of crosslinker concentration on the response of lactose biosensor [β-galactosidase (20 U) and glucose oxidase (10 U), 7.5% gelatin, 0.7 V; pH: 7.0; 25°C].

these results, 0.012 M crosslinker concentration was selected as the most suitable one. Increasing the crosslinker concentration probably first caused an increase in the amount of immobilized enzymes, thus had a positive effect on the amount of lactose reacted but later, began to negatively effect it due to deactivation of the enzyme and increasing the diffusion barrier (by blocking the active sites by excessive bonding and a firmer coating).

Calibration Curve

The calibration curve for the developed biosensor obtained for lactose levels 0.1 to 300 mM is presented in Figure 2. We observed a linear response between 0.1 to 15 mM of lactose concentrations.

As seen from the figure, even 0.1 mM lactose concentrations can be measured with the developed biosensor.

pH Dependency

The pH dependence of the developed lactose biosensor was investigated in a pH range 3.5 to 9.6 by using 20 mM lactose. Results are presented in Figure 3. As seen in Figure 3, the best results were obtained at pH 8.0 for G-β-GAL-GOD electrode. The amperometric signal value obtained for pH 7 was about 87% of that obtained for pH 8. We used pH 7 in the subsequent experiments.

When compared with the literature, best results were obtained at pH 7.3 for free ß GAL by Sungur and Akbulut (14) and at pH 5.1 for free GOD enzyme by Numanoğlu and Sungur (13). Our bienzymatic biosensors best values were obtained at higher pH values than those and this was attributed to immobilization and crosslinking of enzymes. The catalytic activity of the enzyme depends on the presence of a given conformational structure in the folded polypeptide chain, even minor alterations in the

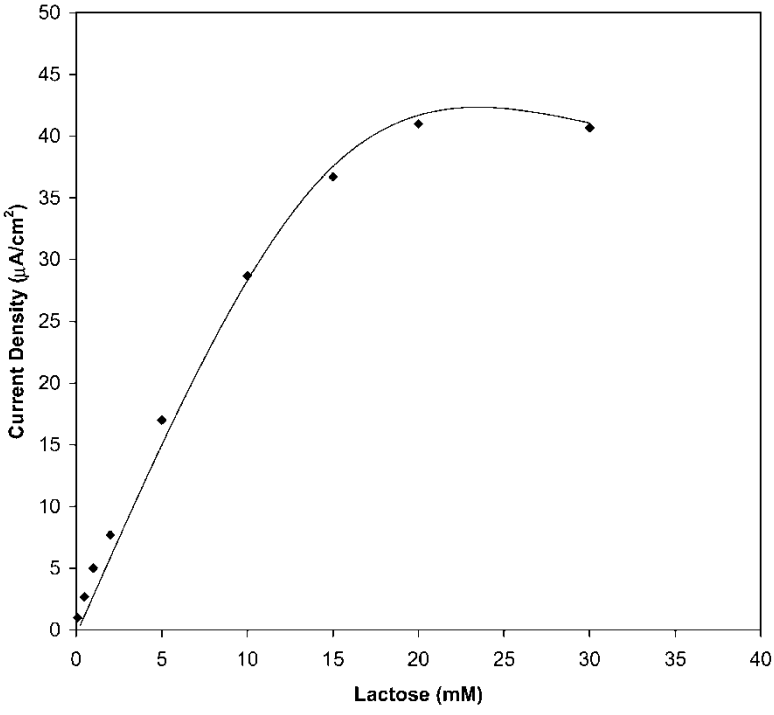

Figure 2. Calibration curve of the developed lactose biosensor [β-galactosidase (20 U) and glucose oxidase (10 U), 7.5% gelatin, 0.7 V; pH: 7.0; 25°C].

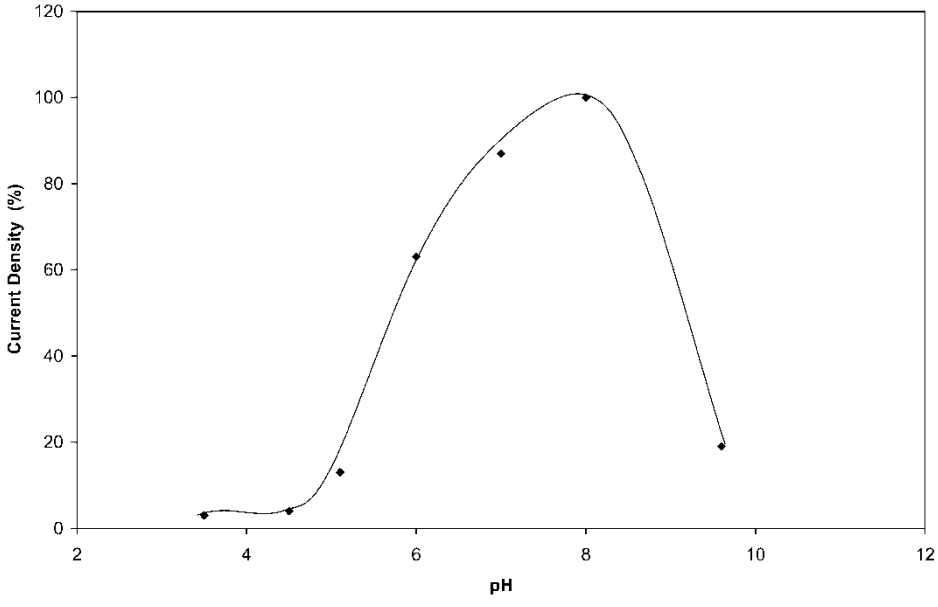

Figure 3. Effect of pH variations on the amperometric activity of the lactose biosensor [β-galactosidase (20 U) and glucose oxidase (10 U), 7.5% gelatin, 0.7 V at 25°C].

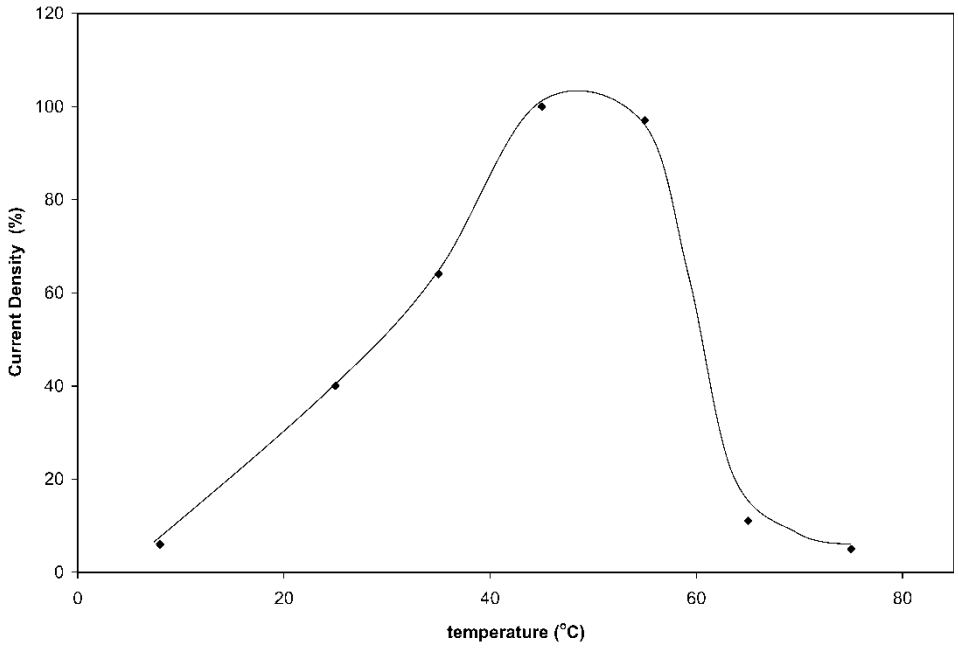

Figure 4. Thermal stability of lactose biosensor [β-galactosidase (20 U) and glucose oxidase (10 U), 7.5% gelatin, 0.7 V; pH: 7.0].

tertiary structure result in a loss of biocatalytic activity. As the tertiary structure of β-galactosidase and glucose oxidase is altered in highly acidic and basic solutions, the electron transfer cannot take place efficiently. Thus, our biosensor is highly dependent on pH.

Our best results fit with the results obtained by Tkac, Sturdik and Gemeiner (15), who found the optimum as pH 8.0 and Gülce et al. (7), pH 7.8 and closer to the neutral pH than the data presented by Adanyi et al. (8) pH 4.5, Eshkenazi, Maltz, Zion, and Rishpon (16) pH 6.0. These results showed that our biosensor could be used very efficiently in milk lactose measurements.

Temperature Dependency

Temperature dependency of our sensor was investigated within a temperature range of 8 to 75°C. Results are presented in Figure 4. As seen from Figure 4, we obtained a maximum current density at 45°C. Activity loss over 45°C can be attributed to the denaturation of enzyme molecules and degradation of the polymer matrix.

The amperometric signal value obtained for 25°C was about 40% of that obtained for 45°C. Since high amperometric signals were obtained at 25°C, we used this temperature in the subsequent experiments.

Stability

Since stability of biosensors for an adequate length of time is very important, our lactose biosensor (prepared by using 0.012 M GA) was used with 3 day intervals to investigate the

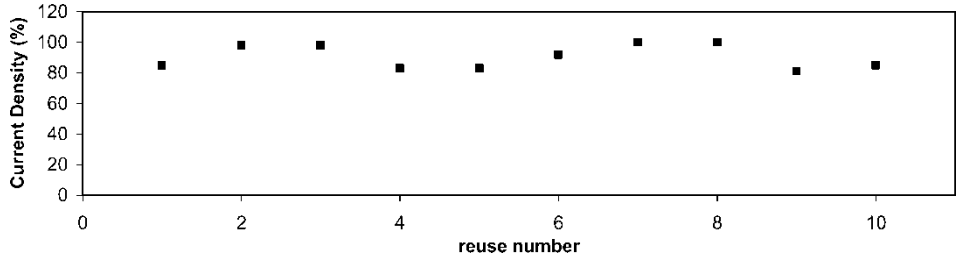

Figure 5. Stability of the developed lactose biosensor [β-galactosidase (20 U) and glucose oxidase (10 U); 7.5% gelatin; 0.012 M GA; 0.7 V; pH: 7:0; 25°C].

effect of reuse and shelf life on amperometric activity. This process was continued for 30 days and 10 uses. These experiments were performed at 25°C with 72 h intervals by using 20 mM lactose. Results are presented in Figure 5.

As seen from the figure, lactose biosensor can be reused 10 times within 30 days with negligible activity loss. Confirming the success of our immobilization method developed biosensor retained almost all of its activity in 30 days of shelf life. To obtain the reusability figures of our lactose biosensor, it was subjected to continuous repeated usage with 1/2 hour intervals. Standard lactose (20 mM) and milk samples with a known amount of lactose were used as substrate solutions. Results are presented in Figure 6.

As seen from Figure 6, most of the activity weas retained even after the 10th use for standard lactose solutions and milk samples.

Conclusions

In this research, we aimed to develop a new biosensor to be used in the food industry and for medical applications. β-galactosidase and glucose oxidase were immobilized into carrier gelatin by crosslinking. Glutaraldehyde was used as a crosslinking agent. Gelatin was found to be a very suitable carrier system and coating material to be used in lactose biosensor manufacturing. With the lactose biosensor developed by immobilizing β-GAL and GOD into gelatin, comparably low concentrations of sugar

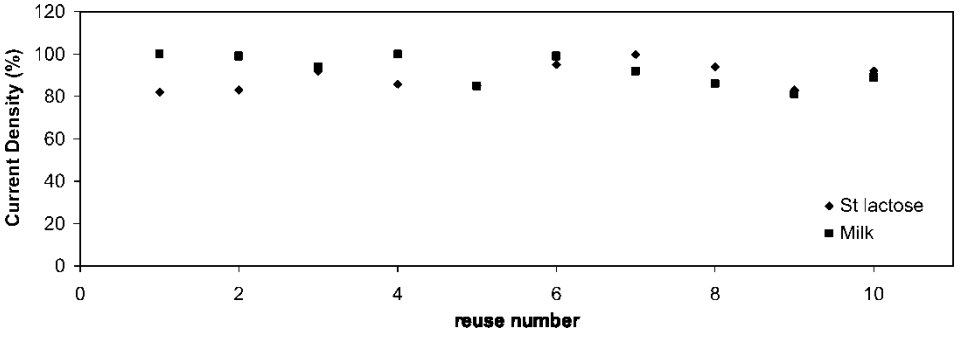

Figure 6. Effect of continuous repeated use on the activity of biosensor. Standard lactose (20 mM) and milk samples were used as substrate solutions [β-galactosidase (20 U) and glucose oxidase (10 U); 7.5% gelatin; 0.012 M GA; 0.7 V; 25°C].

(0.1 mM) could be determined. The developed biosensor could be used repeatedly 10 times and had a shelf life of 30 days without considerably losing its accuracy. This low cost method is very promising, and further experiments on this subject were planned in our laboratory.

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