IJABR

International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-13, Issue1, 2022, pp01-10
http://www.bipublication.com

**Research Article**

# Development of a Polyphenol Oxidase Biosensor from Corn Tassel Extract for Determination of Phenolic Compounds in Industrial Waste Water

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[Received 12 Aug-2021, Accepted 02 Jan-2022, Published 19 Jan-2022] DOI: 10.5281/zenodo.5886683

## ABSTRACT

This work describes the preparation of an polyphenol oxidase biosensor for polyphenols determination in industrial wastewaters. In the present study, corn tassel on the top of the corn plant was used as the enzyme source. The oxygen electrode was used as a transducer to test the sensor responses of developed biosensor. To develop the biosensor, crude enzyme extract was immobilized onto oxygen electrode using gelatin as immobilization material and glutaraldehyde as crosslinker. The optimum values of enzymatic extract amount, gelatin amount and glutaraldehyde percentage to design the biosensor were 100 µl, 10 mg and 1.5%, respectively. This biosensor provides a linear response for catechol in the concentration ranges of 0.5-10 mM. The storage stability and reproducibility of the biosensor was evaluated. The biosensor is a promising tool to detect and quantify phenolic compounds in the environmental monitoring. For comparison, the phenolic content of effluent samples was also measured by using the newly developed biosensor. SPSS program was used for analysis of research data.

**Keywords:** Polyphenol oxidase, biosensor, phenolic compounds, corn tassel

## INTRODUCTİON

Approximately 8,000 phenolic compounds are known in nature. Some of these are phenol, resorcinol, orsinol, pyrogallol, catechol, gallic acid, hydroxy benzoic acids such as syringic acid, flavonoids and other phenol products. A phenolic compound named catechol is used as a standard for the determination of such a wide class of phenolic compounds, especially for the biosensor methods [1]. Phenol (benzo phenol or hydroxybenzene) is known as the first member

of phenolic compounds ($C_6H_5OH$) [2]. Phenolic compounds are small groups of molecules that have at least one phenol unit with their structure. They are phytochemicals found in most plants [3]. Most phenolic compounds are synthesized in the intracellular endoplasmic reticulum of plants and stored in the vacuole [4].

A rapid industrial change has occurred in the world towards the end of the 20th century. As a result of this, a large amount of industrial waste was left to the nature and harmful effects of these wastes started to be seen in time. Phenol and phenol derivatives are among the most important of these waste types [5]. They are important parameters which should be monitored in environmental engineering. There are many industrial waste water streams containing these compouns, such as those of the oil, paint, paper, polymer and pharmaceutical industries. These toxic and carcinogenic compounds are introduced into the environment particularly into the waters [6]. Even at very low concentrations is harmful to human health. They cause various problems in humans because of their irritation effect on the eyes, skin and liver [7].

Detection of polyphenolic compounds has been drawing attention both in food quality analysis and in the environmental control of toxic contaminants [8-12]. Phenols is known to affect the odor and taste of drinking water at very low concentrations (a few µg/L). Chronic toxicity of phenol in humans also result in headache, vomiting, difficulty in swallowin, liver injury, fainting, etc.Thus, both the European Union (EU) and the US Environmental Protection Agency (EPA) placed some phenols, mainly chlorophenols and nitrophenols on their lists of priority pollutants. The techniques such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) in combination with ultraviolet (UV) and mass spectrometry detection (MS) systems are used to analyse the phenols. Analytical techniques commonly used in the determination of phenols are. However, these methods expensive and they involve a long time per analysis [13]. For this reason, the enzymatic biosensors have been widely employed because of their potential to detect a target substance/analyte with high specificity via enzyme-catalyzed reactions [14-17].

Enzyme□based biosensors have been most widely applied to detect phenolic compounds in the industrial, environmental pharmaceutical, biomedical and clinical analysis [18]. Some enzymes sources such as tyrosinase [19], laccase [20,21] and phenol oxidase [22] as biosensors have already been utilised to detect phenolic compounds. Enzyme based-biosensors are utilised as important and promising tools to assess phenolic pollutants, due to the improved selectivity of these devices, as well as low cost and fast analysis [23,24,19].

Polyphenol oxidase (PPO) represents a strong potential to be used as a biosensor. PPO catalyzes the o-hydroxylation of monophenols to o-diphenols and oxidation of the o-diphenols to o-quinones [25]. It belongs to oxidoreductases, which are a set of copper containing metalloenzymes[26]. PPO is the principal enzyme involved in enzymatic browning [27], catalyzing electron-transfer reactions which does not need any additional cofactors, oxidation of phenolics in the presence of oxygen and with good stability [28,20].

Polyphenol oxidase (PPO) is known to be produced by most living organisms, including microorganisms, plants and animals, that catalyze the oxidation of phenolic substances to quinones that produce brown pigments in damaged tissues [29,30]. The proposed mechanism of action for PPO is based on the oxidation capability of phenolic compounds. When the tissue is damaged, the breakdown of the plastids, the cellular compartment where the PPO is located, causes the enzyme to come into contact with the phenolic compounds released as a result of the breaking of the vacuole, the main storage organ of these compounds [31].

In the present study, corn tassel on the top of the corn plant was used as the enzyme source. The normal function of corn tassel is to produce pollens to pollinate female flowers called silk [32].It is rich in anthocyanins, flavonoids and phenolic compounds, as well as having antioxidant activity [33,34]. Corn tassel is viewed as waste by corn processing industry. We have previously shown that it is a good source of

polyphenol oxidase; thus, crude enzyme extracts of the corn tassel can be immobilized on electrochemical materials in order to construct a biosensor.[35] Although several biosensors have been proposed for the detection of phenolic compounds based on the enzyme polyphenoloxidase [19,36,37], there is no biosensor study in the literature where corn tassel as industrial waste is used as an enzyme source. In the present study, a new polyphenol oxidase based biosensor was developed for detection of phenolic compounds in samples from industrial effluent into Tigris River near Elazığ-Turkey.The results were in accordance with Folin Ciocalteu (FC) method. The oxygen electrode was used as a transducer to test the sensor responses of developed biosensor.

**Experimental**

*Reagents, solutions and Apparatus*

An industrial wastewater sample was provided from industrialized region in Elazig–Turkey. Catechol was purchased from Merck (Darmstadt, Germany). Phenol, $Na_2HPO_4$, $NaH_2PO_4$, glutaraldehyde (GA, 25%) and gelatin were purchased from Sigma Aldrich. The measurements were performed Data LineWINLAB (DO) Bench top type dissolved Oxygen meter and its oxygen probes. All experiments and assays were carried out in triplicate.

*Crude Extract Preparation*

The corn tassel on the top of corn plant was obtained from a local market in Diyarbakir City, Turkey. Corn tassel (5g ) was homogenized in the 100 ml of 0.1 M phosphate buffer (pH 7.0) by using a blender. The suspension was stirred for 30 min at room temperature, and the mixture was filtered through cloth and centrifuged at 15000 rpm for 15 min at 4 °C. The supernatant (corn tassel enzymatic extract) was collected and stored at 4 °C until used.

*Determination of PPO Activity*

PPO activity was assessed by a spectrophotometric method based on the initial rate of increase in absorbance at 420 nm. Enzyme activity was measured in 3 ml of reaction mixture consisting of 0.1 mL substrate (0.1 M catechol) and 0.1 mL enzymatic extract in 0.1 M phosphate buffer (pH=7). The blank consisted of buffer and substrate .One unit of enzymatic activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per min.

*Biosensor development*

The oxygen (DO) probes were primarily washed with ethanol and then with distilled water. 10 mg gelatin and 900 µL enzymatic extract were mixed and incubated at 37.5 ºC for 15 minutes. 50-400 µL of this mixture were then dispersed over the cleaned DO surface and allowed to dry at 4ºC for 30 min. The probe loaded with bioactive layer was dipped into glutaraldehyde solution (1.25 % (v/v) in phosphate buffer, pH 7). allowed to stay for 5 min for appropriate cros-linking. The biosensor probe was then washed and stored in distilled water at 4 ºC.

*Measurement procedure for phenolic compounds*

For determination of the phenolic compounds, the biosensor based on corn tassel PPO enzyme extract was dipped in the thermostatic reaction cell containing 30 ml of working buffer (pH 7.0; 0.1 M phosphate buffer) and was fixed at constant speed at 37.5 ºC. A few minutes later, dissolved oxygen concentration was equilibrated. Dissolved oxygen concentration was recorded. Then, the various concentrations of substrate catechol were injected into the reaction cell. The biosensor, which has polyphenol oxidase enzyme, showed activity on catechol and thus the dissolved oxygen concentrations in the reaction cell started to decrease accordingly as per the following reaction. After each application of catechol, the dissolved oxygen concentration was recorded separately. Measurements were carried out by standard curves which were obtained by the determination of dissolved oxygen level (ΔDO) during reaction time. The measurement procedures were used for all biosensor characterizations and real sample measurements. The biosensor was found to to detect phenolic substances in a real sample, in accordance with standard spectrophotometric assays.

*Opimization and Determination of Working Conditions for Biosensor*

In order to determine the effect of crude extract amount in the bioactive layer on the biosensor responses, measurements were carried out using different amounts of (50, 100, 200, 400 µl) enzymatic extract. For determining optimal enzyme extract amount, studies were performed at 37.5 ºC and pH 7.0 in 0.1 M phosphate buffer. In order to determine the effect of the amount of gelatin in the bioactive layer on the biosensor responses, different amounts of (2.5, 5, 10, 20 mg) gelatin were used.

In order to determine the effect of gluteraldehyde percentage on biosensor response, measurements were carried out using different gluteraldehyde percentages (1%, 1.25%, 1.5% and 1.75%) at optimum conditions, described above.

Optimum pH experiments were performed in the pH range of 2.0 to 8.0. The sodium citrate buffer range was pH 2-5, while phosphate buffer was in the pH range of 6 to 8. The optimum temperature experiments were conducted over the temperature range of 20 to 50$^{o}$ C .

The process stability of the sensors was determined in order to determine how many measurements can be made on the same day with the same sensor. In order to determine the stability and reproducibility of the biosensor, measurements were carried out 6 times at 30 min intervals.

In order to determine the storage stability of the biosensor, measurements were performed at different intervals for 30 days and the response of the electrode on the first day and the response on the last day were compared. The biosensor responses of the measurements taken on the following days were plotted in % activity

*Measurement of total phenolic content*

The samples were obtained from an industrial effluent into Tigris River near Elazığ-Turkey. The sample was collected and stored at 4 ℃ before use. Total phenolic substance values of the samples were first measured by Folin Ciocalteu (FC) method [38], in order to compare the results obtained from biosensor. For comparison, the phenolic content of effluent samples was also measured by using the newly developed biosensor. SPSS program was used for analysis of research data.

## RESULTS AND DİSCUSSİON

*Biosensor Construction*

In the present study, the optimum enzyme extract amount, gelatin amount and gluteraldehyde percentage were determined for biosensor development. The optimum amount of corn tassel PPO enzyme extract to construct the biosensor was found as 100 µl, while the amounts of glutaraldehyde (g / mL) and gelatin used were 1.5 % and 10 mg, respectively. (Figures 1-3). The results with gluteraldehyde concentrations found for the immobilization is in agreement with the literature, as the glutaraldehyde concentration (g / mL) was found to be 1.25% in banana peel and fresh bean biosensors developed for the detection of phenolic compounds [39] .

The dependence of fabricated enzyme probe on pH, was investigated over the range of pH 2.0 to 8.0 using 0,1 M sodium phosphate buffer in the presence of 0,1M catechol substrate. The optimum pH was found to be 7.0 (see Figure 4) Therefore, pH 7.0 was selected as the working pH in further experiments. In the previous studes, the gold-graphene-chitosan and tyrosinase-bound biosensor and Tyr-AuNPs / BDD-based biosensor used for the determination of the phenolic compound had also an optimum pH of 7.0 [40-42] .

Furthermore, the temperature effect on the enzyme of the probe was studied across a range of 20 $^{o}$C to 50$^{o}$C. The optimum temperature was found to be 40 $^{o}$C (see Figure 5), which was selected as the working temperature in further experiments. There have been several studies on the temperature effects on biosensors developed for the determination of phenolic compounds in the literature. The optimum temperature of the developed solid-state composite pH sensor was 20 $^{o}$C, while 37.5 $^{0}$ C for the broad bean biosensor and 35 $^{o}$ C for banana peel and ground apple biosensor [39,43,44]. Moreover, it has been found that the optimum temperature for the biosensors by immobilization of polyphenol oxidase inconducting copolymers exactly was 40 $^{o}$C [40].

Different concentrations of the substrate catechol were used to determine the catechol standard plots. Figure 6 shows the results measured with the biosensor using enzyme extract under optimum working conditions, where catechol determination limits were found as 0.5-10 mM. In *Lactobacillus acidophilus*-based biosensor, catechol concentration range was between 0.5 to 5.0 mM [45]. However, in a study using apricot homogenate-based biosensor under optimum conditions, the substrate catechol concentration used was in the range of 0.5-20 mM [46]. Moreover, the linear range for phenol for the carbon paste electrode modified with polyaniline-active carbon composite and tyrosinase enzyme based biosensors was $5{,}0\times10^{-7}$-$1{,}0\times10^{-5}$M. [47].

*The reproducibility and storage stability of the biosensor*

In order to determine the reproducibility of the biosensor, 6 consecutive measurements were carried out with the corn tassel enzyme based biosensor at fixed catechol concentrations. The biosensor was found to maintain its activity by approximately 80 % after 5 measurements performed at 30 min intervals (figure 7). In the literature, it was shown that the banana peel biosensor had 98 % activity at the end of 6 measurements performed at 30 min intervals and approximately 20 % activity loss in the next measurements [39]. Furthermore, the rate of loss of activity of the immobilized enzyme of polyphenol oxidase is high for the first 10 uses retaining 60% of the initial activity [40] .

To determine the storage stability of the immobilised biosensor prepared from corn tassel tissue, 6 times measurements were taken with fixed catechol concentrations. As can be seen in Figure 8, the corn tassel biosensor maintains its activity by 60 % after 30 days. In the literature, the amperometric biosensor developed for cholesterol detection preserved its activity by 35 % after 20 measurements whereas it lost approximately 73 % the initial amperometric response at the end of 78 days [48] . In another study, the biosensor was found to preserve 47 % the initial amperometric response at the end of 35 days [47] .

*Application on Real Sample Analysis*

As can be seen from Table 1, the developed biosensor has been applied for the detection of polyphenols in real samples as industrial effluent. The results based on the proposed method were compared with the results assayed using the spectrophotometric UV-Vis (determination of total phenolic content using Folin-Ciocalteu method). The results show that no major difference is observed between both methods. The standard deviation value was found to be less than the value of the spectrophotometric method. Therefore, the present method developed is considered as reliable.

## CONCLUSİONS

Phenol is toxic substance found in water. Phenol can enter natural water reservoirs with industrial waste. Therefore, it is necessary to determine the amount of phenol in water sources. Methods for the determination of phenolic compounds such as gas chromatography and spectrophotometry are available [49-50].These methods are used for the detection of phenolic compounds using polyphenol oxidases. But these methods are costly equipment, chemicals, time consuming methods and is not portable. Recently, it has been observed that instead of these methods, biosensors which provide fast results and low cost are used for the detection of phenolic compounds.

In this study, a biosensor was developed by using corn tassel enzyme extract with polyphenol oxidase enzyme activity. The optimization conditions, characterization, reproducibility, the interference effects of some chemicals and the aplication of the biosensor to measure phenolic compounds in industrial effluents as real sample were all investigated. The newly designed biosensor was successfully employed to assess phenolic contaminants in a real sample as there was no important difference between that of the improved method used and standard spectrophotometric one. Moreover, the biosensor was precise, accurate, reliable and also in confirmation with analytical standard. This biosensor developed is a reproducible tool to assess phenolic contaminants in industrial effluents. All of these advantages stated are

suitable for commercial and practical applications.

**ACKNOWLEDGEMENTS**

This work was financially supported by Dicle University Research Fund (ZGEF.17.007).

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**Table 1** Comparison between the corn tassel based-biosensor and Folin-Ciocalteu method.

| | **Total phenol (mM) (n=3)** | **Sd** | **p** |
|---|---|---|---|
| Folin-Ciocalteu method | 11.73 | 0.25 | 0.00* |
| Biosensor | 10.16 | 0.15 | |

*p<.05; p<.01

## Figures

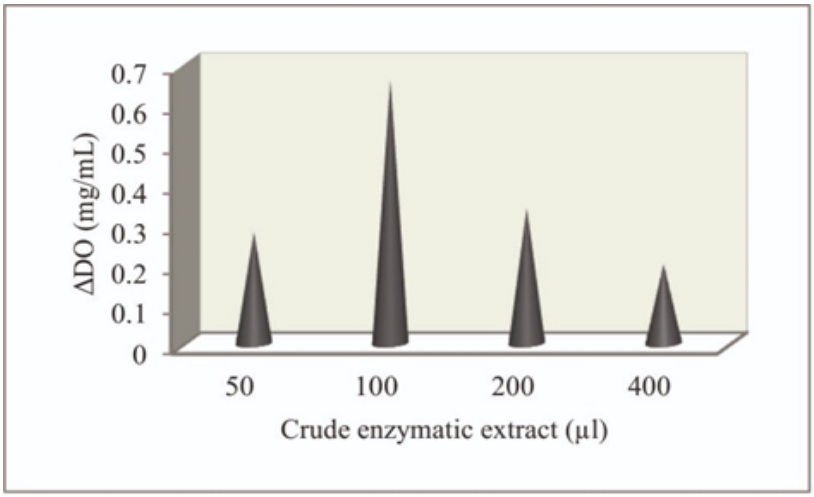


Fig. 1 The optimum amount of corn tassel PPO enzyme extract

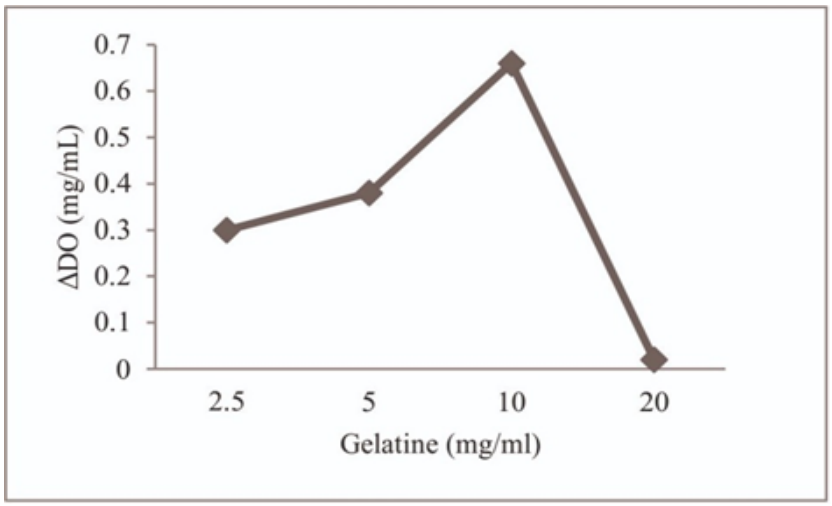


Fig. 2 The optimum amount of gelatin concentrations

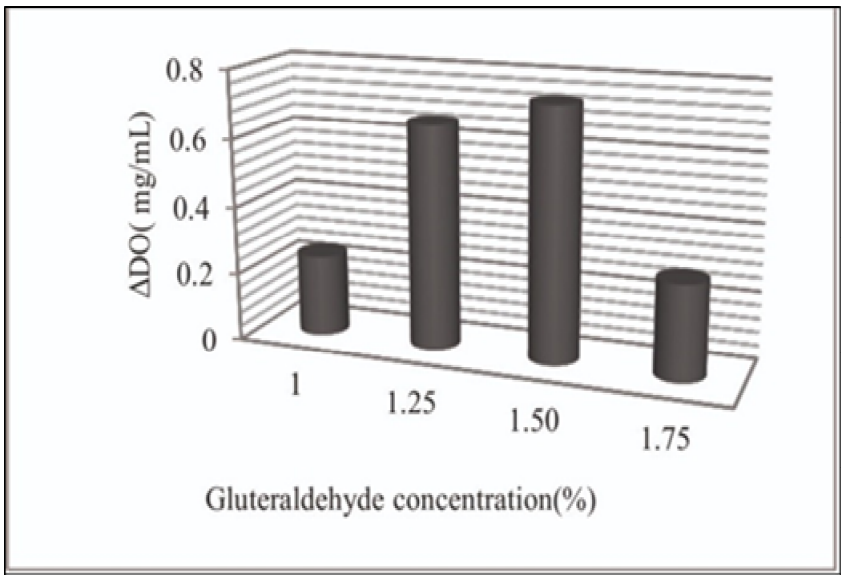


Fig. 3 The optimum amount of glutaraldehyde percentage

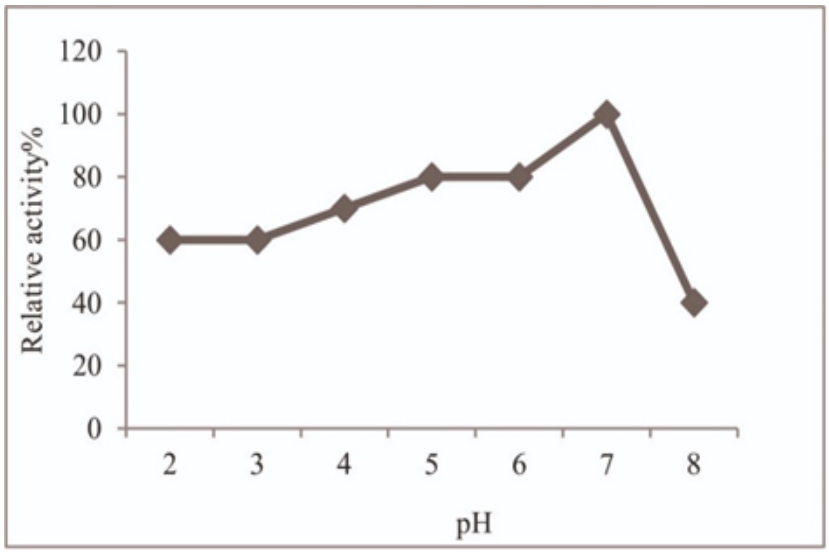


Fig. 4 Effect of pH on the biosensor

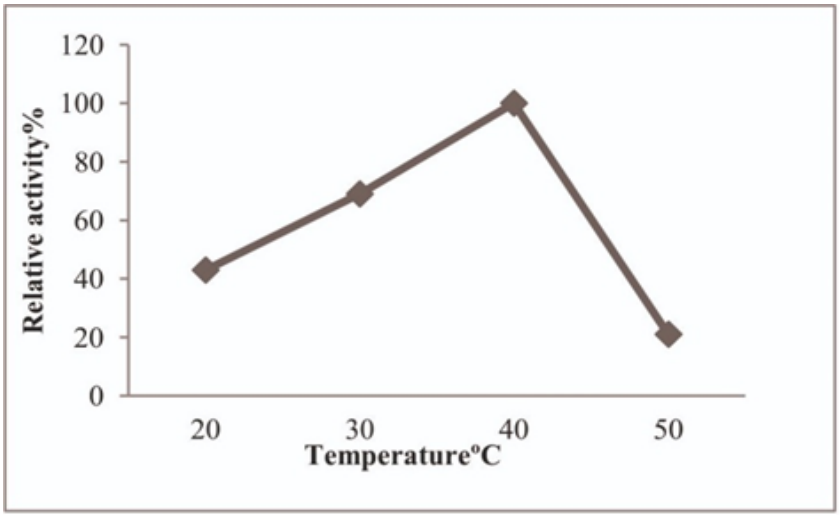


Fig. 5 Effect of temperature on the biosensor

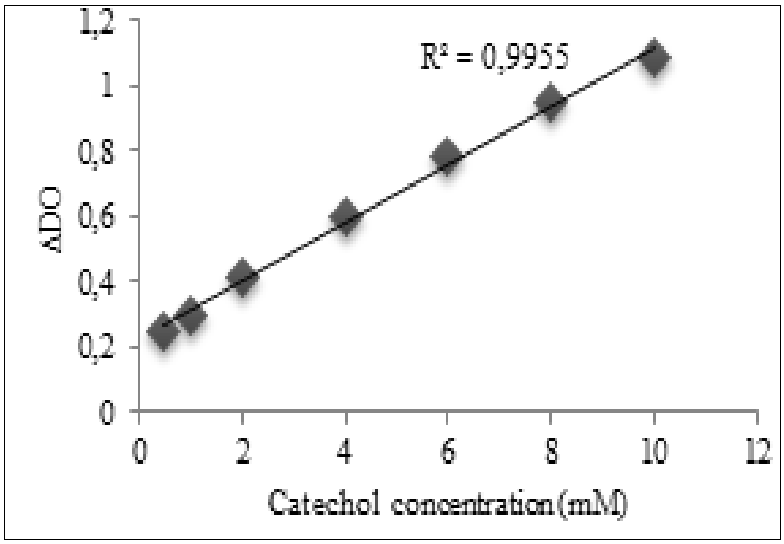


Fig. 6 Calibration curve obtained with catechol

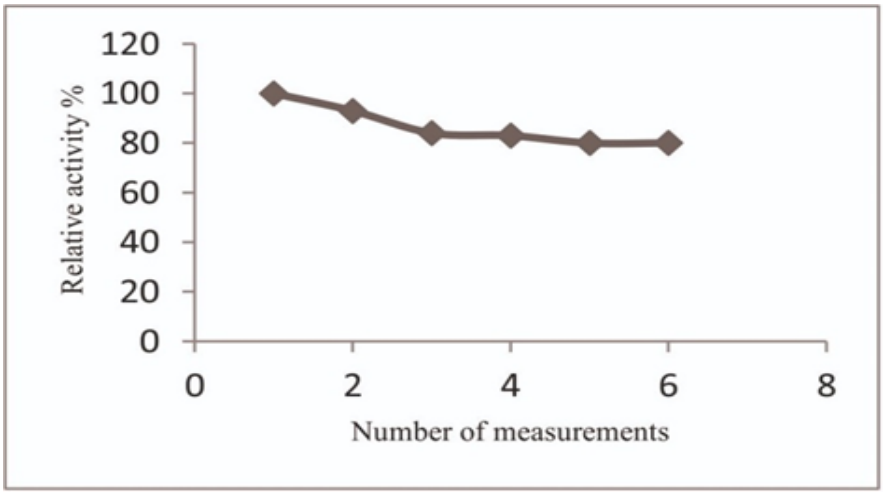


Fig.7 The reproducibility of the biosensor

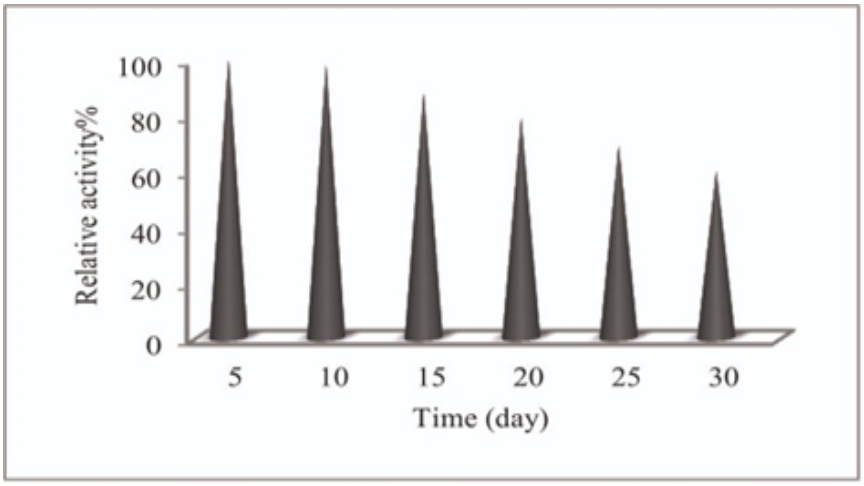


Fig. 8 Storage stability of the biosensor