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Detection of cadmium in water through laboratory designed enzyme inhibition-based bio-electrochemical system

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The work presents designing of an enzyme inhibition-based bio-electrochemical system for the detection of cadmium (Cd) in water. The electrode is fabricated by coating a thin layer of graphene oxide on indium tin oxide (ITO) coated glass substrate and subsequent chemical immobilization of alkaline phosphatase (ALP) enzyme using bovine serum albumin and glutaraldehyde. Different experimental parameters are investigated and optimized to quantify the inhibition of the catalytic activity of the ALP in the presence of Cd. ALP catalytic activity is determined by the voltammetric determination of the electroactive product p-nitrophenol formed after the enzymatic reaction with p-nitrophenyl phosphate which is used as substrate. The cyclic voltammetry showed that under optimized conditions p-nitrophenol could be oxidized electrochemically on the ALP/GO/ITO electrode at around +1.04 V. Optimized incubation time for the signal measurement is found to be 30 min and the relative inhibition of enzyme activity at 10 ppm concentrations of Cd^{2+} is found to be 34%.

Keywords: Alkaline phosphatise, Biosensor, Cadmium detection, Enzyme inhibition, Graphene oxide

Introduction

Monitoring of heavy metals in different environmental components is of great importance for ecological assessments and to understand the dissemination and dissipation of the pollutants. The determination of trace metals is imperative to assess their behaviour in different matrices in order to protect both environment and the consumers. Heavy metals are one of the major environmental pollutants having high toxicity, non-biodegradability that can be bioaccumulated in plants, animals and humans[1-3].

One of these toxic metals is Cadmium (Cd), a non-essential trace element, which is widely distributed in different environmental matrices. Due to atmospheric deposition of combustion emissions, mining and other anthropogenic activities, continuous addition of Cd led to contamination of the soil. Cd can be carried to the lower layers of the soil by chelating agents that can reach the underground water thus contaminating the source of drinking and irrigation water[4]. According to Environmental Protection Agency, maximum permitted level of Cd contamination in drinking water is 5.0 μg/L [Ref.5]. The cadmium exposure has also been linked with glucose metabolism, cerebral infarction, breast and lung cancer, and heart failure[6]. Therefore, determination and monitoring of trace levels of Cd in different environmental components especially in water and food samples is very important.

There are different techniques that have been utilized to analyse Cd and include bioassay, cold vapour atomic absorption spectrometry, inductively coupled plasma mass spectrometry, UV-visible spectrophotometry etc. Though these techniques are sensitive and precise but they have some disadvantages such as high cost and complex analytical processes along with the requirement of a trained personnel. In recent years, biosensors have gained importance for the detection of cadmium and other heavy metal ions in different matrices due to its simplicity, rapidness and selective approach[7]. Biosensors based on the principle of enzyme inhibition have been utilized widely for detection of various analytes including heavy metals (Cd, Hg, Pb, Zn etc.)[8-11]. However, development of sensitive and accurate electrochemical biosensing system depends on the selection of bioreceptor and transducer matrices that are biocompatible, robust, provide high surface-to-volume ratio, and are electrochemically active, with high electron transfer rate. In this direction, graphene oxide (GO) has emerged as an attractive matrix material for biosensing applications due to its mechanical stability, two-dimensional structure, biocompatibility and electronic properties that can be easily changes[12]. Keeping all these facts in mind, the work was carried out to develop an electrochemical biosensor using GO immobilized on indium oxide and alkaline phosphatase (ALP) enzyme for the detection of cadmium in water.

Experimental Section

Chemicals and reagents

Alkaline phosphatase (ALP), Disodium 4-nitrophenylphosphate p-nitrophenyl phosphate (pNPP), HEPES buffer, bovine serum albumin (BSA), and all other chemicals were purchased from Sigma-Aldrich. Deionized water (DI), purified with Milli-Q, Millipore which was having a specific resistivity of 18.2 Ω/cm, was used throughout the experiments. Disodium 4-nitrophenylphosphate p-nitrophenyl phosphate (pNPP) was dissolved in the buffer solution and used as the substrate for measuring alkaline phosphatase activity. The HEPES buffer was diluted with Milli-Q water to prepare solutions of 5, 50 and 100 mM and the required pH was adjusted using 0.1 M NaOH solution. Stock solution of Cd^{2+} was prepared from the standard solution purchased from Sigma-Aldrich. Different stock solutions were stored at 4°C and dilutions were prepared in the buffer solution (HEPES) daily before each series of analysis.

Apparatus

For electrochemical measurements a computer-controlled electrochemical interface (Potentiostat/ Galvanostat) was used to perform voltametric measurements. The pH was measured using a pH-meter.

Synthesis of Graphene Oxide

Graphene oxide was synthesized using modified Hummer's method. Briefly, 0.5 g of graphite and 0.5 g of $NaNO_3$ in 23 mL H_2SO_4 (98%) were mixed with continuous stirring in ice bath (0-5 °C). Finely powdered $KMnO_4$ (1.5 g) was then slowly added to the reaction mixture with continuous stirring, until dark green colour is seen. After 1 h the content was transferred to water bath maintained at 35-40 °C and again stirred for 1 h. Then 100 mL of deionized (DI) water was added along the sides of the flask that yielded a dark brown solution. This solution was sonicated for 30 min after which few drops of H_2O_2 (30%) were added to it. It resulted in a yellow coloured solution indicating the completion of the reaction. Then the solution was centrifuged and washed with DI water several times until the neutral pH of the GO solution was achieved. The neutral GO solution was finally lyophilized to get a highly exfoliated, dry, yellow colour, fluffy GO powder.

Designing of Biosensor

The biosensor was designed using following steps:

Fabrication of Electrode

Indium tin oxide (ITO) coated glass substrate was cleaned by sonicating it for 10 min in soap water, DI water, acetone, ethanol and DI water. Then a thin film of graphene oxide (5 mg/10 mL of DI water) was coated on electroactive side of ITO substrate using a programmable spin coater (Laurel 650). In the similar way, two more layers were coated and the films were annealed at 100°C for 4 h.

Then the enzyme was immobilized on GO electrode by the method reported by Samphao *et al.*, with slight modification[13]. A mixture of bovine serum albumin, solution of alkaline phosphatase (1KU/mL), glutaraldehyde (GA) solution (2.5%) and glycerol using a known amount was prepared whose final volume was adjusted to 250 µL using HEPES buffer solution (pH 8.1). The mixture was gently agitated on a vortex shaker for 10 min at room temperature (RT). The 40 µL of this enzyme casting solution was immobilized on 64 mm^2 area of GO electrode. The fabricated electrode was dried at RT and then stored at +4°C till further use. Fig. 1 shows the schematic representation of the procedure used for fabrication of bio-electrode.

Electrochemical Measurement

Cyclic voltammetry (CV) was performed with a computer-controlled electrochemical interface (Potentiostat, Palmsens3) using a laboratory made three-electrode system. The platinum mesh and Ag/AgCl in 3M KCl was used as counter electrodes and the reference electrode respectively. The biosensor was used as working electrode with a dipping area of 40 mm^2. The working standard solutions of Cd^{2+} were prepared from standard stock solutions. The solutions of 5 mM HEPES buffer (pH 8.1) and 5 mM $MgCl_2$ were used as electrolyte mixture for electrochemical measurements. The substrate was p-nitrophenyl phosphate disodium salt (pNPP), and the substrate working solution was prepared daily. All the solutions were prepared with Millipore water having resistivity ≥18.2 Ω /cm.

Alkaline phosphatase activity and toxicity measurement

The activity of alkaline phosphatase was determined in an electrolyte mixture at optimized pH (8.1) and room temperature, using pNPP as substrate. The

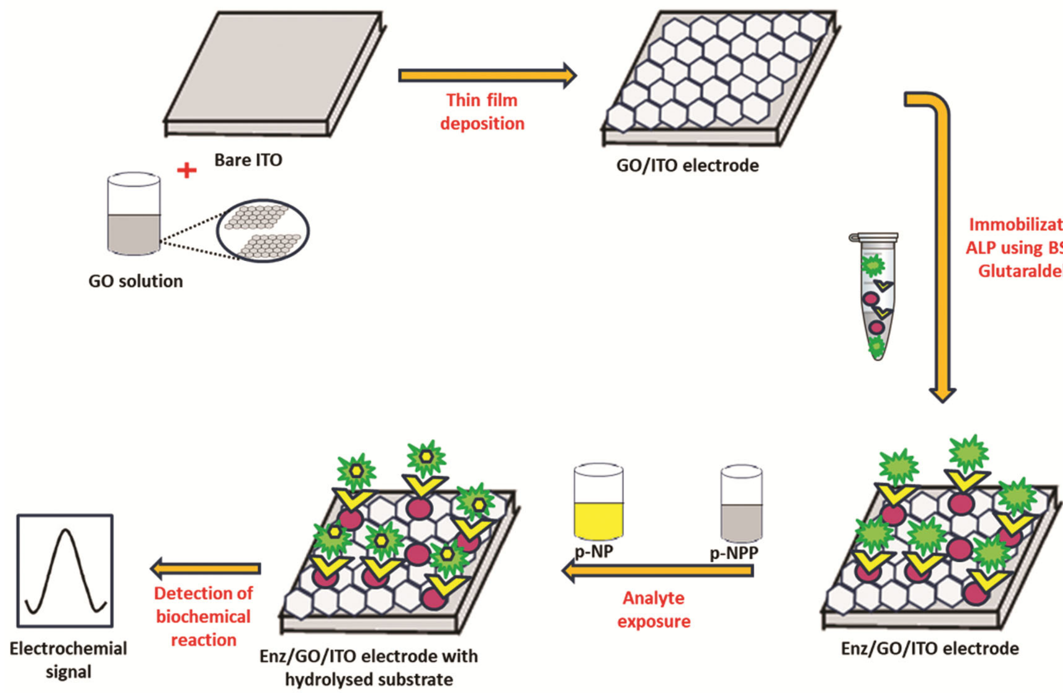

Fig. 1 — Schematic representation of fabrication of Enz-GO/ITO based bioelectrode

enzyme catalysed the hydrolysis of p-NPP to para-nitrophenol (pNP), an electroactive compound which got oxidized on the electrode to respective benzoquinones as shown in (Fig. 2). The amount of current produced was determined by using CV (potential applied was -0.2 V to 1.4 V). Then in a separate set, the metal solution of known concentration was added and incubated for 30 min. The change in the generated current was then determined.

Measurements were performed by gently immersing the biosensor into the buffer solution in a glass cell of 10 mL. After the signal stabilization, different quantities of the substrate were added in the measurement cell. The decrease of the pNPP substrate response was measured from the reduction in current signal on getting exposed to Cd^{2+}solution was used to estimate the enzyme inhibition.

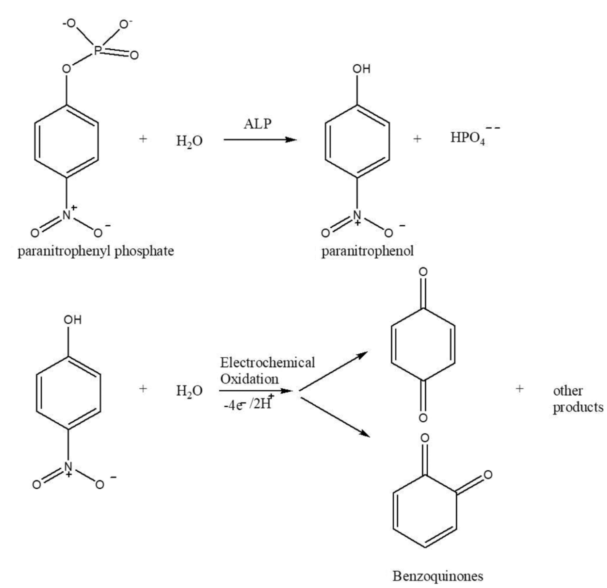

Fig. 2 — Measurement principle of ALP/GO/ITO biosensor

Results and Discussion

GO characterization

The UV-visible spectrum of GO showed the characteristic bands of π-π*(229 nm) and n-π*(300 nm) transitions as shown in (Fig. 3a). The morphology of GO sheets was viewed by HR-TEM as shown in the (Fig. 3b). The wrinkles and folds in the TEM micrograph revealed the formation of single or few layered two-dimensional sheets[14].

Biosensor design

ITO substrate has advantage of ALP sensing as it does not react at positive potential and exhibit little water oxidation current. GO has also been employed by other workers as an attractive matrix material for biosensing applications due to its biocompatibility and electronic properties that can be easily changed[12]. Glutaraldehyde is a bifunctional compound and

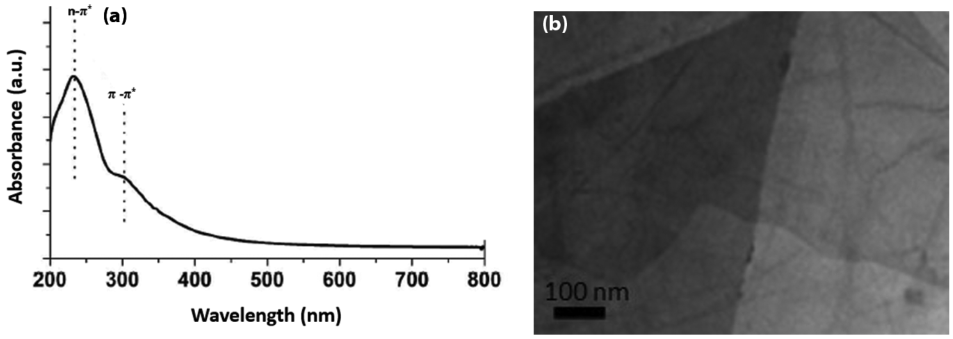

Fig. 3 — (a) UV-visible spectrum and (b) HRTEM image of GO sheet

mainly used for modifications of polymers[15]. It can be used as crosslinker to avoid the enzyme loss during immobilization process[16]. After modifying the ITO substrate with GO, it gave similar pattern of cyclic voltammogram. The successful immobilization of ALP was confirmed by an oxidation current at + 1.04 V. Previous studies have shown that pure enzymes can be immobilised using BSA and GA as a cross-linker[17,18]. Srivastava *et al.*[19] used GA for immobilization of urease enzyme on gelatin beads. Chemical cross-linking using GA and BSA to fabricate a stable and reliable bilayer potentiometric phosphate biosensor had also been investigated by researchers. The inclusion of the BSA–GA layer improves the adhesion of the BSA-GA-ALP layer and ensures stability biosensor[20].

Measurement of enzymatic activity

CVof GO/ITO in buffer showed a clean baseline except for a current near +1.3 V. ITO had an advantage for ALP sensing that being present in an already oxidized form, it does not react at +ve potential and shows a little water oxidation current. There was an oxidation current at +1.04 V in the absence of the substrate (Fig. 4).

ALP immobilized GO/ITO biosensor when placed in HEPES buffer solution having pNPP resulted in the catalytic hydrolysis of pNPP to pNP which is an electroactive species. The CV showed that under the experimental conditions, pNP was electrochemically oxidized at the Enzyme/GO/ITO electrode at around +1.04 V (Fig. 5). The oxidation products, ortho and para-benzoquinone structures were formed finally (after primary oxidation of nitro phenol to ortho or para-dihydroxybenzene) and due to absence of pNPP the peak at +1.04 V was not found (Fig. 4). Measurement principle of Enzyme/GO/ITO biosensor as explained in Fig. 2.

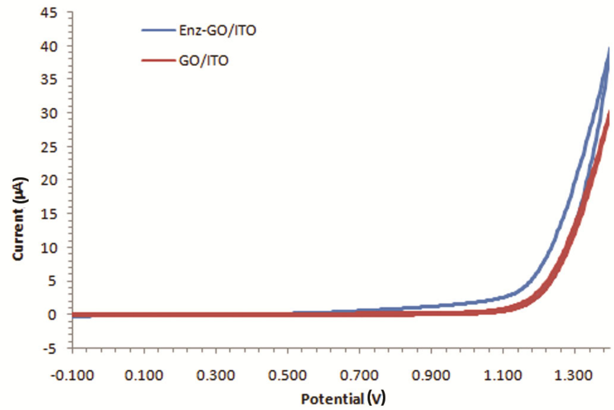

Fig. 4 — Cyclic voltammograms of sensors before and after enzyme immobilization (Enz-GO/ITO)

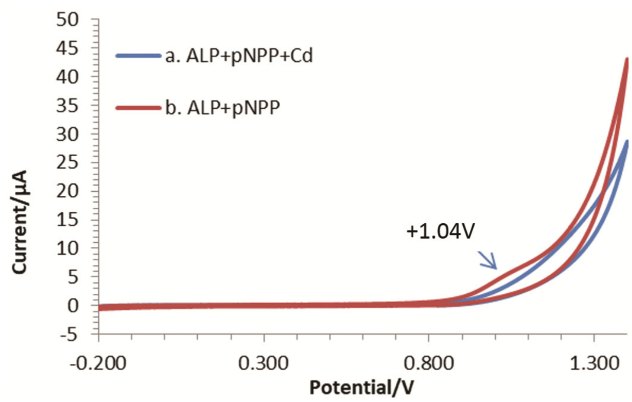

Fig. 5 — Cyclic voltammograms of pNP with designed Enzyme/GO/ITO biosensor; (a) in presence and (b) in absence of cadmium (supporting electrolyte HEPES 5 mM, pH 8.1, potential applied -0.2 V to 1.4 V, E step 0.001 V, scan rate 20 mV/s)

Optimal conditions for enzymatic reaction

The catalytic activity of ALP is highly influenced by the reaction conditions such as reaction pH, reaction time etc. In addition, the concentration of buffer is an important factor for conductometric measurements as higher concentration will lead to larger fluctuations in the biosensor response due to its high conductivity[9]. Some of the buffers that have been proposed to be used with ALP/pNPP are Tris-HCl, PBS, DEA and HEPES. Although Tris-HCl and PBS have been recommended as optimal and widely used buffer solutions, respectively but in present study HEPES was used for ALP/pNPP enzymatic reaction. Tris-HCl buffer has been reported to have complexation with Ag in Ag/AgCl reference electrode whereas PBS buffer shows complexing properties with divalent heavy metal ions although it is widely used buffer. Different factors optimized for the study are discussed below.

Effect of operating potential

In the present study, the dependence of the applied potential on an Enzyme/GO/ITO exposed to a stirred solution of the supporting electrolyte containing pNPP was investigated. The electrode showed an almost linearly increasing current with an increasing potential up to +1.04 V beyond which the signal decreased. It can be due to the formation of oxidized products other than benzoquinone or of polymers which are sorbed on the surface thus reducing its active area.

Effect of pH

The effect of buffer pH on biosensor performance also plays an important role because the three-dimensional structure of the enzyme and biosensor's electrochemical response are pH dependent. The biosensor responses were examined at pH 7.0, 8.1 and 9.0. The results showed that in a HEPES buffer solution of pH 8.1, the peak current was found to be maximum (Fig. 6). Previous studies have also shown that alkaline phosphatases show optimum activity between 7.3 and 9.2 depending on the source and shows highest activity at pH 8.5 in phosphate buffer[13].

Effect of incubation time and enzyme concentration

The incubation time in the substrate solution was optimized using ALP enzyme, immobilized on GO/ITO sensor plate (80 units of ALP per 64 mm^2 area of electrode) and cyclic voltammetry was carried out with 0.74 mg/mL concentration of pNPP solution at various time intervals (Fig. 7). The results showed that as the time increased, the current value also increased upto 15 min of reaction time, after which it became constant. However, the change in current intensity was much higher upto 7 min. The ALP inhibition-based biosensors required incubation time for optimum activity which depends on various factors as a incubation time of 25 min has been reported for acrylamide hydrogels encapsulated, ALP based sensor[21].

Effect of ALP concentration on reaction time was also determined. The incubation time required for enzymatic reaction decreased as the concentration of ALP increased (using same concentration of substrate). It was also found that at lower concentration of ALP, the enzyme reactions took almost 24 h to complete. The incubation time was also found to increase with the increase in substrate concentration[22].

Effect of exposure time for toxicity measurement in presence of heavy metal

The ALP immobilized biosensors were exposed to a known concentration of cadmium nitrate solution for different time intervals and the phosphatase activity was determined using cyclic voltammetry. The current intensity increased up to 30 min after which nearly same signal was obtained. Based on the results, an incubation time of 30 min was used for all toxicity measurements.

Measurement of enzymatic activity inhibition

For the indirect determination of analytes through ALP inhibition, the substrate concentration was held

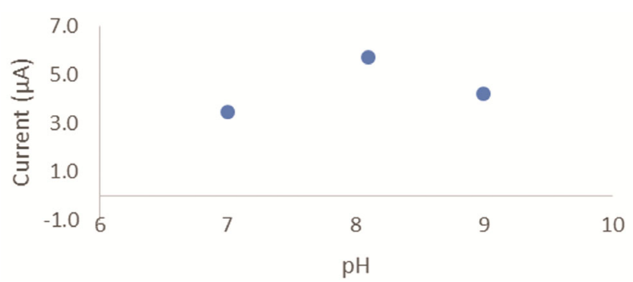

Fig. 6 — Effect of pH on the electrochemical response of Enz/GO/ITO (HEPES buffer 5mM; operating potential +1.04 V)

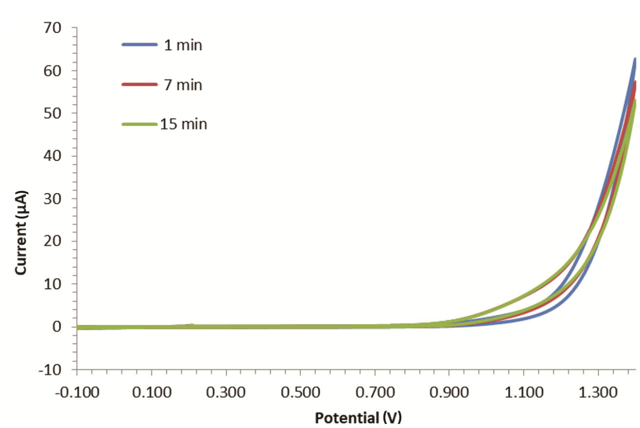

Fig. 7 — Cyclic voltammograms showing effect of incubation time on the response of ALP/GO/ITO biosensor

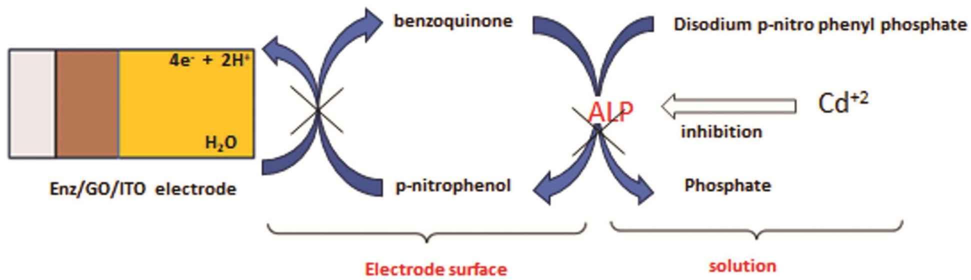

Fig. 8 — Inhibition of the ALP/CPE biosensor with Cd

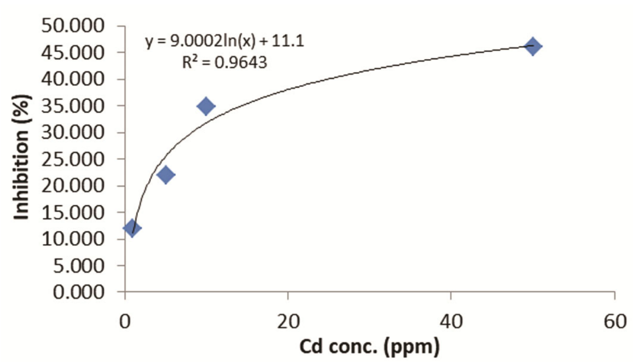

Fig. 9 — Inhibition profile of ALP in presence of different concentration of Cd

constant, and the decrease in the response was monitored after addition of the inhibitor (Cd). The response current dropped when Cd was present in the test solution as the extent of inhibition is proportional to the concentration of Cd. The inhibition of ALP activity due to the presence of Cd has also been reported by other researchers[23].The inhibition of the enzyme by Cd can be attributed to its substitution with zinc and magnesium which are essential cofactors of alkaline phosphatase. Thus, the determination of Cd can be realized according to the degree of inhibition of the enzyme. Inhibition of the ALP/CPE biosensor with Cd is shown in Fig. 8.

The relative inhibition, expressed in percent, was evaluated as $(I_0 - I) / I_0 \times 100$, where I_0 and I are the current response in the absence and in the presence of the inhibitor, respectively[24]. In the present study, relative inhibition at different concentrations of Cd was determined. The inhibition percentage was found to be 12, 21.9, 34.9 and 46 % at the cadmium concentrations 1, 5, 10, 50 ppm, respectively.

Fig. 9 shows the %inhibition in ALP activity due to the presence of Cd in water. The similar trend was reported by Shyuan *et al.*[25]. Jiang and co-workers reported 50% inhibition of enzymatic activity (I_{50}) by Cu, Pb, Cd, Zn and Hg at the conc. of 8.18, 0.10, 0.27, 1.99 and 0.07 µM/L, respectively[26, 27].

Conclusion

The work was carried out to design an immobilized alkaline phosphatase enzyme inhibition-based biosensor to study the enzymatic inhibitory activity in the presence of cadmium at different concentrations using cyclic voltammetry under optimized conditions. The value of the %inhibition at different concentrations of cadmium was 12%, 21.9%, 34.9% and 46% at 1, 5, 10 and 50 ppm, respectively. The detection capability of biosensor was not affected even after 30 days of storage. Thus, the developed biosensor can be used as a tool for its potential application in determining the cadmium level in water for food safety and environmental monitoring after its validation by using them real water samples.

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