Journal of Molecular Liquids 180 (2013) 26–30

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Liquids

journal homepage: www.elsevier.com/locate/molliq

Electrochemical investigations of lipase enzyme activity inhibition by methyl parathion pesticide: Voltammetric studies

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ARTICLE INFO

Article history:
Received 29 August 2012
Received in revised form 18 December 2012
Accepted 20 December 2012
Available online 10 January 2013

Keywords:
Candida Rugosa Lipase
Methyl parathion pesticide
p-Nitrophenol
p-Nitrophenyl Acetate
Voltammetric technique

ABSTRACT

Candida Rugosa Lipase mobilized sensor method was developed for the detection of organophosphorus pesticides like methyl parathion. Here *p*-Nitrophenyl Acetate was used as a substrate which releases *p*-Nitrophenol by the enzymatic hydrolysis and gives an anodic oxidation on electrochemical oxidation peak potential at 0.05 V versus SCE. Methyl parathion is a toxic substance and releases *p*-Nitrophenol when it is hydrolyzed and it also has the property of inhibiting the enzymatic activity and this is taken as a basis for the inhibition studies. The current response under the influence of pH, substrate concentration, enzyme concentration and time variation effects on the hydrolysis process was studied for the reaction between enzyme and substrate which releases *p*-Nitrophenol. A linear calibration for the methyl parathion was obtained in the concentration range of 10–70 ppb with a correlation coefficient of 0.948 under the optimized conditions by following the incubation time of 25 min. The detection limit was found as 26.32 ppb of methyl parathion with optimal conditions of 750 μM substrate concentration, 125 U of enzyme, pH 7.0, 25 min of hydrolysis time and incubation time of 25 min and the proposed method has a quantification limit of 87.72 ppb.

1. Introduction

Organophosphorus (OP) pesticides (herbicides, fungicides, insecticides) are the most toxic compounds and are widely used as pest controllers in agriculture, medicine and industry. Most of the aquatic eco-systems are contaminated by these pesticide residues due to the heavy runoff rain water. The increasing concentrations of organophosphorus pesticide residues are found to be present in many sampled soils, aquatic ecosystems and waste-water streams. It has generated the need to understand and evaluate the biological effects of pollutants on aquatic ecosystems. In this sense, a large number of studies have used biomarkers as functional tools to evaluate the toxicity of such compounds for natural populations [1]. Methyl parathion is an organophosphorus insecticide (Table 1) and it is widely used in the agriculture for the control of most of the pest verities. The mistreatment of these pesticides results in contamination of fields, crops, aquatic environment and air. Methyl parathion is relatively insoluble in water, and readily soluble in most of the organic solvents. The residues of these pesticides in air, aquatic environment, soil and organisms in the environment will influence several chemical and biological factors. Methyl parathion is readily absorbed via all routes of exposure (oral, dermal, inhalation) and is rapidly distributed to the tissues of the living organisms. Most of the traditional analytical methods like High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Liquid Chromatography (LC) coupled to sensitive and specific detectors such as nitrogen–phosphorous detectors (NPD) [2–4], flame ionization detectors (FID), ultraviolet detectors (UV) or diode array detector (DAD) [5–7] and mass spectrometry (MS) [8,9] are used for the quantification of environmental pollutants, pesticide residues in water, soil and other sources. These sophisticated techniques are time-consuming processes; require highly trained personnel, tedious extraction and clean-up procedures prior to instrumental analysis and therefore are not very suitable for routine analysis.

An electrochemical method of sol–gel immobilization acetylcholine esterase enzymatic biosensor method is developed for the determination of pesticide residues in environmental matrices due to their good selectivity, sensitivity and rapid response towards pesticides due to their eco-friendly nature [10]. An immobilizing potentiometric biosensor [11] was developed by using Candida Rugosa Lipase enzyme which shows an intrinsic capability to catalyze carboxylic ester bonds to the corresponding alcohol and acid and the same enzyme was used for the surface acoustic wave impedance sensor for the determination of organophosphorus pesticides [12]. *p*-Nitrophenol is used as a starting substrate for the synthesis of many fungicides, pesticides and pharmaceutical products. In particular *p*-Nitrophenol is a toxic derivative of methyl parathion insecticide [13].

Based on electrochemical response of phenolic compounds [14] towards carbon paste electrode, a simple voltammetric mobilized

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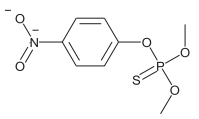
http://dx.doi.org/10.1016/j.molliq.2012.12.032

Table 1
The structure and molecular formulae of methyl parathion.

Sl. no	Selected organophosphorus pesticide	Molecular formula	Chemical structure
01	Methyl parathion	$C_8H_{10}O_5NPS$	

mono-enzyme sensor for the indirect, sensitive and selective determination of methyl parathion is developed. In this work a differential pulse voltammetric lipase enzyme mobilized method is also developed which can be applied for the determination of methyl parathion in the environmental samples.

2. Experimental section

2.1. Reagents and chemicals

All chemicals were obtained from commercial sources and used without further purification. Lipase from Candida Rugosa (EC 3.1.1.3, type VII, ≥700/mg) *p*-Nitrophenyl Acetate, methyl parathion was purchased from Sigma-Aldrich Chemicals. The pesticide stock solutions were prepared by dissolving in acetone (GR grade solution). The fine graphite powder was obtained from Merck chemicals. Silicon oil, acetone (GR grade) was procured from Himedia chemicals. Phosphate Buffer [0.1 M] was prepared by using 0.1 M disodium hydrogen phosphate and 0.1 M sodium dihydrogen phosphate. All chemicals were of analytical grade and aqueous solutions were prepared with double distilled water. The enzyme stock solution was stored at −5 °C and all stock and working solutions were stored at 5 °C.

2.2. Apparatus

Cyclic voltammetric experiments were performed with a CHI model 660c electrochemical work station with a connection to a personal computer that was used for electrochemical measurement and treating of data. A conventional three electrode cell was employed throughout the experiments, with a bare carbon paste electrode (homemade cavity of 3.0 mm diameter) as a working electrode, saturated calomel electrode (SCE) as a reference electrode and a platinum wire as a counter electrode. All the experiments were carried out at room temperature 25 ± 2 °C.

2.3. Preparation of bare carbon paste electrode

The bare carbon paste electrode was prepared by hand mixing of 70% graphite powder and 30% silicon oil in an agate mortar to produce a homogenous carbon paste. The paste was packed into the cavity of homemade PVC (3 mm in diameter) and then smoothed on a weighing paper. The electrical contact was provided by copper wire connected to the paste at the end of the tube [15].

3. Results and discussion

3.1. Cyclic voltammetric studies

The electrochemical behavior of in situ generated *p*-Nitrophenol from the hydrolysis of *p*-Nitrophenyl Acetate substrate in the presence of Candida Rugosa Lipase enzymatic hydrolysis was examined. Fig. 1 shows a cyclic voltammogram of the *p*-Nitrophenol in the absence and presence of 125 units of Lipase enzyme with 750 μM substrate in 0.1 M phosphate buffer (pH 7.0) at a scan rate of 30 mV/s. In the absence of enzyme and substrate (blank) the electrode gave no response and only a small background current was observed (peak a). In the presence of only 750 μM substrate a little back ground peak current was observed (peak b). When enzyme was added to 750 μM of substrate after 25 min a relatively large anodic peak current at a potential of 0.05 V (peak c) was obtained. The *p*-Nitrophenol molecule readily undergoes electrooxidation. Primarily, it is oxidized to nitro-phenoxy radical as shown in Eq. (1) according to literature report [14].

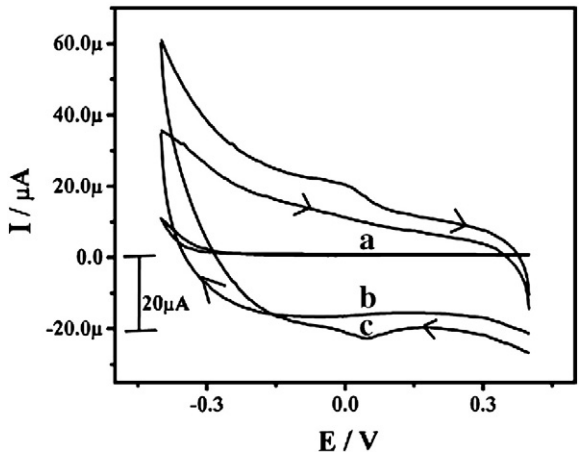


Fig. 1. Cyclic voltammograms of in situ generated *p*-Nitrophenol in 0.1 M phosphate buffer, pH 7.0 a) blank; b) 750 μM substrate alone; and c) substrate in the presence of 125 U of enzyme.

$$C_6H_4NO_2OH \longrightarrow C_6H_4NO_2O^{\bullet} + H^+ + e^- \quad (1)$$

This radical intermediate subsequently undergoes polymerization leading to the formation of a non-sticky thin film on the electrode surface. For further studies the non-sticky polymeric film electrode surface was removed by physically smoothing against a tissue paper [14]. When the concentration of enzyme was gradually increased from 25 U to 250 U there was a gradual increase in the peak current response and this response was finally saturated at 125 U of enzyme concentration where the current values observed were almost constant (Fig. 2) and the optimized enzyme concentration is presented (Table 2).

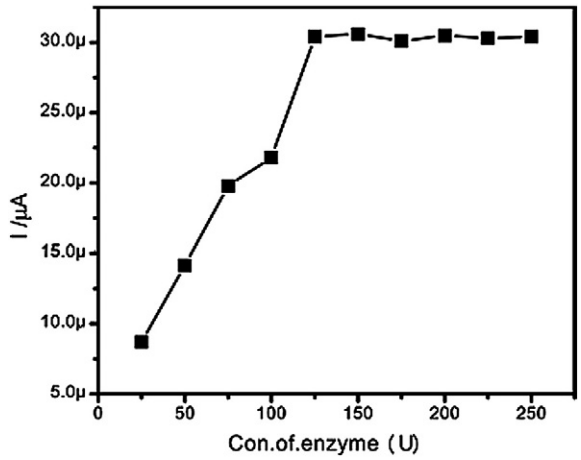


Fig. 2. Response of increasing concentrations of Lipase enzyme in 0.1 M phosphate buffer pH 7.0.

Table 2
Optimized study conditions for Candida Rugosa Lipase enzyme activity.

Sl. no	Name of the optimized parameters	Optimized value
01	Substrate concentration	750 μM
02	Enzyme units	125 U
03	Hydrolysis time	25 min
04	Selected pH	7.0 pH

3.2. The effect of scan rate on the peak currents of p-Nitrophenol

The effect of scan rate for *p*-Nitrophenol was studied at carbon paste electrode. It showed an increase in the oxidation peak current with an increase of scan rates from 10 to 400 mVs^{-1}. The graph of current (Ipa) vs square root of scan rate ($\nu^{1/2}$) was plotted. The result of the graph obtained is with a good linearity (Fig. 3) which indicates that the electrode reaction is a diffusion controlled process [16–18].

3.3. The effect of pH on hydrolysis of substrate and oxidation of p-Nitrophenol

To establish optimum conditions the effect of pH on 750 μM of substrate hydrolysis by mobilized Lipase enzyme is studied in (0.1 M) phosphate buffer with pH 6.0–8.0. The resulting profile showed the maximum hydrolysis of substrate by mobilized Lipase enzyme at pH 7.0 (Fig. 4) [19]. The obtained optimized pH value of substrate hydrolysis by Lipase enzyme is tabulated (Table 2). The potential diagram was constructed by plotting the graph of anodic peak potential Epa vs pH of the solution (Fig. 5). The pH dependence of oxidation peak potential of in situ generated *p*-Nitrophenol reveals that there is a potential shift towards positive and the Ep = 0.5752 + 0.071 and this graph is almost linear with a slope of 71 mV/pH, this behavior is nearly obeying the Nernst equation for an equal number of electron and proton transfer reaction [20,21].

3.4. The effect of substrate concentration on enzyme catalysis nature

The effect of the substrate concentration on 125 U of Lipase enzyme in 0.1 M phosphate buffer was investigated. Choosing of a proper substrate concentration is important for enzyme inhibition studies. Fig. 6 shows various concentration levels of substrate hydrolysis by the enzyme. From this it can be inferred that the hydrolysis of *p*-Nitrophenol Acetate to *p*-Nitrophenol was linearly increased from 125 μM to 750 μM, and then after that the hydrolysis was continuously decreased from 750 up to 1750 μM. The obtained optimized concentration of substrate hydrolysis by Lipase enzyme is tabulated (Table 2).

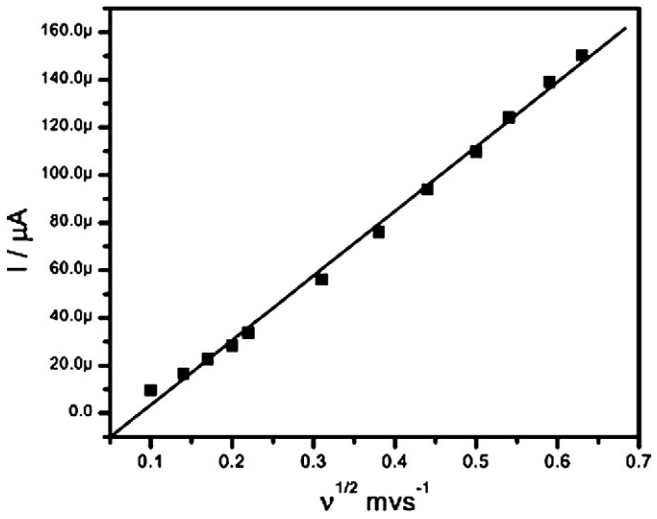


Fig. 3. The effect of square root of scan rate on anodic peak current for *p*-Nitrophenol in 0.1 M phosphate buffer of pH 7.0.

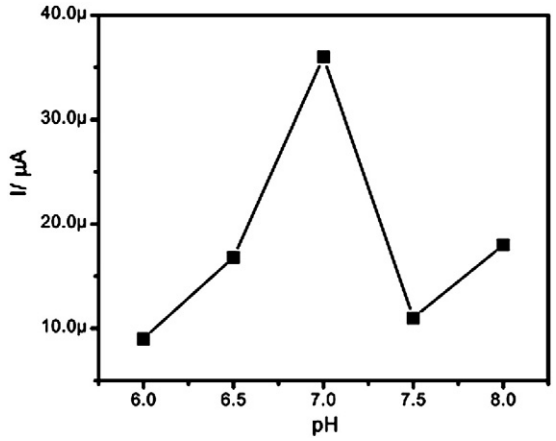


Fig. 4. The effect of pH on hydrolysis of 750 μM substrate in 0.1 M phosphate buffer.

3.5. The effect of time on the hydrolysis of substrate

The effect of time variation on *p*-Nitrophenyl Acetate (substrate) hydrolysis by 125 U of mobilized Lipase enzyme is studied in 0.1 M phosphate buffer with 750 μM substrate. The time vs substrate hydrolysis product (Fig. 7) showed a gradual increase in current from 5 min to 25 min of hydrolysis and this increase was continued till a plateau level was reached and after that there was no significant increment in the hydrolysis of substrate up to 1 h. The obtained optimized time for the hydrolysis of the substrate by Lipase enzyme is presented (Table 2).

Table 2 shows the optimized parameters for Lipase enzyme activity on *p*-Nitrophenyl Acetate (substrate) in 0.1 M phosphate buffer solution. These optimized experimental parameters for Lipase enzyme were applied for further pesticide inhibition studies.

3.6. Pesticide study

The Candida Rugosa Lipase enzyme was used to carry out inhibition studies by incubating with pesticide solution up to 25 min to obtain lower detection limits. Inhibitor was mixed in 1:1 ratio with

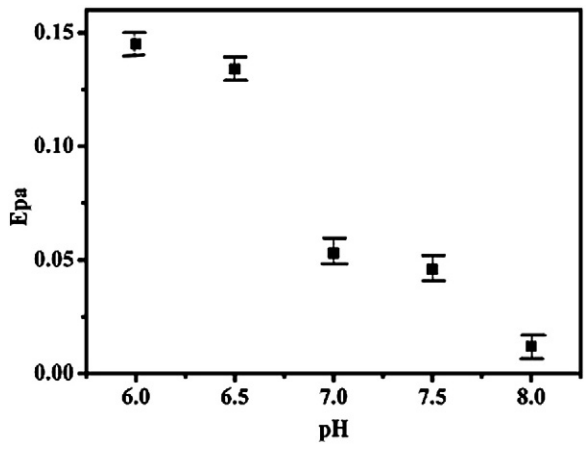


Fig. 5. The effect of pH on anodic peak potential (Epa) of *p*-Nitrophenol in 0.1 M phosphate buffer.

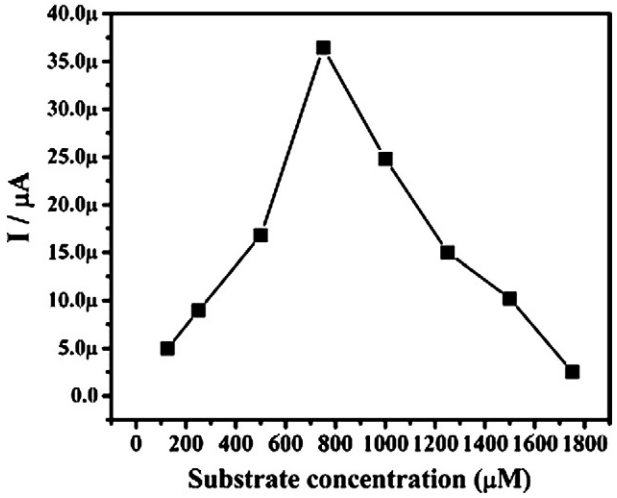


Fig. 6. The effect of substrate concentration on enzymatic hydrolysis.

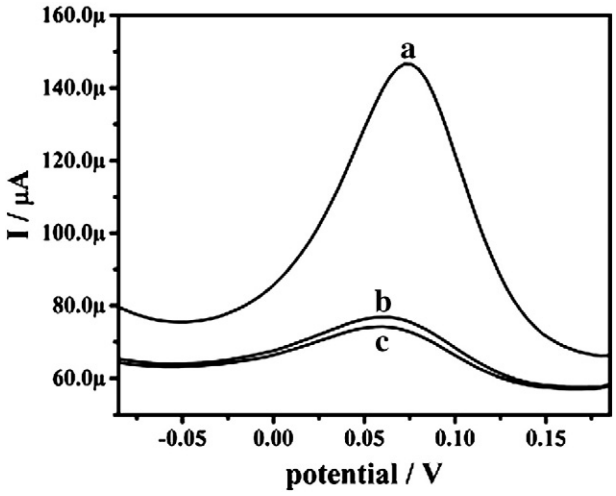


Fig. 8. Differential pulse voltammograms of (c) substrate alone, (b) enzyme inhibition in the presence of pesticide, and (a) complete hydrolysis of substrate in the presence of enzyme.

125 U of enzyme stock solution and incubated at a room temperature of 25 ± 2 °C. To obtain an inhibition plot for methyl parathion the percentage of inhibition method was studied. The detection was based on the measurement of initial (I_i) steady state current response for the product obtained due to complete hydrolysis of liquid phase mobilized enzyme towards the selected concentration of 750 µM substrate in 0.1 M phosphate buffer solution at (pH 7.0). The 1:1 ratio of enzyme and pesticide solution was incubated for 25 min, followed by the transfer of the inhibited enzyme into the electrochemical cell for the measurement of final (I_f) steady state current response in the presence of inhibited enzyme towards hydrolysis of 750 µM substrate. The rate of inhibition (%) and residual enzyme activity was determined according to the following Eqs. (2) and (3) [10,22].

$$\text{Inhibition } \% \ (I\ \%) = [(I_i - I_f)/I_f] \times 100 \quad (2)$$

$$\text{Residual enzyme activity } \% \ (REA\ \%) = [I_f/I_i] \times 100 \quad (3)$$

Methyl parathion pesticide is known to inhibit the catalytic nature of lipase enzyme towards substrate therefore toxic reference to test the lipase enzyme activity was chosen for inhibition studies. Fig. 8 shows the differential pulse voltammogram of substrate alone (peak c) indicating the presence of little amount of *p*-Nitrophenol, a starting substance for the synthesis of *p*-Nitrophenyl Acetate, (peak b) indicates the inhibition of enzyme catalytic activity in the presence of pesticide which is evidenced by the reduction in current response. (Peak a) represents the complete hydrolysis of substrate in the presence of enzyme.

Calibration plots based on the dependence of the percentage of inhibition on concentration are linear (Fig. 9), the detection limit and limit of quantification values were 26.32 ppb and 87.72 ppb respectively for methyl parathion. Various concentrations ranging from 10 to 70 ppb were tested in terms of their effect on enzyme activity at different incubation times (5, 10, 15, 20 and 25 min) in pesticide solutions. When the concentration of methyl parathion increased the residual enzyme activity of the enzyme was decreased with time as shown (Fig. 10). Determination of detection limit (DL) and quantification limit (QL) was carried by using the following formulae (4) and (5) [23–25].

$$DL = 3\ S_b/S \quad (4)$$

$$QL = 10\ S_b/S \quad (5)$$

where S_b is standard deviation, S is the slope of the working curve, DL is the detection limit and QL is the quantification limit. Table 3 shows the various parameters determined for methyl parathion.

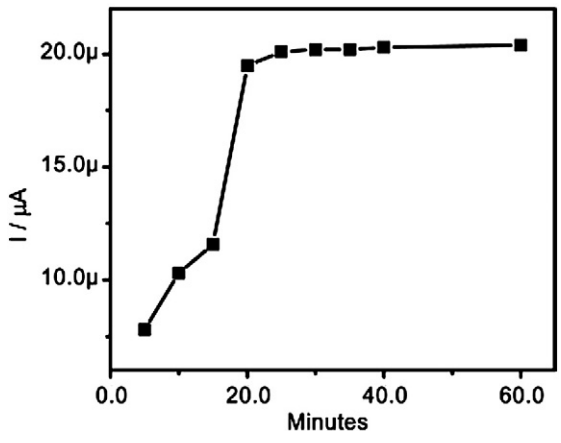


Fig. 7. Time variation on enzymatic hydrolysis.

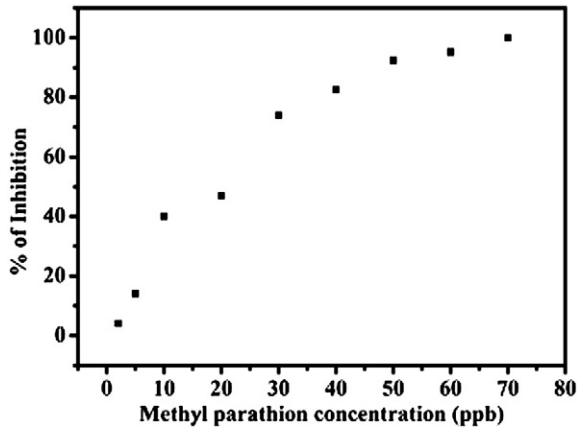


Fig. 9. Inhibition plots of methyl parathion after 25 min incubation time with the measurement condition in 0.1 M phosphate buffer.

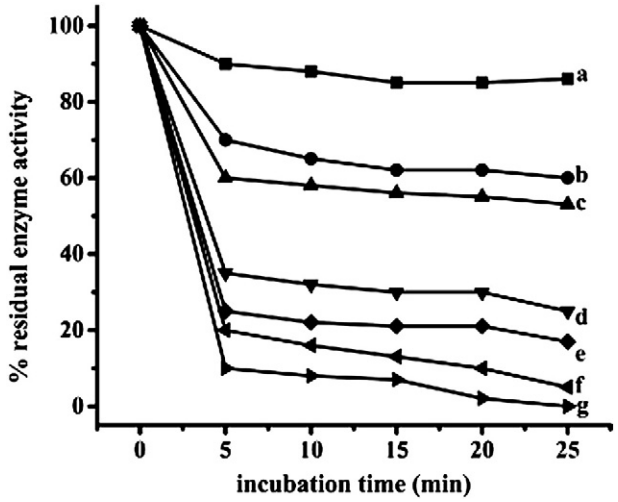


Fig. 10. The effect of incubation time at various inhibitor concentrations on the activity of mobilized Candida Rugosa Lipase enzyme in 0.1 M phosphate buffer pH 7.0 for methyl parathion: (a) 10 ppb, (b) 20 ppb, (c) 30 ppb, (d) 40 ppb, (e) 50 ppb, (f) 60 ppb, and (g) 70 ppb.

Table 3
The various parameters determined for methyl parathion.

Sl. no	Parameters	Methyl parathion
1	Incubation time (min)	25 min
2	Response time	05 min
3	Linear range (ppb)	10–70
4	Correlation coefficient	0.948
5	Standard deviation	12.1607
6	Detection limit (DL) (ppb)	26.32
7	Quantification limit (QL) (ppb)	87.72

4. Conclusions

The present study described the preparation of lipase based mobilized electrochemical sensor within the electrochemical cell to determine the concentration of organophosphorus pesticides. This method is based on the study of inhibition percentage of lipase enzyme activity. Electroanalytical investigation of methyl parathion is achieved down to 10 ppb (correlation coefficient = 0.948 and slope = 1.3862) at pH 7.0. The plot obtained with concentration of methyl parathion vs inhibition percentage yields a straight line almost passing through the origin rendering it suitable for electroanalysis by enzyme inhibition method. The proposed detection method is simple, cost effective, eco-friendly and can be safely used for the determination of methyl parathion in environmental samples.

Acknowledgment

The authors are thankful to the University Grants Commission, New Delhi Government of India for providing the financial assistance through major research project F. no. 39 744/2010 (SR).

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