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Kinetic Evaluation of the Inhibition of Acetylcholinesterase for Use as a Biosensor

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The release of pesticides into the environment has increased, and there is a lack of monitoring of these contaminants. Since the conventional methods of monitoring these contaminants are complicated, costly and time-consuming, mechanisms based on acetylcholinesterase inhibition have emerged as simple and rapid tools for such applications. However, the acetylcholinesterase's effectiveness as a sensing element in such biosensor systems depends on the conditions selected to measure acetylcholinesterase activity and the concentration of substrate or inhibitor, which in turn affect the reaction rates. Therefore, in the present work, the factors affecting the acetylcholinesterase activity were investigated and inhibition experiments were carried out to evaluate the kinetic parameters. The inhibition rate constant for acetylcholinesterase K_i was found to be 1.9 ppm. The kinetic parameter K_m was found to be 3.8mM and V_{max} was found to be 1.3µM/min from the Eadie-Hofstee plot. The kinetic study using Lineweaver-Burk method showed mixed type of inhibition of acetylcholinesterase with carbofuran.

Keywords: Acetylcholinesterase, Biosensor, Inhibition, Kinetics

INTRODUCTION

Pollution of the environmental water and soil by pesticides had become a significant area of concern (Roy 1990, Abhilash and Nandita 2009, Ghorab and Khalil 2016) due to the use of a large number of pesticides in agriculture and related activities. Pesticides are generally determined using liquid (Jose and Ana 2008, Fuad 2016) or gas chromatography (Bruce et al. 1983, Zhi 2017) methods which are sensitive but require sample pretreatment such as extraction, preconcentration, clean up, skilled techniques and also are time-consuming. In this context, enzyme inhibition based sensing systems (Vangelis and Yannis 2002, Siriwuan et al. 2005, Shuaping et al. 2008, Valber et al. 2008, Bhuvanagayathri et al. 2018) are considered as the best choice. These systems have the potential to replace the classical analytical methods by simplifying or eliminating sample preparation protocols and making field testing easier and faster with a significant decrease in costs per analysis. Organophosphates and carbamate

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pesticides are potential inhibitors of Acetylcholinesterase (EC 3.1.1.7), and hence the development of biosensors using acetylcholinesterase has been in focus for monitoring of organo-phosphorus and carbamate pesticides.

An amperometric based biosensor was developed by immobilizing cholinesterase on the chemically modified electrode for the selective determination of carbamate pesticides (Gilvanda et al. 1998) to carbofuran levels up to 10^{-4} ppm. Multiwalled carbon nanotubes with immobilized cholinesterase have been investigated for detection of carbofuran levels of 10^{-11}g/l in the sea water. Fatma et al. (2002) studied the inhibition mechanism of the biosensor with carbaryl. The biosensor was preincubated with the pesticide for varying time, and then the substrate was added to measure an inhibited enzyme activity. The inhibition was found to be non-competitive. The selection of substrate concentration [S] is essential to study the inhibition and determine the percentage inhibition. Higher inhibitions was observed at high [S], but at low substrate concentration, the detection of pesticides at low concentrations was not achieved. Biosensors work based on the inhibition of an enzyme; the degree of inhibition depends on the concentration of pesticide and substrate. Variation of these parameters by keeping other constant helps to decide the type of inhibition and, thereby, inhibition constant. The rates of inhibition also depend on the nature and type of inhibitor. Therefore, establishing the operational ranges of all these parameters is essential for the development of such enzyme-based mechanisms. The development of a biosensor based on inhibition requires a detailed study of the inhibition kinetics (Lanny et al. 2017).

In the present work, the effect of temperature, pH, and substrate concentration on acetylcholinesterase activity was investigated. The influence of substrate and carbofuran on acetylcholinesterase activity by keeping one constant at a time _ was established. Further, the mode of inhibition was explored through a kinetic study.

MATERIALS AND METHODS

Acetylcholinesterase (AChE (electric eel)), acetylthiocholine iodide (ATChI) and 5,5′-dithiobis (2-nitrobenzoic acid, dinitro thio benzoic acid (DTNB)) and carbofuran were procured from Sigma-Aldrich (India). Bovine serum albumin, methanol, phosphate buffer saline (PBS). and sodium hydroxide carbofuran, were obtained from SRL (Mumbai, India) and were of AR grade. Deionized water was used for the preparation of all reagents and chemicals. Acetylthiocholine iodide was stored in the freezer (-4ºC).

Acetylcholinesterase Assay

The enzyme activity was determined using modified Ellman's method as reported elsewhere (Ellman et al. 1961, Seyed 2018), with slight modifications. Fifty µl of the enzyme was added to 3 ml of phosphate buffer and incubated for 5 minutes. Then, 100 µl of DTNB was added to the enzyme solution, followed by 20 µl of acetyl thiocholine

iodide (ATChI) solution to give the final concentration 1 mM of the substrate. The increase in absorbance at 412 nm was recorded for the blank solution (without enzyme) and test sample (with enzyme). In the former case, 50 µl of buffer solution was added instead of enzyme solution. One unit of acetylcholinesterase activity was defined as the amount of enzyme which catalyzed the reaction to produce one micromole of thiocholine per minute per ml of enzyme solution.

Effect of pH on AChE

Experiments to study the influence of pH on the activity of acetyl-cholinesterase were carried out as per the following procedure. Phosphate buffer solution (0.1 M) was prepared in the range of pH 5.5 – 8.5. 50 µl of the enzyme solution was incubated for 5 minutes in each buffer solution at room temperature. After incubation, 100 µl of DTNB was added to the enzyme, followed by the addition of 20 µl of ATChI. The increase in absorbance at 412 nm was recorded for blank and test solutions. The readings were taken every 20 seconds.

Effect of Temperature on free AChE

Temperature ranges from 15°C - 40°C were selected to investigate the effect of temperature on the activity of acetylcholinesterase. Fifty µl of enzyme solution was added to 3 ml of phosphate buffer (optimum pH) and incubated at every temperature for 5 minutes. After 5 minutes, DTNB followed by substrate was added to the enzyme solution. Absorbance was recorded at 412 nm for both blank and test solutions. The readings were taken every 20 seconds.

Effect of Substrate on free AChE

The substrate concentration range was varied from 1 mM to 100 mM. Substrate concentrations of 100 mM, 75 mM, 50 mM, 5 mM, 1 mM were prepared, and the activity of AChE was determined as per procedure mentioned earlier for each substrate concentration at optimum pH and optimum temperature established.

Inhibition of AChE with Carbofuran

Inhibition of acetylcholinesterase was studied using carbofuran, an inhibitor. The stock solution of carbofuran was prepared in methanol at 100 mg/ml. The intermediate stock solution was made at 10 mg/ml. Dilutions were made with water at different concentrations (10 mg/ml, 8 mg/ml, 6 mg/ml, 4 mg/ml and 2 mg/ml). The effect of inhibitor concentration on enzyme activity was studied by incubating carbofuran with enzyme solution for 5 minutes. After incubation, 100 µl of DTNB was added to the enzyme-inhibitor mixture, followed by 20 µl of ATChI. The absorbance at 412 nm was recorded with and without carbofuran solution. The inhibition was calculated and expressed as a percentage of the ratio of the change in enzyme activity before and after inhibition.

RESULTS AND DISCUSSION

Initial experiments demonstrated the importance of reaction concentrations. Maintaining an appropriate relationship between enzyme concentration, substrate concentration, and conditions are

paramount in obtaining the linearity of the assay. The enzymatic reaction of acetylcholinesterase with acetylthiocholine was found to be highly instantaneous. The formed product thiocholine was colorless, and in the presence of coloring agent DTNB, it formed a yellow color that was absorbed in 412 nm using a UV-Vis spectrophotometer. The reaction was recorded for 10 minutes due to a rapid reaction. The solution needed to be kept in the icebox before the experiment and brought to the required temperature in the water bath. Special care was taken while using acetyl thiocholine iodide as it was auto degradable at ambient temperature. Absorbance values were plotted as a function of time in seconds.

Effect of pH

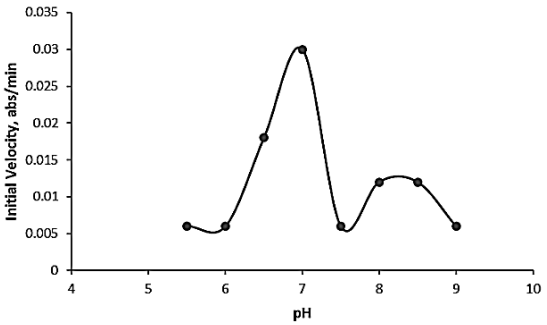

Fig. 1: Effect of pH on the activity of free acetylcholinesterase enzyme

Figure 1 shows the pH profile dependency on the activity of acetylcholinesterase. The characteristics of ionizable groups of amino acid side chains depend on the pH, hence the activity of enzyme varied. At very low pH, a fraction of enzyme attained required form for its catalytic activity. The complete form was required to obtain an optimum pH of 7.0. Further increase of pH, damaged the tertiary structure of enzyme and activity of enzyme further decreased.

It is well understood that the active site of acetylcholinesterase is formed of histidine, serine, and glutamine. Histidine and glutamine are ionizable groups whose pKa values were 6 and 8, respectively. The degree of ionization of their side chain of amino acid residues changed and reached the complete required form at pH 7.0 to give maximum catalytic activity. This is due to the presence of histidine residue, which buffered any change in pH during the reaction and maintained near the neutral pH. This value agrees well with the report by Ferat et al. (2005). However, different optimum pH for AChE has been reported in the literature, which may be due to the presence of ionizable groups in the active site (source of enzyme) and choice of the buffer (microenvironment, which is in the vicinity to the enzyme). Marinov et al. (2009) had obtained a pH of 6.0, whereas Serge (1985) obtained a value of pH 8.0. Cometa et al. (2005) obtained an optimum pH at 7.2. Songci et al. (2007), obtained 7.6 as optimum pH. These variations in optimum pH may be due to a change in the degree of ionization of the side chains of amino acid residues.

Effect of temperature

Figure 2 demonstrates the effect of temperature on enzyme activity. When the temperature was increased, the rate of enzymatic reaction increased until it reached the optimum. The number of collisions of molecules increases with temperature, hence the rate of reaction increases.

The optimum temperature obtained in this work was 25°C, then it was decreased activity after it. Increasing temperature may lead to the breaking of the bonds, which is responsible for the structure of enzyme, thereby its activity. Cometa et al. (2005) and Songci et al. (2007), obtained similar results, whereas Serge (1985) observed optimum at 18°C. Ferat et al. (2005) and Marinov et al. (2009) also have reported optimum temperatures of 30ºC and 38ºC, respectively.

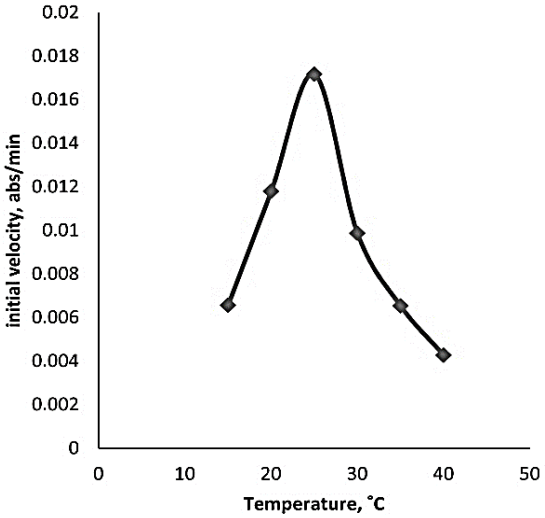

Fig. 2: Effect of temperature on the activity of free acetylcholinesterase enzyme

Effect of substrate concentration

The effect of substrate concentration on the acetylcholinesterase activity was studied at optimum pH and temperature in the range of 1 mM to 100 mM. The profile was hyperbolic in the range investigated. The activity of the enzyme attained saturation at a substrate concentration of 75 mM (Figure 3).

An explicit understanding of kinetic behavior is essential to understand the kinetic aspects of the reaction. The Lineweaver–Burk (LB) plot (or double reciprocal plot) is a graphical representation of the enzyme kinetics (Lineweaver and Burk, 1934) for a quick, visual impression of the form of enzyme inhibition and is expressed as $1/V = (K_m/V_{max})(1/[S]+(1/V_{max})$. Further, the Eadie-Hofstee relation expressed as $V = -Km (V/[S]) + V_{max}$ also was used to construct the linear plots (Figs. 4 and 5) and to evaluate the kinetic parameters.

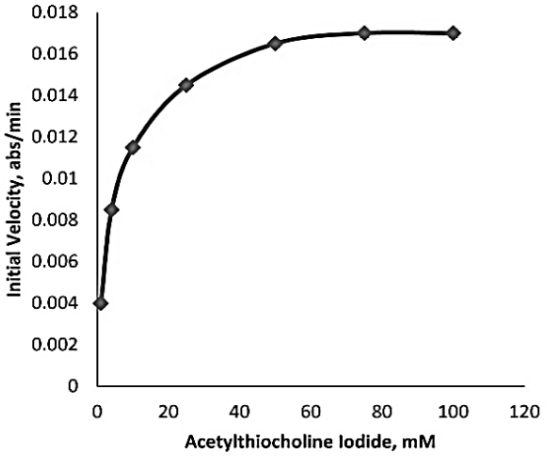

Fig. 3: Effect of substrate concentration on acetylcholinesterase without inhibitor

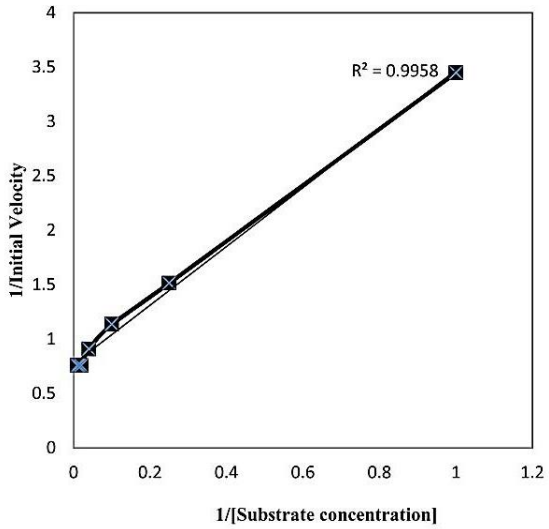

Fig. 4: Lineweaver-Burk Plot plot for the effect of substrate concentration on enzyme activity

The K_m and V_{max} were evaluated from the Lineaweaver-Burk plot. The data

points at higher values of 1/[S] are closer. This is because this plot was found to distort the experimental error in initial reaction velocity with error amplified at low substrate concentration. Eventually, this result will yield to inaccuracy. Since in this plot, the intersecting at the axes was very close to the origin, when the substrate range is higher than K_m, an Eadie-Hofstee plot was constructed and is shown in Figure 5, which approached linearity. Hence K_m and V_{max} values were estimated from Eadie-Hofstee linearized plot. The values of K_m and V_{max} from the Eadie-Hofstee plot were found to be 3.8 mM and 1.3 µM/min, respectively (Table 1).

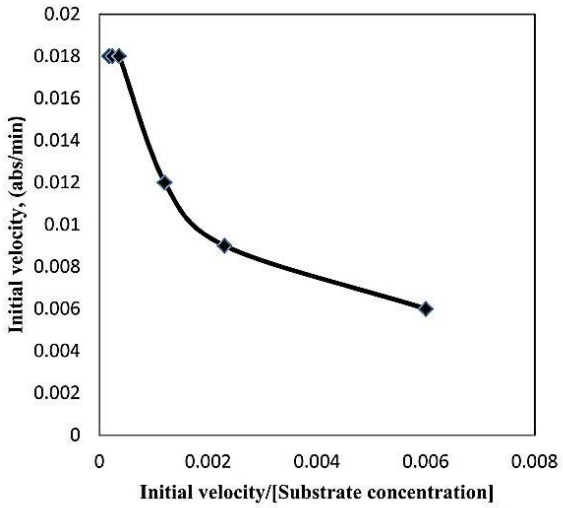

Fig. 5: Eadie-Hofstee plot for the effect of substrate concentration on enzyme activity

Table 2 depicts the comparison of the kinetic parameters estimated in the present work with those carried out by earlier investigators. The results of the current work compare well with the same.

Table 1. Comparison of K_m and V_{max} values from Lineweaver-Burk plot and Eadie- Hofstee Plot

Plot	K_m (mM)	V_{max} (µM/min)
Lineweave-Burk	3.5	1.3
Eadie-Hofstee	3.8	1.3

Table 2. Comparison of Kinetic Parameters

Range of substrate concentration [S], mM	K_m	V_{max}	Reference
0.001 - 0.05	117.3±3.1 µM	-	Songci et al. 2007
0.5 - 18.75	6.35 mM	50 mM/min	Ferat et al. 2005
0.02 - 0.2	0.9 mM	23.1 µmol /min	Marinov et al. 2009
1 - 100	3.5 mM	1.3 µM /min	**Present work**

Effect of carbofuran on the enzyme activity at [S] – 50 mM

Figure 6 shows that as the concentration of carbofuran increased, the percentage inhibition of acetyl-cholinesterase activity increased. The lowest detection limit for carbofuran was found to be 1.7 ppm. Hence, with acetylcholinesterase concentration of 0.25 U/ml, detection of carbofuran at 1.7 ppm was established from the laboratory studies. However, a detailed investigation was needed to understand the nature of inhibition and to determine the inhibition rate constants**.**

Inhibition Kinetics

In general, inhibition of an enzyme follows Michalies-Menten equation. Inhibition studies were carried out by varying substrate concentration at a

specific carbofuran concentration and also by varying inhibitor concentration at a particular substrate concentration under specified conditions. Figure 7 depicts the change in the initial velocity with varying substrate concentration and inhibition levels. Increasing the inhibitor level from 2 ppm to 4 ppm resulted in an 80.8% reduction in the initial velocity.

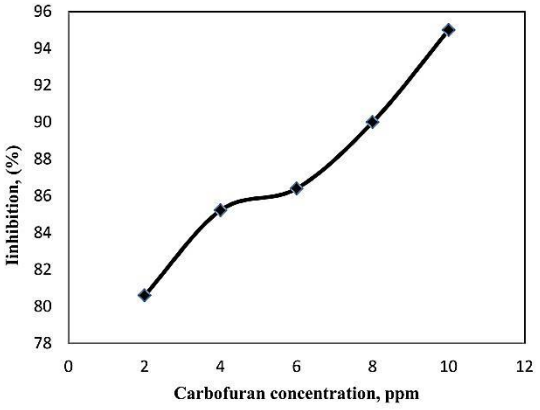

Fig. 6: Effect of carbofuran concentration on enzyme at substrate concentration [S] – 50 mM

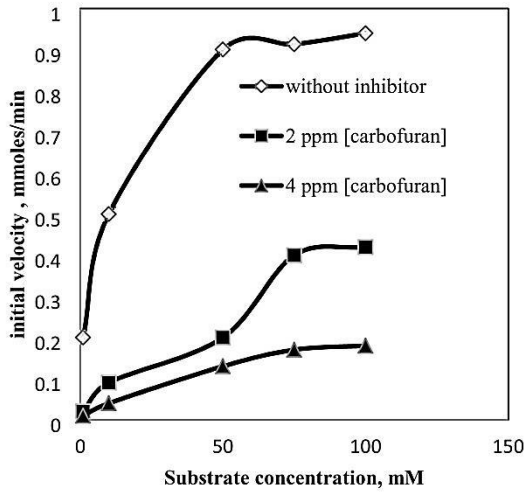

Fig. 7: Effect of substrate concentration on acetylcholinesterase with and without carbofuran at 2 ppm and 4 ppm

From the Lineweaver-Burk plot (Figure 8), V_{maxapp}= 0.55 µmol/min for 2 ppm of carbofuran, V_{maxapp} = 0.2 µmol/min for 4 ppm of carbofuran , V_{max} = 1.43 µmol/min (without inhibition) whereas K_{mapp} = 26 mM for 2 ppm of carbofuran , K_{mapp} = 35.6 mM for 4 ppm of carbofuran and K_m = 3 mM (without inhibition). The maximum reaction velocity and Michaelis-Menten constant from the Eadie-Hofstee plot with 2 ppm and 4 ppm of carbofuran also yielded values for the kinetic constants that agreed well with the earlier ones (Figure 9). Hence it was inferred that V_{max} decreased as the inhibitor concentration increased, whereas K_m value increased as inhibitor concentration increased.

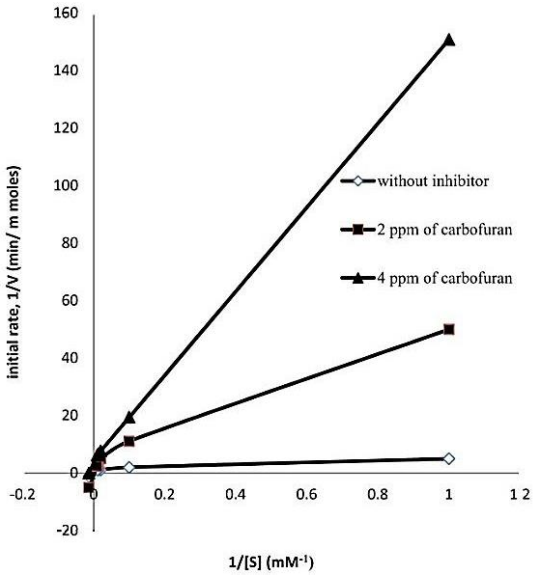

Fig. 8: Lineweaver-Burk Plot with and without carbofuran

Carbofuran was also a structural analog to the substrate. Hence, the inhibition of acetylcholinesterase by carbofuran appeared to be mixed-type inhibition from this study. The mechanism of inhibition demonstrated by the LB plot showed mixed-type inhibition. Many studies show that acetylcholinesterase has beta-amyloid aggregation property, which

can be inhibited by mixed-type mode inhibition. It is due to the binding capacity of carbofuran on the peripheral anionic site of acetylcholinesterase enzyme. The mechanism of inhibition reveals the carbofuran might compete with the substrate or acylated enzyme. It could compete with the enzyme-substrate complex.

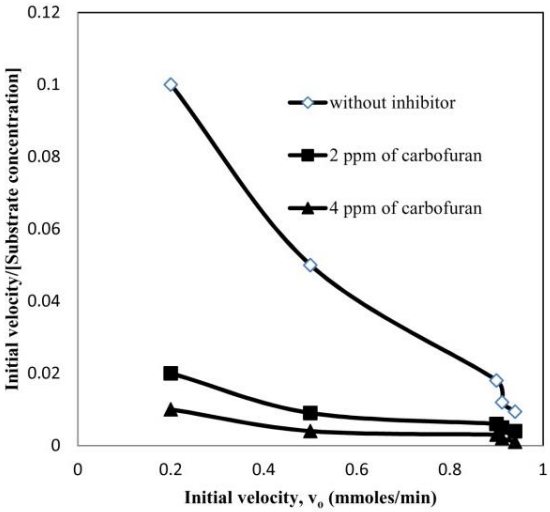

Fig. 9: Eadie-Hofstee plot for effect of on the acetylcholinesterase activity

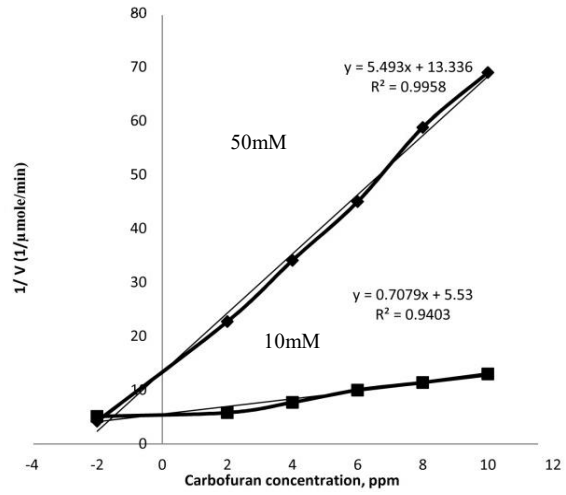

Fig. 10: Dixon plot for inhibition at varying substrate concentration

Thus, the mode of enzyme inhibition was derived from the Lineweaver-Burk, and from EH plots, the constants determined. It was confirmed by plotting the Dixon graph (Figure 10), in which the intersection occurred neither in the x-axis nor in the y-axis, but above the x-axis (second quadrant). The K_i value was calculated as – 1.9 ppm. The inhibitor has strong inhibition potency at this concentration. The free enzyme kinetics and inhibition studies helped to predict the lowest detection limit of pesticide. The results obtained from this study would help in designing the biosensor based on the inhibition principle for the detection of pesticides and other environmental pollutants.

CONCLUSION

The effect of pH and temperature was investigated to fix the condition for further studies of enzyme kinetics. Substrate concentration on acetyl-cholinesterase was investigated in the range of 1 – 100 mM. The free enzyme kinetic parameters were evaluated and established for this system. The observed decrease in the activity of enzyme provides a way to develop the biosensor for monitoring the pesticides in a challenging environment where multiple pollutants are present. Inhibition kinetic studies reveal that carbofuran inhibition follows mixed-type inhibition with the corresponding K_i values being established.

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NOMENCLATURE

μ	: micro
M	: molar
nm	: nanometer
S	: substrate
l	: litre
C	: celsius
g	: gram
mg	: milligram
R	: regression coefficient
abs	: absorbance
K_i	: inhibition constant
K_m	: michaelis-menten constant
V	: reaction velocity
V_{max}	: maximum velocity
min	: minutes
ATChI	: acetyl thiocholine iodide
DTNB	: 5, 5'- dithiobis (2-nitrobenzoic acid)
PBS	: phosphate buffer saline
SRL	: sisco research laboratories
ppm	: parts per million

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