



Review Paper

## Current trends in enzymatic biosensors for pesticides determination

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### Abstract

*Owing to the documentation of worldwide surveys of pesticides in different food products and high mortality rate associated with its exposure to environment and human, pesticides has become a serious public health concern. Maximum residual limits for pesticides as a legal requirement have been laid down by several regulatory bodies throughout the world. It is very important to quantify pesticide residues by using different analytical methods which are extremely susceptible and accurate due to the trace level of pesticides. Although conventional analytical methods, based on different chromatographic techniques like GC, HPLC coupled with mass selective detectors, fulfil these requirements. Despite, these have intrinsic demerits e.g. limited scope of application under field conditions, time-consuming, cost-effective and are not ease for the direct analysis of pesticides residue in real samples. To address this issue, development of biosensors as rapid alternative techniques for pesticides determination is predominant goal. Enzyme based biosensors has become very popular for their sensitivity and high efficient analysis of pesticides over few past decades. The present article mainly demonstrates the recent advancement in the development of enzymatic biosensors for pesticides determination. Enzyme based biosensors have been classified according to their catalytic and inhibition mechanism for sensing of pesticides. Their construction, mode of immobilization and analytical characteristics are discussed. Applications in the field of environmental safety, food safety and future prospects for development of more superior enzyme based sensing technologies for pesticides determination are also delineated.*

**Keywords:** Pesticides, analytical methods, enzyme, biosensors, catalytic, inhibition.

### Introduction

Pesticides are used globally in agriculture sector to manage the problems of pest to enhance the productivity of crops and overcome to several health effects. The nomenclature and classification of these pesticides are very vast; major groups may be classified on the basis of organism they control, for e.g. insecticides, fungicides, herbicides, etc. or other depends on chemical structure i.e. organophosphate, carbamate, organochlorine<sup>1-4</sup> etc. (Table-1). Currently, around 800 pesticides active ingredients are present in the form of commercial products. These substances generally belong around more than hundred classes<sup>2</sup>. Pesticides are used worldwide with their production and a billion dollar market in the form of sale. Europe secures 1<sup>st</sup> rank globally in pesticides sales market followed by Asia, North and Latin America (Figure-1). Presently the excessive use of pesticides has become dangerous to the environment as well as human health and also beneficial soil microorganisms to plants, insects, birds and fish etc<sup>1</sup>. Mostly they are supposed to be primarily dangerous environmental contaminants because of their behaviour to bioaccumulate and their mobility as well as long term effects on the environment<sup>4</sup>. Basically, the residues of pesticide can be enter into the food chain through soil, water and air.

Therefore, their content could be produce bone marrow and nerve disorders, infertility, immunological and respiratory diseases and are also known to inhibit fundamental metabolic pathways<sup>5,6</sup>. Thus, the use of such xenobiotic in food production certainly leaves few residues and clearly represents a potential risk for consumers<sup>7</sup>. There is growing concern about risk and safety of pesticides in food and environment due to the environmental contamination associated with pesticides<sup>8</sup>. In order to guarantee consumer safety, MRL and ADI values for pesticide residues in various types of foods like fruits, vegetables, milk and other dairy products etc. have been set by several organizations e. g. European Union, Codex Alimentarius and Food Safety and Standards Authority of India etc and thus requiring effective methods for enforcement<sup>9</sup>. Current increases in the threat of food contamination of pesticides have led the food industry to analyze rapid and cost effective methods for the health and safety of consumer. Although sophisticated AOAC approved standard analytical methods i.e. GC-MS, LC or HPLC-MS allow sensitive, efficient and more reliable detection of pesticides qualitatively and quantitatively<sup>10-13</sup>. But these methods have some inherent disadvantages like time-consuming and not cost effective. Therefore, there is need to develop effective screening methods at a lower cost and with less operator training.

Biosensor based analytic methods could become most promising tools for detection of very low level of pesticide residue. Use of enzymes as biorecognition molecule has been proved to be very effective in pesticides analysis. Several authors reported different types of analytical methods including biosensors based on enzyme inhibition especially those based on acetylcholinesterase (AChE) inhibition for pesticides detection. In order to avoid repetitions of already existing research work, the present article emphasized on enzymatic biosensors based on both inhibition and catalytic principle. This is the first article that demonstrate all the enzymes used over past decades for the development of pesticides biosensors, their construction, analytical performance and their beneficial applications. Factors which affect the performance of enzyme based biosensors could be useful for design and improvement of sensors. The major aim of this article is to explore the potential of other enzymes, like AChE (widely used) for their use as biorecognition molecule for the development of biosensors for the determination of pesticides in order to ensure environmental and food safety. Recently, Campana et al. studied electrochemical based biosensors for microfluidic platforms to detect the residues of pharmaceutical in wastewater<sup>14</sup>.

**Enzymes:** Enzymes are well known globular proteins composed amino acids ranging from 65 to over 2,450 based on their origin. These are biocatalysts and have the unique property to react selectively with substrate. Activity of enzymes is calculated by their biochemical composition and tertiary structure. Their activity is affected by two main types of modifiers e.g. first inhibiting molecules which reduce the activity and activator molecules which increase activity. These external modifiers can be either microbial or non-microbial contaminants present in food and environmental samples. Most of the enzymes may be denatured and lose their secondary and tertiary structure in their native state through application of few external modifiers<sup>15</sup>. Unique characteristics of enzymes e.g. specificity towards its substrate and change of activity make them an attractive tool for biosensor applications because of a variety of measurable reaction products such as electrons, protons light, and heat, arising from the catalytic process in response to modifiers<sup>16</sup>.

**Biosensors:** These are based on the detection of signals produced from the catalyzed reaction of enzyme. Depending on the type of signal generated, several types of transducers are used for the designing of biosensor. These transducers are primarily of electrochemical, amperometric, optical and potentiometric types which detect depletion of electroactive species, voltage applied, absorbance or fluorescence and change in electrode potential respectively (Figure-2). Moreover, a signal processor connected to a transducer collects, amplifies, and displays the signal. Enzymes can either be used singly (monoenzymatic biosensors) or in a group of two (bienzymatic) or set of more than three enzymes (multienzymatic) to recognize more than one type of pesticides in similar biosensor. In context to the present article, Kumar et al.<sup>17</sup> Also studied the detection of organophosphate pesticides through biosensors.

Bucur et al.<sup>18</sup> also reported the efficient enzyme based methods for the detection of pesticide.

## Principle of enzyme based biosensors

Enzyme based biosensor devices were developed using two different types of approaches as given below.

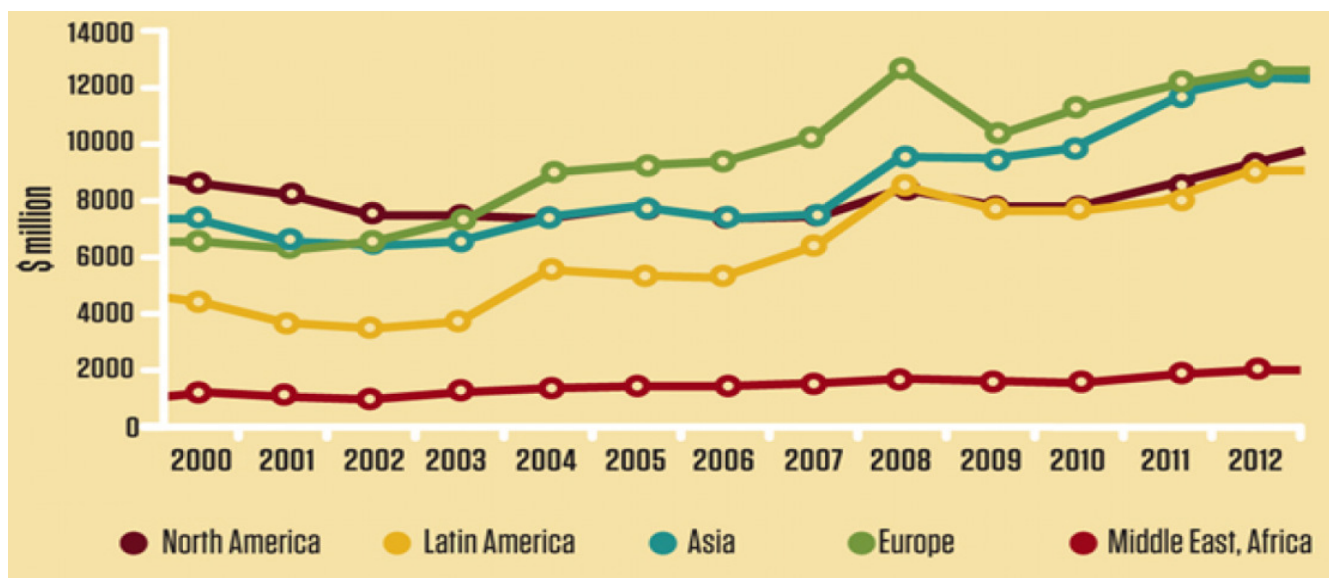
**Catalytic approach:** Enzymes possess very high catalytic activity and can catalyze different compounds effectively. This strategy was successfully used to design enzymatic biosensors to detection the contamination of pesticides. Several enzymes are characterized to metabolize different types of pesticides. Depending upon the quantity of pesticides, the end products of enzymatic reactions are produced and calculated by electrochemical or optical means (Figure-3).

**Enzyme inhibition based approach:** Biosensors based on enzyme inhibition was found large range of applications to trace the pesticide residues in different types of samples viz., water, milk, juices, fruits and vegetables. The working principle of the inhibition based biosensor was used to calculate the activity of enzyme in the presence or absence of pesticide residue as shown in Figure-4. The activity of enzyme decreases in the presence of pesticides could be further related with the amount of pesticides present in the sample. Inhibition of enzyme activity could be calculated with the formula given equation.

$$I\% = \left( \frac{A_o - A_i}{A_o} \right) \times 100$$

Where, I stand for percent inhibition,  $A_o$  for activity of enzyme in the absence of pesticide,  $A_i$  for activity of enzyme in the presence of pesticide. Several authors observed the LOD value for inhibition based sensor that was between 10-20%<sup>19-22</sup>. Moreover, Arduini et al. studied the formula which can calculate both types of enzymatic inhibition<sup>23</sup>.

**Enzyme immobilization:** To develop an enzyme based biosensor, the selection of right type of transducer is very important. As functioning of biosensor in conditions of quantisation of the analyte is achieved by coupling the biorecognition element i.e. enzyme with the transducer. This idea is served through proper choice of an immobilization technique. In the designing of biosensor, immobilization of enzyme plays an important step for its overall performance. The immobilization of the enzymes can give several advantages e.g. thermal and storage stability, greater pH, easier recovery of enzyme and separation of product. Immobilization permits the reuse of enzyme over an extensive time which could be cost effective. Several different approaches were used for the immobilization of enzymes in the devices of biosensor. However, all approaches are not appropriate for the construction of biosensor. But, the most commonly used immobilization techniques with some merits and demerits for designing and development of enzymatic sensors comprise either physical or chemical method<sup>24-27</sup> and these are listed in Table-2.


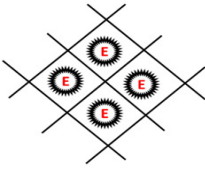
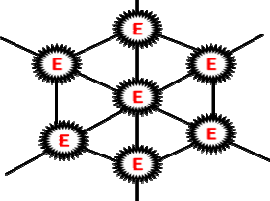
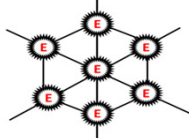


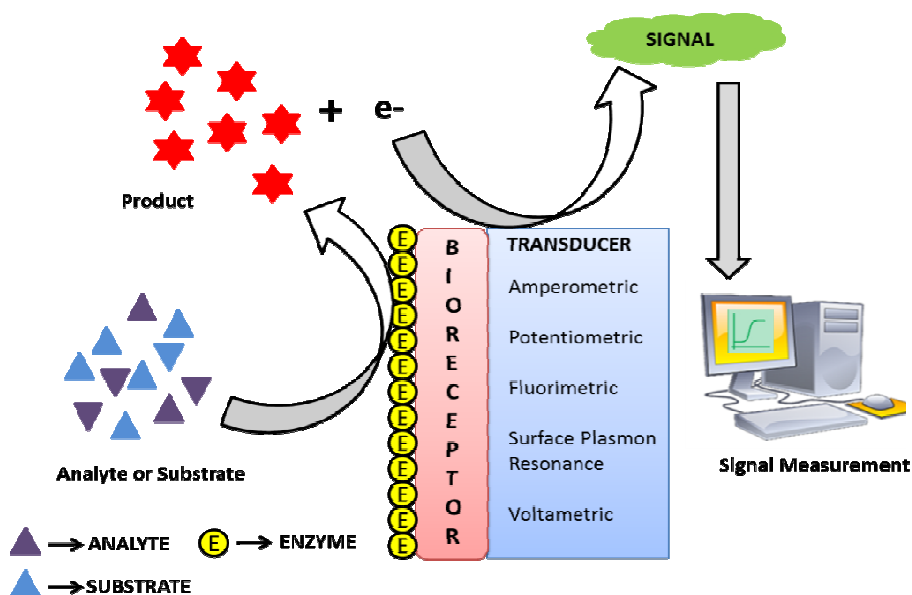
**Figure-1:** Global pesticides sales by region as a function of the year of production and sales from 2000-2012<sup>133</sup>.

**Table-1:** Types of pesticides on the basis of chemical nature, their general formula and members most commonly used in development of enzyme based biosensors.

Group	General Formula	Members used as targets in biosensors
Organophosphorous	$\begin{array}{c} \text{O(S)} \\    \\ \text{R1}-\text{P}-\text{R2} \\   \\ \text{X} \end{array}$	Paraoxon, Methyl Parathion, Malathion, Diazinon, Dichlorvos, Trichlorfon etc.
Carbamates	$\begin{array}{c} \text{O} \\    \\ \text{R}^1-\text{O}-\text{C}-\text{N}-\text{R}^2 \\   \\ \text{R}^3 \end{array}$	Carbaryl, Carbofuran, Thiodicarb etc.
Organochlorine		Aldrin, Heptachlor, Endosulfan etc.
Phenylurea		Diuron, Siduron etc.
Pyrethroides		Pyrethrin, Deltamethrin etc.
Phenoxykanoic acid		2,4-D, 2,4-T etc.

**Table-2:** Methods used for immobilization of enzymes.

Physical Methods				
Method	Principle	Substrate/Reagents	Advantages	Disadvantages
<p>Adsorption</p> 	Electrostatic interaction, Hydrophobic interaction, Vander Waal's forces, Hydrogen bonding	Cellulose, Silica gel, Glass, hydroxyapatite, Collagen etc.	Simple, Easy, Inexpensive, Activity retained	Easily exude upon repeated use, Denaturation of relatively unstable enzymes on hydrophobic surfaces
<p>Entrapment</p> 	The polymer lattice structure is made such that the large enzyme molecule cannot permeate out of the matrix but small substrate molecules can diffuse into the polymer	Polymeric gel such as Polyacrylamide, Starch, Nylon, Hydrogel, Siliastic gel etc.	Simple, Large amount of enzyme can be trapped	Difficult to mass produce, Diffusion
<p>Cross-linking</p> 	Cross linking of enzyme to other enzyme molecules to form a three dimensional complex or to functional groups on an insoluble support matrix	Glutaraldehyde (GA), Hexamethylene diisocyanate, 1,5 difluoro-2,4- dinitrobenzene, Avidin biotin	Simple, Strong chemical bonding, Reduced non- specific interactions	Possibility of activity loss due to the distortion of the active enzyme conformation and the chemical alterations of the active site
<p>Covalent binding</p> 	Initial activation of the carrier, using multifunctional reagents, followed by enzyme coupling to the activated surface	Nucleophilic functional groups present in amino acid side chains of proteins such as amino, carboxylic, imidazole, thiol, hydroxyl are mostly involved	Stable coupling, Intimate contact with transducer, Low diffusion barrier, Rapid response	Complexity and cost of derivatization steps, Limited sites for attachment leads to shorter lifetime



**Figure-2:** Schematic diagram of working of enzymatic biosensor.

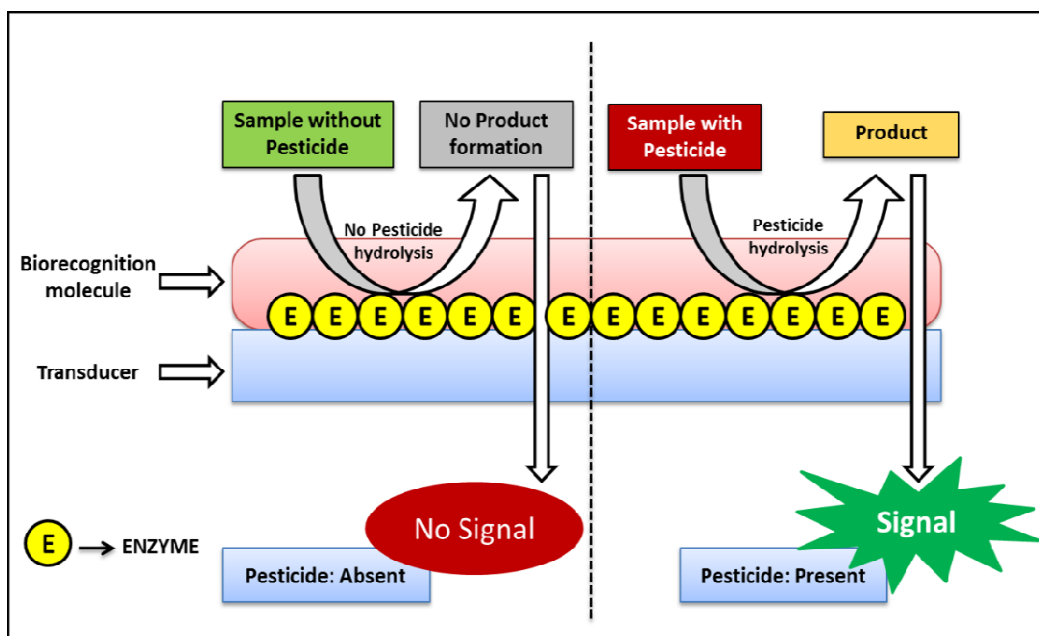


Figure-3: Schematic diagram of enzymatic biosensor based on catalytic approach.

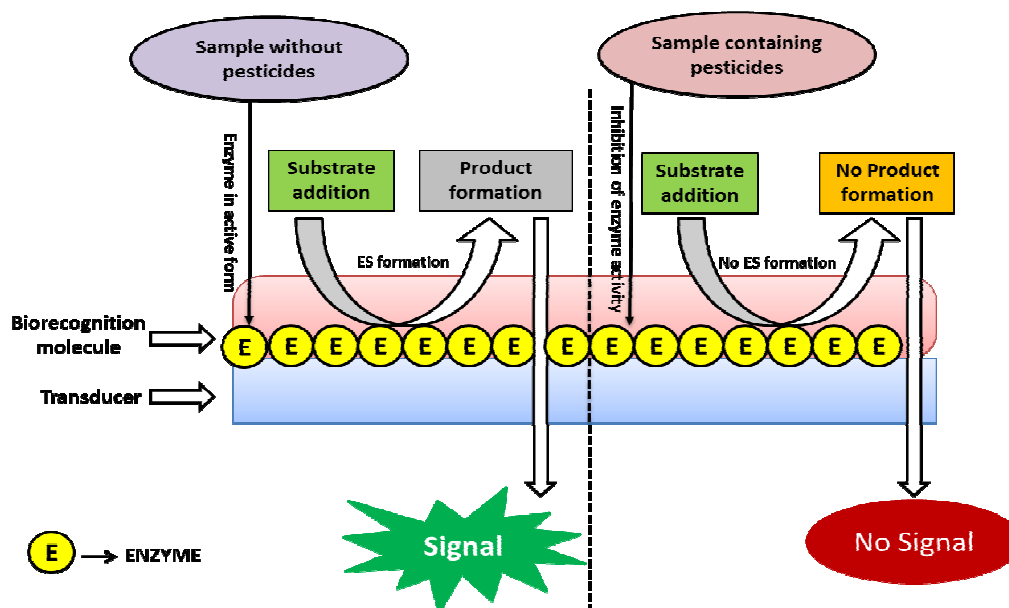


Figure-4: Schematic diagram of enzymatic biosensor based on inhibition approach.

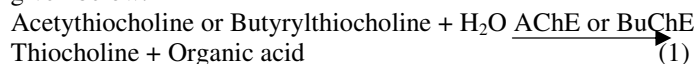
## Enzyme Inhibition based pesticides biosensors

**Cholinesterase based biosensors:** Cholinesterases (ChE) are one of the most commonly used enzymes for the detection of pesticides e.g. AChE, BuChE etc. They both have same molecular organization but dissimilar in the specificity of substrate. Hosea and Berman<sup>28</sup> reported that BuChE hydrolyzes butrylcholine, while AChE predominantly hydrolyses acetyl esters like acetylcholine. Both types of ChE show different degree of sensitivity towards dissimilar inhibitors. Inhibitors are able to chelate different sites of ChE like esteratic part of active site, aromatic gorge and anionic part of the site<sup>29,30</sup>. Esteratic

part is composed of glutamate, histidine, and serine. Organophosphorous and carbamate inhibitors bind to active serine hydroxyl in the esteratic part<sup>31</sup>. ChE are well known to be irreversibly inhibited by organophosphorous pesticides. Hence, biosensors based on this enzyme are primarily known as disposable<sup>32</sup>. However, in case of carbamates like carbofuran, fenoxycarb, methiocarb, the mechanism of action is pseudoirreversible<sup>33,35</sup>. Furthermore, spontaneous hydrolysis could play important role in the splitting of bounded carbamate moiety in the active site.

Therefore, to develop biosensors for analytical applications, ChE are accessible from different sources i.e. electric eel, bovine or human erythrocytes, human blood and horse serum. Among all sources, ChE from insects have been found to be more sensitive than those from any other source<sup>36,37</sup>. ChE based biosensors were developed either based on the use of this enzyme singly or use of this enzyme in combination with other enzymes. Therefore, biosensors based on ChE can be classified into mono-enzymatic, bi-enzymatic and tri-enzymatic. Various such types of biosensors were developed for detection of pesticides using AChE and BuChE, analytical characteristics of some of them are demonstrated in Table-3.

**Mono-enzymatic biosensors:** ChE hydrolyse acetylcholine and butyrylcholine substrate with the production of choline and corresponding organic acid. The same reaction catalyse when artificial substrates e.g. acetylthiocholine and butyrylthiocholine are used with the production of thiocholine and organic acid as given below.



The working of AChE biosensors depend on the measurement of the end product detection in the presence or absence of pesticides using various types of transduction methods like optical, fluorimetric, amperometric, potentiometric and thermometric etc<sup>10</sup>. The most popular used ChE based biosensors for pesticides detection exploit the potentiometric determination of the amount of acid formed in terms of pH change due to the electrochemically non-active nature of choline produced in the enzymatic reaction<sup>38</sup>. Using fibre optic approach a portable AChE biosensor for detection of carbaryl and dichlorvos was developed by taking acetylcholine as a substrate. In order to construct this sensor, the immobilization of AChE was carried out on the outer layer of polyvinylidene fluoride membrane. The membrane was in contact with bromocresol purple on an inner glass disk. Colour of indicator changed as a result of substrate hydrolysis through carbaryl and dichlorvos inhibited enzyme due to local pH alterations. The optical signal was measured spectrophotometrically at 600nm<sup>39</sup>. Activity of ChE can also be taken by an amperometric device when acetylthiocholine and butyrylthiocholine both are used as a substrate. Thiocholine produced as a result of substrate hydrolysis is further oxidized through voltage to dithiocholine. Amperometry based biosensor was developed by immobilization of AChE on TiO<sub>2</sub> (Titanium dioxide) NPs (Nanoparticles) for carbaryl with LOD of 0.0014  $\mu\text{M}$ <sup>40</sup>. As far as substrates are concerned, ChE have also been reported to show good affinity for some chromogenic esters like indoxyl acetate, indophenyl acetate, 2, 6-dichloroindoxyl acetate and 2, 6-dichloroindophenyl acetate<sup>41,42</sup>. Using indoxyl acetate, an AChE based dipstick assay for detection of neurotoxic compound like paraoxon was developed with the detection of 1  $\mu\text{M}$  upon the exposure of naked eye<sup>43</sup>.

**Bi-enzymatic biosensors:** In this biosensor, ChE are usually coupled with choline oxidase (ChO). Bi-enzymatic biosensors catalyze two-step reaction. In first step, acetylcholine or butyrylcholine is hydrolysed to choline and acetic or butyric acid. While in second step, choline generated in first step is oxidised to hydrogen peroxide. Thus, consumption of O<sub>2</sub> or production of H<sub>2</sub>O<sub>2</sub> is measured to determine the activity of enzyme to detect pesticides. Several different transduction approaches in combination with various modes of immobilization were used to develop this biosensors. A biosensor based on the quenching of CdTe (Cadmium telluride) QDs (Quantum dots) by the produced H<sub>2</sub>O<sub>2</sub> for detection of dichlorvos was developed by Meng and co-workers. The LOD of this biosensor was about 0.0044  $\mu\text{M}$  for dichlorvos<sup>44</sup>. Another potentiometric bi-enzymatic biosensor composed of AChE and ChO, co-immobilized on platinum electrode was optimized for carbamates with a detection range of  $9.04 \times 10^{-4} \mu\text{M}$ <sup>44</sup>. Amperometric biosensor was designed with co-immobilization of AChE/ChO on poly (2-hydroxyethyl methacrylate) membranes. The working principle of this biosensor was based on the measurement of O<sub>2</sub> depletion in the medium due to the activity of AChE/ChO by using acetylcholine<sup>45</sup>.

**Tri-enzymatic biosensors:** These were developed by addition of a third enzyme i.e. peroxidase to ChE and ChO based bi-enzymatic biosensor. The working principle of this biosensor involves three steps. In first step acetylcholine is converted to choline by AChE. While, in second step choline is converted to H<sub>2</sub>O<sub>2</sub> by ChO<sup>46</sup>. This H<sub>2</sub>O<sub>2</sub> oxidized 3, 3'-diaminobenzidine to an insoluble product by horseradish peroxidase (HRP). Several transduction and immobilization techniques, like mono and bi-enzymatic biosensor was also used to develop tri-enzymatic biosensors (Table-3). A quartz crystal microbalance (QCM) sensor was developed to determine organophosphorus and carbamate<sup>47</sup>. In the presence of pesticides very less QCM-detectable precipitate produced due to the inhibition of AChE. This sensor was employed for successful detection of carbaryl and dichlorvos<sup>6</sup>. Faxon and co-workers also developed a potentiometric biosensor to detect traces of organophosphorus pesticides. The sensing element was consisted of three different co-immobilized enzymes i.e. ChO, peroxidase and ChE. The detection limit of ChE is primarily dependent on the principle of molecular mechanism. Furthermore, Faxon et al. studied the catalysis reaction of H<sub>2</sub>O<sub>2</sub> through direct electron transfer method<sup>48</sup>.

**Tyrosinase based biosensors:** Tyrosinase belongs to the group of oxidoreductase and also called polyphenol oxidase (PPO). This enzyme consists of copper metal and catalyzes oxidation of diphenols and monophenols to the o-quinones at the expense of oxygen reduction to water<sup>49,52</sup>. This translation of monophenols by tyrosinase is a two-step reaction. In 1<sup>st</sup> step, monophenol gets hydroxylated to o-diphenol and after that o-diphenol gets oxidized to o-quinone<sup>53</sup>. Consumption of oxygen or production of quinones forms the basis of determination of tyrosinase activity in order to detect inhibition by pesticides<sup>53,54</sup>.



Therefore, the electrochemical mode is the most commonly employed transduction system in development of tyrosinase based biosensors for the detection of pesticides. Determination of signal for the production of quinone is beneficial as the quinones are decreased at low potentials. Hence, this in turn leads to amplification of signal in order to enhance the sensitivity of enzymatic sensor<sup>53</sup>. Quinones are unstable in water to form intermediate radicals during their electrochemical reduction. Nistor<sup>55</sup>, Chang et al.<sup>56</sup> explained the intermediate radicals which polymerize the polyaromatic compounds and catalyze different electrochemical reactions. Hence, most of the research work focussed on the stability and sensitivity of tyrosinase based biosensors. Further, the work has been carried out to improve the electrode configurations with the use of suitable redox mediators such as tetracyanoquinodimethane<sup>53,57</sup> to improve the analytical characteristics of tyrosinase electrodes. This redox coupling allows the faster conversion of the enzymatically formed o-quinone to catechol and thus contributes to the electrochemical signal amplification. One such amperometric tyrosinase biosensor of quinoid enzymatic products was developed. The electroreduction was mediated by osmium (4,4'-dimethyl 2,2'-bipyridine) 2 (1,10-phenanthroline-5,6-dione) mediator which was incorporated in the carbon paste material of enzyme electrode. This electrode showed a 3-fold raise in current density and almost two orders of magnitude decrease in the detection limit<sup>53</sup>.

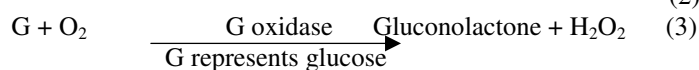
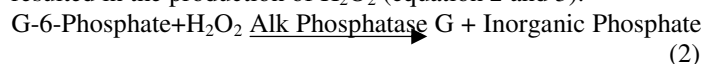
To determine the phenolic compounds and herbicides, an amperometric sensor was developed by immobilization of tyrosinase using PEDT on a glassy carbon electrode (GCE). Here, PEDT film was very efficient PPO immobilization method to detect the concentration of atrazine and diuron<sup>58</sup>. Several different efficient biosensors for detection of organophosphorous, carbamates and other pesticides based on tyrosinase were developed by using different immobilization and transduction techniques as shown in Table-4.

### Alkaline phosphatase based biosensors (ALP)

ALP is an enzyme which catalyses non-specific hydrolysis of orthophosphoric monoesters to alcohols. Various ALP based biosensors using this principle and several immobilization and transduction approaches have been developed as illustrated in Table-5. Transduction techniques mainly included fluorimetric, amperometric and voltametric etc. A fluorimetric, free and sol-gel immobilized ALP biosensor was developed. The substrate used for the development of ALP biosensor was 1-naphthyl phosphate which is hydrolysed by ALP to 1-naphthol, a highly fluorescent product and phosphate. ALP enzyme was found to be inhibited from metham-sodium and tetradifon pesticides. Activity of enzyme in the presence or absence of pesticide was measured in terms of decrease or increase of fluorescent signals produced as a result of formation of 1-naphthol. The LOD with free ALP based sensor were found to be 91.2µM and 4.1µM for metham-sodium and tetradifon respectively.

While, on the other hand with sol-gel immobilized ALP based sensor detection limit was recorded 4.9µM and 292.3µM for metham-sodium and tetradifon respectively<sup>59</sup>. An amperometric biosensor was designed through immobilizing ALP enzyme in a chitosan film and deposited on the carbon paste electrode. Ascorbic acid 2-phosphate was used as a substrate which is hydrolyzed by ALP enzyme. The activity of ALP was measured amperometrically in terms of current change as a result of oxidation reaction. Inhibition of ALP by herbicides resulted in lower ascorbic acid production which is determined by reduction in the current generated. A linear response range of 0.0045-0.27µM was constructed for 2, 4-D and 2, 4, 5-T. The rate of maximum inhibition was 45% and 32% for 2, 4-D and 2, 4, 5-T respectively<sup>60</sup>. A Chemiluminescence biosensor was developed using macrocyclic phosphate compound, hydrolysed by ALP generating a chemiluminescence signal. Detection limit of this biosensor was found at ppb level for paraoxon and methyl parathion<sup>61</sup>. The chemiluminescent biosensor was designed by using alkaline phosphatase in bulk solutions and then immobilized form for reactions on optical fibers. A macrocyclic compound, chloro-3-(4-methoxy spiro [1,2 dioxetane-3-2'-tricyclo-"3.3.1.1"-decan]-4-yl) phenyl phosphate was used as a substrate which releases light as a result of dephosphorylation by ALP. This enzyme was observed to be inhibited by organophosphorous and reduced its activity due to weak chemiluminescent signal. Therefore, quantitative analysis of pesticides is possible with correlation between signal strength and pesticide concentration<sup>61</sup>. Another ALP inhibition based biosensor was developed for malathion and 2, 4-D pesticides. The reduced activity of ALP in the presence of these pesticides was analysed by amperometry and voltametry. For amperometric detection, 3-indoxyl phosphate was taken in the form of enzyme substrate and for voltametric analysis; two substrates PP and A-2-P were used<sup>62</sup>. Amperometric biosensor was recorded with high efficiency to detect the amount of carbofuran. PP was used as substrate to determine the quantity of carbofuran. The rate of enzyme inhibition was based on the concentration of the carbofuran. Inhibition of the activity of immobilized ALP due to carbofuran was determined by decreased anodic current. ALP biosensor has been showed successful detection of carbofuran<sup>63</sup>.

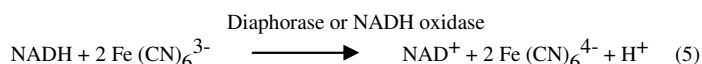
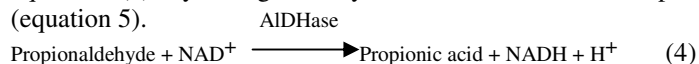
**Acid phosphatase based biosensors (AP):** AP in combination with (GOD) has been used to develop bienzymatic biosensors to detect amount of organophosphorous and carbamate group of pesticides as shown in Table-6. This biosensor catalyze the hydrolysis of glucose-6-phosphate by AP resulting in production of glucose which in turn was oxidized by GOD and resulted in the production of H<sub>2</sub>O<sub>2</sub> (equation 2 and 3).



H<sub>2</sub>O<sub>2</sub> produced as demonstrate in equation 3, was detected amperometrically by a H<sub>2</sub>O<sub>2</sub> sensitive electrode. Organo phosphorous (methyl parathion, malathion and paraoxon) and

carbamates pesticides inhibit the activity of AP, as a result the production of glucose will be reduced and the activity of the  $H_2O_2$  by amperometric sensor will also be affect. For development of AP biosensor, two types of amperometric  $H_2O_2$  electrodes have been employed. In one type, both enzymes AP and GOD were taken in pure form. While, AP with a thin layer of potato (*Solanum tuberosum*) tissue with pure form of GOD were immobilized on the tip of electrode. These biosensors revealed higher sensitivity for organophosphorus compounds. On the other hand, a higher detection limit was obtained for carbamates, as a weaker inhibition power<sup>64</sup>. Gouda and co-workers also developed a dual enzyme ampermetric biosensor based on AP and GOD using the same principle. AP biosensor composed of bienzymatic membranes one of potato tissue slice (rich in acid phosphatase) and another immobilized GOD. The response of this biosensor in the presence of organophosphorous and carbamate pesticides was measured in terms of oxygen depletion by clark type dissolved  $O_2$  electrode<sup>65</sup>. Plant tissue based bioelectrodes exhibited better reliability of amperometric results.

**Aldehyde Dehydrogenase based biosensors (AIDH):** Dithiocarbamate fungicides are known to inhibit aldehyde dehydrogenase (AIDH). This enzyme carry the the oxidation of aldehydes (e.g. propionaldehyde) by using  $NAD^+$  and produces propionic acid and NADH as end products (equation 4). Diaphorase or NADH oxidase reoxidise NADH generated in equation (4) by using hexacyanoferrate as an  $e^-$  acceptor (equation 5).

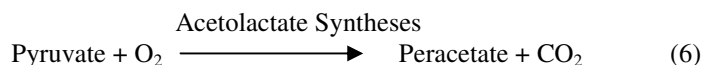


Based on this principle, an electrochemical bienzymatic (AIDH and NADH oxidase) biosensor, for detection of dithiocarbamate was developed. These two enzymes were embedded in a photocrosslinkable PVA-SbQ, attached to a Pt electrode. The activity of AIDH was dependent on the oxidation of hexacyanoferrate at a potential of 250mV vs SCE. The amount of fungicide was measured by a difference of the induced current in the absence or presence of the pesticides. Using this bienzymatic biosensor, the detection of  $0.0055\mu M$  of maneb was achieved by Noguer and Marty<sup>66</sup>. Marty and co-workers designed an amperometric biosensor with the use of Pt electrode for dithiocarbamate detection operated according to the same reactions as given above. The rate of Detection limit was observed to be  $0.188\mu M$  for maneb by Marty et al.<sup>67</sup>. Using same principle, another disposable bienzymatic sensor was also developed. In this biosensor, in place of diaphorase, a highly stable NADH oxidase was taken in combination with AIDH. The two enzymes were immobilized on a Pt sputtered carbon paste SPE by using photocrosslinkable PVA-SbQ. Due to poor solubility of ethylenebis (dithiocarbamate), its disodium form was produced using EDTA to make it highly soluble (zineb). Commercial disodium form of ethylenebis (dithiocarbamates)

i.e. nabam was used as a calibration compound. The observed detection limit was  $0.031\mu M$  for the disodium salt<sup>68</sup>.

**Esterase based biosensors:** Esterases are a group of hydrolases which catalyze the cleavage and formation of ester bonds. They are widely found in animals, plants and microorganisms<sup>69</sup>. This group of enzyme is well known to metabolize organophosphorous pesticides which could be divided three different groups- A,B,C. Types of esterases are not inhibited by organophosphorous pesticides, but are capable of hydrolysing them e.g. phosphatases. B type of esterases is primarily inhibited by organophosphorous group namely carboxyl esterases and C type of esterases are neither inhibited nor degraded by this group of pesticides<sup>70</sup>. Cutinase from *Fusarium solani pisi* was used for validation of a spectrophotometric enzyme assay in 96-well plate to detect the concentration of organophosphates and carbamates. The range of detection for chlorpyrifos and paraoxon with inhibitory rate constant (ki) of  $1.6 \times 10^4 L/mol \cdot min$  and  $2.0 \times 10^3 L/mol \cdot min$  was measured to  $0.145\mu M$  and  $7.41\mu M$  respectively. The spectrophotometric assay was further extended to two esterases derived from *Bacillus subtilis* (BS2) and rabbit liver (RL). These esterases were strongly inhibited by organophosphorous thions and showed ki in the range of  $5.3 \times 10^5 - 2.3 \times 10^4 L/mol \cdot min$ <sup>71</sup>. Spectrophotometric assay developed using RLE, BS2 and cutinase from *Fusariumsolani pisi* was transfer to HPLC. LOD for paraoxon obtained by this assay were 0.0013, 0.004 and  $0.590ng/zone$  with RLE, BS2 and cutinase, respectively. The observed detection limits were 1.5, 6.4 and  $936ng/zone$  for malaoxon, and 69.6, 29.9 and  $1632ng/zone$  for carbofuran using RLE, BS2 and cutinase, respectively. Therefore, esterases from *B. subtilis*, rabbit liver and cutinase from *Fusarium solani pisi* gets inhibited by organophosphorous and carbamates pesticides<sup>72</sup>.

**Acetolactate Synthase based biosensors (ALS):** ALS enzyme is very essential for the biosynthesis of the branched amino acids. Pesticides of sulfonylurea (e.g. sulfomethuron methyl and thifensulfuron methyl) and imidazolinones groups are mainly known to inhibit the activity of ALS<sup>73</sup>. ALS biosensor to detect the concentration of sulfonylurea herbicides was based on the entrapment of ALS enzyme on a PVA-SbQ polymer membrane. ALS enzyme catalyzed  $O_2$  consumption in which pyruvate acts a substrate as given in equation (6)<sup>74</sup>.



In the presence of herbicide, this side  $O_2$  consumption reaction of ALS was inhibited. Hence, decrease in oxygen consumption was employed to measure the concentration of herbicide using oxygen electrode. The sensitivity of ALS biosensor for sulfonylurea was observed to be  $1\mu M$ <sup>74</sup>. Another biosensor using ALS was developed by Marty and co-workers, where the reduction in activity of ALS was taken spectrophotometrically. The detection range of ALS biosensor was recorded to  $7.27 \times 10^{-5}\mu M$  for paraoxon,  $0.188\mu M$  for maneb and  $0.049\mu M$  for thifensulfuronmethyl<sup>67</sup>.



**Peroxidase based biosensors:** Several inorganic and organic compounds are already reported for the inhibition of peroxidase enzyme. Peroxidases are oxidized by  $H_2O_2$  and then could be reduced with phenolic compounds. Phenolic compounds get converted to quinones or free radical products after oxidation reaction and electrochemically reduced on the surface of electrode. Ivanov et al.<sup>38</sup> developed the voltametric biosensor for the detection of thiodicarb, a carbamate pesticide. Hydroquinone was oxidized by  $H_2O_2$  to p-benzoquinone and finally reduced to hydroquinone. The presence of inhibitor compounds causes a reduction in biosensor current response. The developed biosensor showed a linear response of thiodicarb with the measured LOD of  $0.6\mu M$ . The same principle was also applied for the determination of thiodicarb from vegetable extracts<sup>75</sup>.

**Ascorbate oxidase based biosensors:** Gallo and Lawryk reported the inhibition of ascorbate oxidase activity by organophosphorous pesticides<sup>76</sup>. Ascorbate oxidases use ascorbic acid or L-ascorbate as a substrate and oxidize it to dehydroascorbate and  $H_2O$  (equation-7).

Ascorbate oxidase (AOD)



On the basis of above principle, an amperometric biosensor was developed to detect the amount of paraoxon-ethyl using cucumber tissue as a source of ascorbic acid oxidase. The tissue of cucumber was sandwiched among Teflon and nylon net membrane which adhered to a Clark dissolved  $O_2$  electrode. When the given equation 7 was allowed to take place on Clark dissolved  $O_2$  electrode, local  $O_2$  depletion was observed which causes the voltage response of the electrode to reduce. A decrease in ascorbate oxidase activity in the presence of paraoxon-ethyl was traced by reduction of above reaction and was detected in form of a change in output voltage. This voltage change was proportional to the concentration of pesticide. 10% inhibition was observed with incubation of the enzyme electrode for 10 min. with different concentrations of the pesticide. The linear response between substrate reaction and pesticide (paraoxon-ethyl) was reported in the range of  $3.63\text{--}36.33\mu M$  by Rekha et al.<sup>77</sup>.

**Table-3:** Characteristics of cholinesterase based pesticides biosensors.

Mono-enzymatic cholinesterase based biosensors							
Target Analyte	Transducer	Immobilization	Detection Limit ( $\mu M$ )	Linearity ( $\mu M$ )	Detection Time (min.)	Lifetime (days)	Ref.
Carbaryl and Dichlorvos	Fiber optic	Covalent coupling via GA & isothiocyanate on a polymeric membrane	0.53 Carbaryl, 0.023 Dichlorvos	0.54-39.8 Carbaryl, 0.022-0.13 Dichlorvos	12	21 retained 70% activity	39
Carbofuran	Amperometric	Electrostatic interaction on glass carbon electrode	$4.0 \times 10^{-3}$	$4.8 \times 10^{-3}$ –0.09	9	21	106
Paraoxon	Amperometric	Diffusion of enzyme using Bovine Serum Albumin (BSA) and Glutaraldehyde (GA) inmembrane	1.80	10-170	20	30	107
Organophosphorous and Carbamates	Amperometric	Cross linking using GA on Prussian Blue-modified Screen-printed electrodes	0.126 Aldicarb, 0.124 Carbaryl	0.063–0.315 Aldicarb, 0.124-0.525 Carbaryl	30	21	108
Paraoxon	Amperometric	Crosslinking using cellophane membrane	1.45	1.45–7.26	15	NR	109
Paraoxon, Chlorpyrifos-ethyl oxon	Amperometric	Entrapment on polyvinyl alcohol-bearing styryl pyridinium groups (PVA–SbQ) polymer on SPE	$1.91 \times 10^{-2}$ Paraoxon; $1.24 \times 10^{-3}$ Chlorpyrifos oxon	NR	10	NR	110
Methyl parathion	Electrochemical	Entrapment on Au NPs polypyrrole nanowires composite film modified glassy carbon electrode	$7.5 \times 10^{-3}$	0.017– 0.41 and 1.7–15.44	12	30 retained 60% activity	111

Paraoxon	Amperometric	Electrostatic interactions with zinc oxide sol-gel/SPE	0.127	0.127– 5.01	10	90	112
Butyrylcholinesterase based biosensors							
Methyl Paraoxon, Methyl Parathion	Chemiluminescence	BuChE with dextrose in protein standard solution dispensed in microwell plate and dried to form thin film like layer	4 Methyl Paraoxon	$2.02 \times 10^{-5}$ -0.202 Methyl Paraoxon, $0.17 \times 10^{-2}$ –0.034 Methyl Parathion	12	15 more than 90% activity, 42 retained 74% activity	113
Paraoxon, Dichlorvos	Amperometric	Cross linking using BSA and GA on SPE	0.01	Curvilinear 0.01 – 100	30-60	NR	112
2-methylthio-4H-1,3,2-benzodioxaphosphorin-2-oxide; Dichlorvos	Potentiometric	Immobilized on plasticized poly(vinyl chloride) membrane	0.18	0.01-10	10	NR	113
Trichlorfon	Potentiometric	Cross-linkage with BSA-GA membrane on Polyethyleneimine thin film electropolymerized at the electrode surface	0.1	NR	15	NR	113
Paraoxon	Potentiometric	Entrapment within hydrophilic polyurethane film over a PU based ion-selective membrane	0.01	0.01-10	60	7	114
Bi-enzymatic cholinesterase based biosensors (AChE/ChO)							
Carbaryl, Carbofuran, Aldicarb	Amperometric	Co-immobilization by cross linking onto a pre-activated pHEMA membrane	$1.8 \times 10^{-3}$ Carbofuran, 0.052 Aldicarb, $9.4 \times 10^{-3}$ Carbaryl	0-0.09 Carbaryl, Carbofuran	5	NR	46
Paraoxon	Amperometric	AChE adsorption onto a nylon membrane, ChO cross linking via GA/BSA	$4.7 \times 10^{-3}$	$3.6 \times 10^{-3}$ – $5.4 \times 10^{-2}$	30	NR	115
Carbofuran	Amperometric	Adsorption of ChO adsorbed/AChE in solution	$0.9 \times 10^{-3}$	NR	12	NR	116
Diazinon-oxon	Amperometric	Entrapment on hybrid mesoporous silica membrane/Pt electrode	$1.2 \times 10^{-3}$	$1 \times 10^{-3}$ –0.3	15	80	117
Tri-enzymatic cholinesterase based biosensors							
Trichlorfon	Potentiometric	Adsorption of peroxidase and co-immobilization of ChO and AChE by GA	$0.5 \times 10^{-2}$	0-100	20	30	48
Trichlorfon	Potentiometric	Co-immobilization on composite carbon electrodes using GA	$2 \times 10^{-7}$	$1 \times 10^{-4}$ -10 soluble enzyme; 0-10 <sup>-4</sup> immobilized	15	28	118

Note. NR, not reported.

**Table-4:** Characteristics of tyrosinase based pesticides biosensors.

Target Analyte	Transducer	Immobilization	Detection Limit ( $\mu\text{M}$ )	Linearity ( $\mu\text{M}$ )	Detection Time (min.)	Lifetime (days)	Ref
Phenolic compounds and herbicides	Amperometric	Entrapment in PEDT on GCE	4.63 Atrazine, 2.14 Diuron	NR	0.8	12 Retained 30% activity	58
Organophosphorous and carbamates	Amperometric	Cross-linking with BSA and GA on a composite electrode	0.10 Diazinon, 0.26 Methyl parathion, 0.06 Carbaryl, 0.1 carbofuran	0.02-0.34 Methyl parathion, 0.06-0.16 Diazinon, 0.02-0.4 Carbofuran, 0.049-0.24 Carbaryl	2	10	119
Dimethyl- and diethyldithiocarbamate	Amperometric	Adsorption on the surface of a graphite-disk electrode	0.074 Ziram, 1.3 Diram, 1.7 Zinc diethyl dithiocarbamate	0.2-2.2 Ziram, 4.0-44 Diram, 4.0-40 Zinc diethyldithiocarbamate	0.5	2	120
Thiodicarb	Square Wave Voltammetric	Entrapment in chitosan crosslinked with cyanuric chloride	0.199	0.399-2.2	1	10	121
Organophosphorous and carbamates	Amperometric	Adsorption on gel-like disk of kappa-carrageenan gel	0.01-0.001	0.01-10 <sup>4</sup>	<22-35 min. depending upon the type of pesticide	NR	122

**Table-5:** Characteristics of alkaline phosphatase based pesticides biosensors.

Target Analyte	Transducer	Immobilization	Detection Limit ( $\mu\text{M}$ )	Linearity ( $\mu\text{M}$ )	Detection Time (min.)	Lifetime (days)	Ref
Organochlorine, Organophosphorous and Carbamates	Fluorimetric	Entrapment in Sol-gel	4.9 Metham sodium, 292.3 Tetradifon	194-774 Metham- sodium 3.5-28Tetradifon	8	60	59
Chlorophenoxyacetic acid herbicides, Carbofuran and Endosulfan	Amperometric	Entrapment in hybrid sol-gel/chitosan film deposited on the surface of a screen-printed carbon paste electrode	-	0.0045-0.27 (2,4-D)	NR	NR	60
Organophosphorous	Chemiluminescent	Biotin and streptavidin	0.181 Paraaxon	NR	NR	NR	61
2,4-D and Malathion	Amperometric	Immobilization on nylon 6.6 membrane with carboxyl groups on the surface, by means of polyazetidine	2.2x10 <sup>-3</sup> (2,4-D), 0.3x10 <sup>-3</sup> Malathion	0.0067-0.271(2,4-D), 0.6x10 <sup>-3</sup> -0.136 Malathion	30-60	60 assays for 2,4-D and 80 assays for Malathion	62
Carbofuran	Amperometric	Cross linking with BSA and GA onto the surface of the carbon paste electrode	4.5x10 <sup>-2</sup>	4.5x10 <sup>-2</sup> - 0.43	NR	15 retained 50% activity	63

**Table-6:** Characteristics of acid Phosphatase based pesticides biosensors.

Target Analyte	Transducer	Immobilization	Detection Limit ( $\mu\text{M}$ )	Linearity ( $\mu\text{M}$ )	Detection Time (min.)	Lifetime (days)	Ref
Malathion, Methyl Parathion, Paraoxon, Aldicarb	Amperometric (Bienzymatic; second enzyme: GOD)	Absorption on Polyazetidine prepolymer membrane	0.009 Malathion, 0.0017 Methyl Parathion, 0.018 Paraoxon, 0.21 Aldicarb	0.013-0.045 Malathion, 0.002-0.041 Methyl Parathion, 0.022-0.066 Paraoxon, 0.24-0.65 Aldicarb	20	5	64
Malathion, Methyl Parathion, Paraoxon, Aldicarb	Amperometric (Hybrid)	Absorption of GOD on potato tissue	0.0045 Malathion, Methyl Parathion, 0.0018 Paraoxon, 0.21 Aldicarb	0.009-0.06 Malathion, 0.002- 0.027 Methyl Parathion, 0.011-0.0726 Paraoxon, 0.241-0.65 Aldicarb	20	15	64
Paraoxon	Amperometric (Bienzymatic; second enzyme: GOD)	Crosslinking of GOD on cellophane membrane using gelatin and slice of potato was placed on the electrode surface using Teflon and cellophane membranes	3.63	0-36.33	15	8	65

### Glutathione-S-transferase based biosensors (GST)

GST is very well known isoenzyme family of plants origin. GST has been used by plants to detoxify many xenobiotics including pesticides. A tripeptide glutathione, an endogenous compound in the plants acts as a cellular nucleophile and GST catalysis a wide range of detoxification reactions. The products produced due to the process of conjugation were reported to be much water soluble in contrast to parent xenobiotic<sup>78-79</sup>. A fiber-optic biosensor was also developed to detect atrazine by using GST I from the expression of maize in *E. coli*. The enzyme was immobilized on a hydrophilic polyvinylidene fluoride membrane which was supported on an inner glass disk. The detection of atrazine was carry out by GST based nucleophilic attack in which  $\text{H}^+$  ion get change the colour of bromocresol. Andreou and Clonis<sup>39</sup> reported the detection limit for atrazine which was around  $0.84\mu\text{M}$ . The conjugation reaction catalyzes the release of protons and could be measured by using pH indicator dye. The principle of this sensor is generally used to develop a colourimetric assay for the detection of pyrethroids. The amount of pyrethroids was determined by measuring different range of pH change through the inhibition of enzyme<sup>80</sup>. Analytical characteristics of some of the GST based biosensors as reported by authors in literature are summarized in Table-7.

### Organophosphorous hydrolase based biosensors (OPH)

OPH catalyze the degradation of organophosphorous elements and produces two protons by the cleavage of the P-O, P-F, P-S or P-CN bonds and an alcohol. These products of hydrolysis could be analysed using effective transducers such as amperometric, optical and potentiometric. To construct potentiometric biosensors OPH are combined with pH electrode to determine the concentration of  $\text{H}^+$  ions. A biosensor working

on this principle was developed for determination of organophosphorous pesticides. OPH enzyme was immobilized through cross-linking with BSA and GA on a pH electrode. The concentration of pesticides was determined in terms of amount of  $\text{H}^+$  released<sup>81</sup>. Using the similar approach, Mulchandani and co-workers also designed another potentiometric biosensor with improved sensitivity using OPH. The LOD of OPH was reported to be as less as  $2\mu\text{M}$  for paraoxon, diazinon and methyl parathion<sup>82</sup>. OPH could also be combined with amperometric transducer to monitor the oxidation or reduction reactions. An amperometric sensor to detect the concentration of organophosphorous pesticides was designed through a bilayer approach with OPH and carbon nanotube (CNT) film. The CNT layer was with a great improved anodic detection and end product of hydrolysis with higher stability<sup>83</sup>. Lee and co-workers designed an efficient sensor which was composed of mesoporous carbon (MC) and carbon black (CB). This layer was having amperometric response relative to a CNT-modified electrode<sup>84</sup>. Determination of chromophoric products generated as a result of hydrolysis form the basis of OPH based optical biosensors. A recombinant methyl parathion hydrolase (MPH) based biosensor was developed by immobilization of this enzyme on agarose. MPH carried out the hydrolysis of organophosphorous compounds and the end product of hydrolysis i.e. p-nitrophenol was measured optically. The absorbance was determined linearly correlated to methyl parathion<sup>85</sup>. A quantitative detection of organophosphorous compounds e.g. paraoxon and diisopropyl fluorophosphates (DFP) was possible using this sensor<sup>86</sup>. However, lower sensitivity values and higher LOD is generally obtained by OPH based biosensors than ChE sensors. However, these can detect only specific organophosphorus compounds. Numerous OPH based biosensors have been developed and Table-8 is summarizing the performances of some of these biosensors.

**Table-7:** Characteristics of glutathione-S-transferase based pesticides biosensors.

Target Analyte	Transducer	Immobilization	Detection Limit ( $\mu\text{M}$ )	Linearity ( $\mu\text{M}$ )	Detection Time (min.)	Lifetime (days)	Reference
Atrazine	Fibre-optic	GA crosslinking using Hydrophilic olyvinylidene fluoride membrane supported on an inner glass disk by binder sol-gel layer	0.84	2.52-125	8.33	30 25% activity reduction	39
Pyrethroids	Colorimetric	NR	50 $\mu\text{M}$	0-98.96 Deltamethrin	NR	> 180 retained 80-100%	80
Pyrethroids and Organochlorine	Colorimetric	NR	7.66 Permethrin, 39.58 Deltamethrin, 26.67 Cyhalothrin, 5.64 (DDT)	0-100 Permethrin & Deltamethrin, 0-220 Cyhalothrin, 0-140 (DDT)	10	30	123
Phenylurea, Endosulfan, Malathion	Potentiometric	Crosslinking with GA trapped behind a semipermeable membrane in front of the pH electrode	8.4 IC <sub>50</sub> for Malathion	0-20	10	16-18 retained 50% activity	124
Molinate	Differential Pulse Voltametry	Covalent attachment via aminosilane-GA on GCE	0.34	1.01-42.17	NR	15	125

## Organophosphorus acid anhydrolase based biosensors

This enzyme is a monomer and a metalloprotease of 60 kDa and consists of Mn element in the natural form. It was first time identified in rabbit tissue by Abraham Mazur in 1946, as a hydrolysing enzyme of DFP and then was purified from *Alteromonas* species strain JD 6.5.19 bacterium<sup>87-90</sup>. This enzyme was reported with very high specificity for hydrolysis of P-F and a P-CN bond<sup>91</sup>, but is not capable of hydrolyzing organophosphorus like parathion, malathion and demeton-S containing P-S and P-O bonds<sup>92</sup>.

Simonian and co-workers developed an organophosphorus acid anhydrolase (OPAA) to detect the quantity of organophosphorous fluorine containing pesticides. Hydrolysis rate of DFP (P-F bond), paraoxon (P-O bond) and demeton-S (P-S bond) has been studied using OPAA. In order to develop OPAA based catalytic biosensor, two different approaches were carried with same principle. OPAA showed selective hydrolysis of organophosphorous pesticides containing P-F bond i.e. DFP. While hydrolysis rate of paraoxon and demeton-S was negligible. Developed biosensor was able to detect the concentration of DFP below 25  $\mu\text{M}$  with the glass electrode. Thus, OPAA biosensors could be very effective for the selective detection of fluorine containing organophosphorus<sup>93</sup>.

## Factors affecting the performance of enzyme based pesticides biosensors

There are numerous parameters viz., pH, substrate concentrations, which are primarily known to affect the performance of enzymatic biosensors developed for pesticides detection. Optimization of these parameters is a key factor to decide the efficiency of biosensors.

Among all, important factors are needed to take precaution during the development of pesticides biosensors.

**pH:** It is an important parameter which can alter the activity of enzyme by catalyzing specific reactions. Each enzyme has an optimum pH below which its activity diminish. Enzymes are of proteinaceous nature, like proteins they possess native tertiary structure which is highly sensitive and prone to denaturation by variation of pH from the optimum. Thus, amino acid side chains act as weak acids and bases to perform critical functions in the active site of enzymes. Basically, the activity of enzyme gets changed due to the pH of the solutions containing substrates. Extreme pH conditions could lead to poor binding with substrate and reduce the catalytic efficiency of enzyme. Therefore, pH is very important factor to develop the enzyme based biosensor<sup>94</sup>. Catalytic or inhibition reactions of enzymes by pesticides should not interfere with optimized pH. Stoytcheva and their group studied the effect of pH range (2-9) on the activity of acetylcholinesterase and reported 72% inhibition in the activity of enzyme at pH 2 in contrast to pH 7<sup>95</sup>.

## Substrate concentration

This is also an important factor that needs greater attention to get best analytical performance with enzyme inhibition based biosensors. Benilova and co-workers demonstrated that reversible competitive inhibition based biosensors get inhibit with increase the concentration of the substrate i.e. BuChE was found to be inhibited by  $\alpha$ -chaconine<sup>96</sup>. The reduced sensitivity of BuChE biosensor for tomatine was also observed with the increase in the concentration of substrate<sup>95,97</sup>. Joshi et al.<sup>98</sup> also observed the reduced degree of inhibition before and after incubation with paraoxon through double concentration of acetylcholine to that of apparent  $K_m$ .

Biosensors working on this principle could be work effectively when substrate concentration used is less than that of inhibitor. Different substrates could also be identifying to get better sensitivity towards enzyme from one substrate to other<sup>99</sup>. Shan and co-workers also screened different phenolic compounds e.g. catechol, p-cresol, phenol, m-cresol and p-chlorophenol as substrate to see the inhibition of tyrosinase by benzoic acid.  $K_m$  value of catechol was found to be higher as compared to other four substrates and thus inhibition to a great extent was reported with this substrate. This showed lesser affinity of the enzyme

for catechol. Thus, performance of a biosensor gets affected from Concentration and the nature of substrate<sup>100</sup>. A high substrate concentration was observed with high output signal for irreversible inhibition based biosensor<sup>23</sup>.

**Enzyme concentration:** The concentration of an enzyme is an important factor which could affects the detection limit of a biosensor. Higher sensitivity of enzyme inhibition rate was reported with low enzyme loading<sup>44,95,99-102</sup>.

**Table-8:** Characteristics of organophosphorous hydrolase based pesticides biosensors.

Target Analyte	Transducer	Immobilization	Limit of detection ( $\mu\text{M}$ )	Linearity ( $\mu\text{M}$ )	Detection Time (min.)	Lifetime (days)	Reference
Organophosphorous	Potentiometric	Cross linking using BSA and GA on pH electrode	2 Paraoxon and Parathion	2–400 Paraoxon and Parathion	< 3	>30	81
Organophosphorous	Amperometric	Entrapment of enzyme in Nafion on CNT-modified electrode	0.15 Paraoxon, 0.8 Methyl parathion	0- 4	NR	NR	83
Organophosphorous	Amperometric	Entrapment on MC and CB modified electrode	0.12 Paraoxon	0.2-0.8	~10 seconds	NR	84
Organophosphorous	Optical	Metal-chelate affinity-based immobilization	4 Methyl parathion	0–100	15	NR	85
Organophosphorous	Fibre-Optic (Fluorescence)	Conjugation with biotin and CNF (Affinity)	0.05 $\mu\text{M}$	1-800 Paraoxon and 2 - 400 (DFP)	10-40 seconds	28 retained 80% activity	86
Methyl Parathion	Optical	Adsorption on Glass fiber filter paper	0.3	4–80	3	30	126
Organophosphorous	Amperometric	Entrapment between Polycarbonate and teflon membrane of the dissolved oxygen electrode	0.1 Paraoxon	0 - 49.96 Paraoxon	<5	7	127
Organophosphorous	Amperometric	Dried cells mixed with carbon paste packed in electrode	0.2 Paraoxon, 1 Methyl parathion	0-40 Paraoxon, 0-175 Methyl parathion	2	45	128
Organophosphorous	Conductometric	Using BSA and GA on screen printed carbon electrode	0.59 Diazinon	0-3.28 Diazinon	45 seconds	NR	129
Organophosphorous	Optical	Gold NPs	20 Paraoxon	20-240 Paraoxon	NR	NR	130
Organophosphorous	Potentiometric	Cross-linking OPH with BSA and GA on pH electrode	2 Paraoxon, Ethyl parathion, Methyl parathion and Diazinon	150–700	2-10	30	82
Paraoxon	Amperometry	Cross-linking using GA	0.314	0.5-2.0	3	>90	131
Methyl parathion	Amperometry	Covalent attachment through CdTe QDs on a GCE	0.0038	0.019- 0.76	10	30	132

Different concentrations (40, 50, 60, 80  $\mu\text{L}$ ) of AChE were dissolved in 1.0 mL of 0.1 M phosphate buffer (pH 7.2) solution during the designing of biosensor and a higher sensitivity for pesticides was recorded with 60  $\mu\text{L}$ <sup>103</sup>. Suwansa-ard and his co-workers used different concentrations 150, 200, 250 units of enzyme/mL. silica gel and 100, 150, 200, 250 units of enzyme/mL. of silica gel, for the flow-injection potentiometric and conductometric biosensors respectively. Higher sensitivity for potentiometric and conductometric biosensors obtained with 195 and 150 units of enzyme respectively<sup>104</sup>.

**Organic solvent:** Most of the pesticides are soluble in organic solvents and rarely are found for their solubility in water. This is the reason for detection of pesticides in different matrices; these pesticides are extracted using organic solvents e.g. acetone, methanol, acetonitrile, hexane etc. Extracted organic solvent samples are usually used to determine pesticide either qualitatively or quantitatively through enzymatic biosensors. Activity of enzymes gets affected to a greater extent by organic solvents. Activity of enzymes differs with the polarity of solvent. Three enzymes viz., choline, choline oxidase, esterase and peroxidase were co-immobilized for the development of these biosensors. Ciucu et al.<sup>105</sup> demonstrated that the activity of these biosensors were found inhibited with non polar solvents. While, polar solvents like ethanol and acetone doesn't affect the activity of biosensor. Therefore, selection of organic solvent is an important step during the development of biosensor. Somerset studied the influence of pure and different aqueous polar solvent mixture for the activity of AChE. Inhibition of enzyme activity was found upto 93%, 96% and 77% with pure acetonitrile, acetone and ethanol respectively. The smallest degree of inhibition (10%) of the electrocatalytic effect of AChE in the gold/mercaptobenzothiazole/polyaniline/acetylcholinesterase/ polyvinylacetate biosensor obtained, when 90% aqueous-solvent mixture of acetone was used<sup>103</sup>.

**Applications:** Several biosensors were developed using enzyme as a biorecognition molecule. But, the potential of all these biosensors for estimation of pesticides in real samples was not evaluated. The application of biosensors from standard solutions to real samples further needed optimization of different factors like evaluation of matrix effect and matrix matched calibration etc. Fortunately, very few reports are found to the development of enzyme based sensors along with its validation. Few of these have successfully been used to detect the concentration of organophosphorous, organochlorine and carbamate in environmental (water) and food samples viz., fruits, eggs vegetables, milk, honey etc. Enzyme, AChE based biosensors are also available commercially for the estimation of pesticides from different matrices. Some of the cholinesterase based testing kits for the detection of pesticides are the MAIA Pesticide MultiTest (Liofilchem, Roseto D.A. (TE), Italy), Pesticide Residue Meter (Welfull Group Co., Ltd, Zhejiang, China (Mainland), AGRI STICK (AR Brown Co., Ltd., Chuoku, Tokyo), NIDS@ ACE Rapid Pesticide Test. Similarly other enzymes described in this article have shown higher sensitivity

for pesticides monitoring at laboratory scale analysis. These could also be find potential applications for pesticides analysis in various other types of real samples including food, environmental and for also clinical analysis.

## Conclusion

Enzyme based biosensors have become very efficient tools for the detection of pesticides in food items and other environmental samples. These have very good advantages like cost effective, easy measurement, high throughput and their high sensitivity. Till date, the mostly AChE based biosensors showing high sensitivity and lower limit of detection for pesticide residues are available commercially. The primary objective of this article was to highlight the scope of other enzymes like AChE in the form of biorecognition molecule for the development of pesticides biosensors. Attempts have been made to summarize the analytical performance of enzyme based biosensors for determination of pesticide residues reported so far. Both catalytic and enzyme inhibition based biosensors are simple, rapid devices and have potential of good recoveries, linear response and high limit of detection for pesticides on laboratory level. This shows all the enzymes used as biorecognition molecules in sensor development have the ability to monitor pesticide residues and to commercialize them for field application. Still, some challenges like regeneration, reproducibility, matrix effect, shelf-life needed to be faced. Practical approaches required for successful development of sensing technologies for pesticides monitoring have also been explored. Thus, the improvement of developed enzymatic based sensors could be lead to their faster commercialization by transferring them from laboratory to industry and hence replacement of costly and time consuming traditional methods.

**Future Perspectives:** Developments of enzyme based biosensors are very much selective and easily discriminate the monitoring of pesticides in multicomponent sample. To full fill these objectives, enzymatic biosensors could be coupled to artificial neuron network and data can be analyzed chemometrically to identify the pesticide present in the sample. Miniaturization is another aspect which can also play an important role in conversion of enzymatic biosensors to marketable devices for food and environmental applications. Use of microfabrication and nanofabrication techniques can contribute immensely to miniaturize enzyme based biosensors in order to achieve fast and automatic analysis of pesticides in real samples. In addition to this type of advancement, work can also be done to make enzyme based technologies more cost-effective for the determination of pesticides. To accomplish this task, in recent years work has been done to develop enzymatic biosensors based on inhibition and catalytic principle using bacterial spores and whole cells as a source of enzymes to target different types of analytes like aflatoxin M1, pesticides and antibiotics. These approaches to use whole cells and bacterial spores could further be explored to target analysis of broad range of pesticides.



As development of this type of bio-sensing systems can be effectively reduce the cost of enzyme purification and will not suffer from the loss of activity during storage for longer period of time. Therefore, less cost of sensors fabrication can encourage quality control laboratories to check more no. of samples in order to ensure safe and wholesome products. Moreover, these techniques have the potential to offer several advantages to the growing field of enzyme based sensing technologies like higher sensitivity, selectivity, faster response, small sample requirement and portability which in turn will increase the versatility and will also reduce the time and cost of analysis.

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**Abbreviations used:** MRL, maximum residue limit; ADI, acceptable daily intake; AChE, acetylcholinesterase; LOD, limit of detection; GA, glutaraldehyde; ChE, cholinesterase; BuChE, butyrylcholinesterase; BSA, bovine serum albumin; SPE, screen printed electrode; PVA-SbQ, polyvinyl alcohol-bearing styrylpyridinium groups; PU, polyurethane; TiO<sub>2</sub>, titanium dioxide; NPs, nanoparticles; ChO, choline oxidase; CdTe, cadmium telluride; QDs, quantum dots; pHEMA, poly (2-hydroxyethyl methacrylate); HRP, horse radish peroxidase; QCM, quartz crystal microbalance; PPO, polyphenol oxidase; PEDT, poly 3,4-ethylenedioxythiophene; GCE, glassy carbon electrode; ALP, alkaline phosphatase; PP, phenyl phosphate; A-2-P, ascorbate-2-phosphate; AP, acid phosphatase; GOD, glucose oxidase; AIDH, aldehyde dehydrogenase; BS2, *Bacillus subtilis*; RL, rabbit liver; ALS, acetolactate synthase; GST, Glutathione-S-transferase; GSH, glutathione; OPH, organophosphorous hydrolase; CNT, carbon nanotube; MC, mesoporous carbon; CB, carbon black; MPH, methyl parathion hydrolase; LEDs, light-emitting diodes; CNF, carboxynaphthofluorescein; DFP, diisopropyl fluorophosphates; OPAA, organophosphorous acid anhydrolase; pH-FET, pH-sensitive field effect transistor.

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